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Pharmacology and Therapeutics

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PHARMACOLOGY AND THERAPEUTICS

Edited by **Sivakumar Joghi Thatha Gowder**

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Meet the editor



Dr. Sivakumar Gowder received his academic training and carried out his research in institutions of high academic ranking in India and US (University of Madras -Chennai, India; All India Institute of Medical Sciences -New Delhi, India; UT Southwestern Medical Center -Dallas, TX, US; LSH Health Sciences Center, Shreveport, LA, US and University of Pittsburg School of Medicine, Pittsburgh, PA, US). Currently, he is working as an Associate Professor at the College of Applied Medical Sciences, Qassim University, KSA. Dr. Gowder received prizes and awards in different levels of his academic career. He has developed his own research methods and techniques relevant to his research disciplines, and has published several journal articles and book chapters. Currently, he serves as an author and editor for books; editor in chief for an international journal; editorial member and reviewer for journals; fellow and advisory board member of international organizations and external examiner of doctoral thesis work for international universities. He has also served as an invited speaker and chairperson for international conferences.

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Preface

In recent years, pharmacology is considered to be an important aspect for physicians and biomedical scientists due to industrialization (a source for pollution), commercialization of several products (food products, pesticides, herbal drugs, etc.) and the occurrence of various diseases. In this context, it is an ample opportunity for me to present this book "Pharmacology and Therapeutics" to the audience. This book comprises six sections: Molecular Modeling and Biomolecular Pharmacology, Immunopharmacology, Environmental Pharmacology and Toxicology, Nanotechnology and Chemotherapy, Drugs and Drug Delivery System and Addiction Pharmacology. It also covers important discoveries in the recent years.

The first section deals with molecular modeling and the pharmacological effects of bio-molecules. In the first chapter, the author has discussed about the missense mutations and development of therapies for many genetic illnesses including hearing loss. From the second chapter, we can understand that bio-molecules can also act as modulators for pathogens. In the section "Immunopharmacology," the authors have narrated the importance of adenosine receptor signaling pathway in the development of drugs, and also the role of wasp venom in controlling diseases that will pave the way to develop bio-pesticides. In the third section (Environmental Pharmacology and Toxicology), the authors have claimed the importance of our present environment with reference to human health. In this section, we can also unravel the pharmacological activities of the plant *Moringa oleifera* and the nephrotoxic potential of anticalcineurins. In the section "Nanotechnology and Chemotherapy," the authors have discussed nanotherapeutics and potential drugs in the treatment of cancer and also their limitations in therapeutic use. In the fifth section, the authors describe the potential role of phytol as a psychiatric drug and the importance of chitosan in drug delivery. The last section summarizes drug experiences in relation to drug addiction and dependence.

In-depth analysis of this book "Pharmacology and Therapeutics" depicts intriguing findings and innovative theories and concepts. Though it is a simple and ready to read book, we can infer different aspects of pharmacology with an interdisciplinary approach. The authors have handled advanced techniques to characterize drugs for their mode of action. In brief, this book will be a significant source for scientists, physicians, health care professionals and students. In this era - the age of health care, basic knowledge in medicines/drugs is essential for all to survive in this modernized and industrialized world without many difficulties. Herb is the healing of a nation, and alcohol is the destruction - *Bob Marley*.

I extend my gratitude towards my mother, my late father and my brothers for introducing me to higher education. My thanks are due to Dr. Yousef Aldebasi (Dean of College of Applied Medical Sciences - Qassim University) for encouraging me to carry out this project. I should also thank Dr. Sulaiman Al-Yahya (Vice President-Qassim University), Dr. Naser Al-

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Dr Sivakumar Joghi Thatha Gowder

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Molecular Modeling and Biomolecular Pharmacology

Molecular Modelling-Based Investigations of a Mutant Protein in Patients with Hearing Loss

Kazunori Namba

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/58398>

1. Introduction

The inner ear is a most complicated and highly organized anatomical region that possesses an elaborate system for hearing. It requires a unique structure for discriminating between the physical vibrations caused by various sounds and specific cellular mechanisms to convert the physical sounds received into chemical signals that must be transmitted to the brain correctly. For normal hearing, the highly optimized environment is controlled by the concerted function of an enormous number of expressed proteins. If proteins comprising the elements of hearing are damaged, it is possible that the normal hearing function might be impaired.

So, how do sound waves travel through the ear canal and integrate in the inner ear? Vibrations that come into contact with the tympanic membrane travel through the air-filled middle ear cavity via auditory ossicles: namely, the malleus, incus and stapes. These ossicles convert lower-pressure eardrum sound vibrations into higher-pressure sound vibrations at a smaller membrane called the oval window. This window is a membrane-covered opening that leads from the middle ear to the vestibule of the inner ear or cochlea (Figure 1a, b, c). The sound waves are transduced into nerve impulses, which are perceived by the temporal lobe of the brain. A core component of the cochlea is the Organ of Corti—the sensory organ of hearing—which is distributed along the partition that separates fluid chambers in the coiled, tapered tube of the cochlea (Figure 1d). Sound waves passing through the scala timpani in the cochlea vibrate the tectorial membrane and stimulate stereocilia, which are located on the periplasmic region of hair cells (Figure 2). A shearing movement between the tectorial membrane and the basilar membrane deflects the stereocilia, affecting tension on the tip-link filaments, which then open and close non-

specific ion channels [1]. When the tension increases, the flow of ions across the membrane into the hair cell also rises. This is the first step of the mechano-electrical transduction system and causes receptor depolarization, which subsequently excites the cochlear nerve afferents that are located at the base of the hair cell. In the cochlea, many specific genes such as ion channels, mechanical proteins and signal transduction-related molecules, among others, are expressed and function in hearing [2]. In the case that more than one of these proteins is mutated, hearing function might be impaired or lost. This is one of the main causes of congenital hearing loss, which can be inherited.

It is widely known that deafness is a frequently inherited sensory disorder: one in every 500 new-borns has bilateral sensorineural hearing loss (SNHL) and 70% of these cases are congenital [3, 4]. Hereditary hearing loss is classified as either syndromic or nonsyndromic [5]. It is assumed that hearing-associated genes amount to over 400 [6]; to date, 76 genes involved in syndromic and nonsyndromic hearing loss have been identified [7]. The genetic causes of nonsyndromic hearing loss are autosomal dominant (27 identified genes), autosomal recessive (40 genes), X-chromosome-linked (three genes) and mitochondrial (six genes) [7].

The expression patterns of genes in the inner ear can be visualized on the Hereditary Hearing Loss Homepage [7]. In terms of genes associated with deafness, many different types of mutation have been identified, including missense, nonsense, splicing, regulatory, deletions, insertions, indels and duplications. So far, missense/nonsense mutations have been shown to account for 55.4% of all mutation types [2]. Identification of phenotype-genotype correlations is crucial for determining the aetiology of congenital hearing loss and has implications for prognostic and therapeutic outcomes. For missense mutations in the coding regions of a gene, it is assumed that the mechanism of hearing loss is based on functional disorders of the gene product. Thus, to understand congenital hearing loss it is essential to study extensively the functions of proteins expressed in the inner ear.

Hearing levels and patterns are represented by an audiogram, which is a graph showing the results of pure-tone hearing tests (Figure 3). Hearing loss can be categorized in terms of which part of the auditory system is damaged. There are three basic types of hearing loss: conductive hearing loss, SNHL and mixed hearing loss. In most cases, hearing loss is SNHL and nonsyndromic [8]. It is clear that some correlations between hearing loss and specific genes are robust, such as the low-frequency audioprofile associated with WFS1-related hearing loss [9] and the mid-frequency audioprofile associated with TECTA-related hearing loss [10]. High-frequency hearing loss, by contrast, can be the consequence of mutations in a large number of different genes such as KCNQ4, DFNA5, COCH and POU4F3 [9]. As part of an approach to determining how mutations of these proteins cause dysfunction in the inner ear, analysis of their three-dimensional structures is indispensable.

Analysis of protein structures (i.e., by X-ray crystallography, NMR, cryo-EM, etc.) is very effective in elucidating the mechanisms of hearing loss. However, the reported protein structures that are related to hearing loss account for only 16 of the 97,789 structures deposited in the Protein Data Bank (PDB) [11]. Most hearing-related proteins are adhe-

sion molecules (e.g., Gap junction, CDH23), ion transporters (e.g., KCNQ4, SLC26A4) or proteins involved in vesicular transport such as SNARE proteins (e.g., Otoferlin) [12, 13], for which it is difficult to elucidate the three-dimensional structure owing to technical difficulties such as protein expression, purification and crystallization, among others. Thus, the protein structures of hearing-related proteins are not well elucidated.

However, it is possible to generate computer-predicted structures of disease-related proteins if appropriate template structures are available in the PDB and also to elucidate the mechanism of protein function impairment by comparing wild-type and mutant structures. This alternative approach has an advantage in that the association of ligands such as sugar chains with a protein is extremely difficult to examine by crystallization, due to the structural mobility of the sugar chain, whereas it can be easily investigated by docking simulations. Thus, *in silico* modelling can help explain the functional impairment caused by mutation in terms of protein structure.

In this chapter, we describe a protein, namely a voltage-gated potassium channel, which is implicated in hearing loss. We discuss how mutants of this protein from patients cause hearing impairment from the point of view of molecular structure. Several types of software are freely available for these kinds of study and are useful for pharmacologists, molecular biologists and physicians. In addition, information on the three-dimensional structure is essential for discovering seed compounds in the field of drug design. Elucidation of structural data and the mechanisms of functional impairment due to mutations, coupled with computer-aided drug design, can lead to clinical benefits.

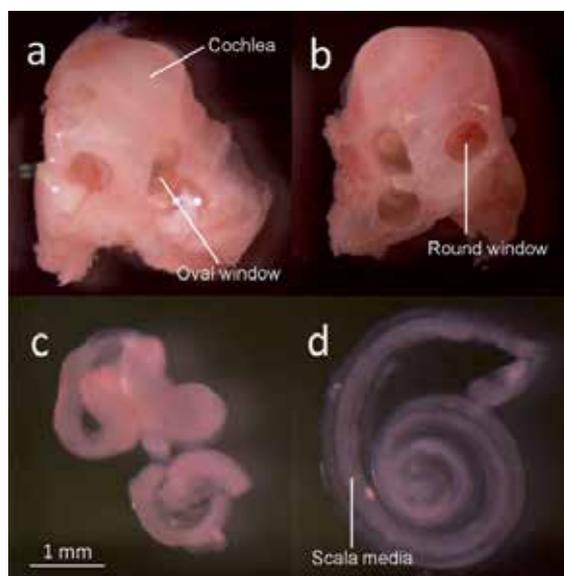


Figure 1. Structures of the rat's inner ear. (a, b): Whole-mount structure of the left (a) and right (b) inner ear with bone tissue dissected from rat. (c, d): The vestibular organ (c) and cochlea (d) extracted from bone tissues.

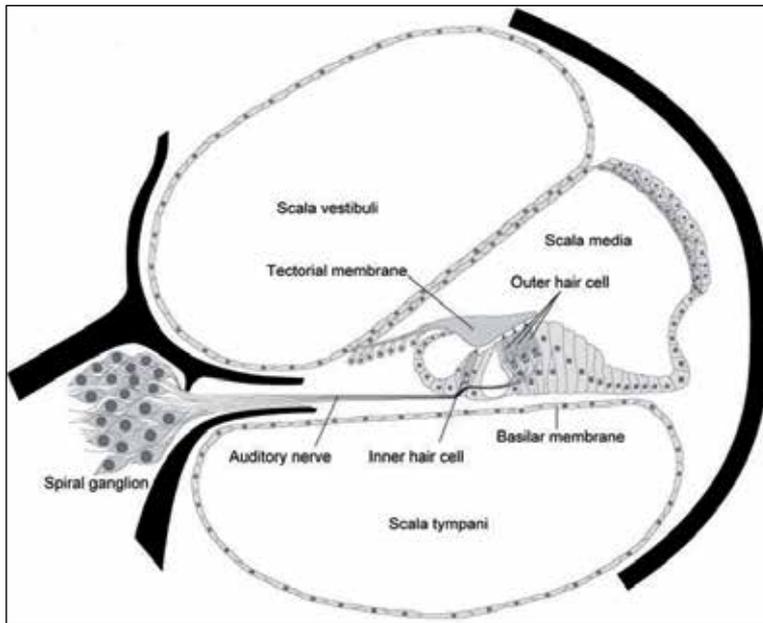


Figure 2. Schematic diagram of the coronal section of the cochlear duct. The majority of hearing loss-related genes are expressed in the cochlea. Hereditary Hearing Loss Homepage [7]

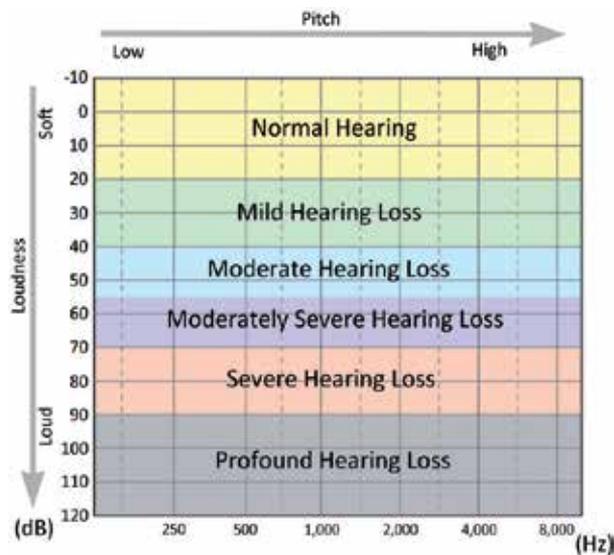


Figure 3. Hearing levels expressed on an audiogram. Frequency is expressed in terms of the number of cycles per second, or "Hertz". The range of most of the sounds in speech is 250Hz to 8000Hz. Loudness or "level of sound" is measured in units called decibels (dBs). The audiogram shows hearing loss across a range of frequencies; both the hearing level and the threshold of hearing level are indicated.

2. The voltage-gated potassium channel KCNQ4

KCNQ4, which encodes the voltage-gated potassium channel KQT-like subfamily member 4, has been identified as a causative gene in hearing loss [14]. The gene encodes a protein of 695 amino acid residues in its longest isoform and contains six transmembrane α -helices, (S1–S6), a pore helix (PH), a pore-loop (P-loop), a short N-terminal region and a long C-terminal region (Figure 4). As in other KQT-like channels, the ion-selective channel formed by KCNQ4 comprises a tetramer of identical subunits in which the highly conserved P-loop of each subunit combines to form the pore structure [15]. The pore region of the KCNQ4 channel consists of contiguous structures of S5, PH, P-loop and S6 elements. Functional and structural analyses of Kv channels have demonstrated that the PH and the P-loop are responsible for the selective K^+ penetration, whereas S4 regulates the open-closed state of the channel as a voltage sensor [16–18].

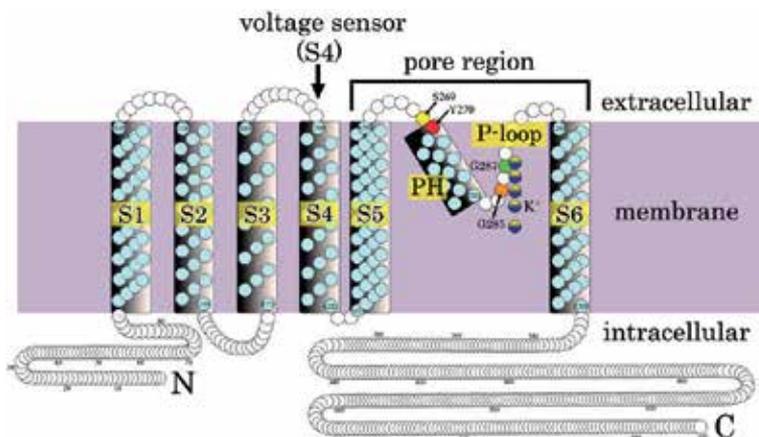


Figure 4. Schematic representation of the six transmembrane helices (S1–S6) and the K^+ -selective channel pore region (S5, PH, P-loop and S6) of KCNQ4. Yellow, red, orange and green circles indicate the p.Ser260del, p.Y270H, p.G285S and p.G287R mutations, respectively. Dark blue spheres indicate potassium ions.

KCNQ4 is expressed predominantly in the basolateral membrane of outer hair cells, a type of auditory sensory cell in the cochlea (Figure 5) and plays an important role in the proper electrophysiological function of these cells [19]. KCNQ4 is also expressed in spiral ganglion (SG) cells, although its function in SG cells is unknown. A mouse model expressing a dominant-negative form of KCNQ4 demonstrates hearing loss with slowly progressive degeneration of the outer hair cells owing to chronic depolarization caused by loss of the major K^+ efflux pathway [20]. The clinical features—namely, congenital, progressive high-frequency sensorineural hearing loss (Figure 6) without substantial loss of speech recognition during the first decade of life—exhibit a very strong hereditary tendency for patients with mutated KCNQ4 proteins [21, 22]. Since patients with KCNQ4 mutations show progressive hearing loss, the development of a drug to improve the function of the KCNQ4 channel might attenuate the symptoms.

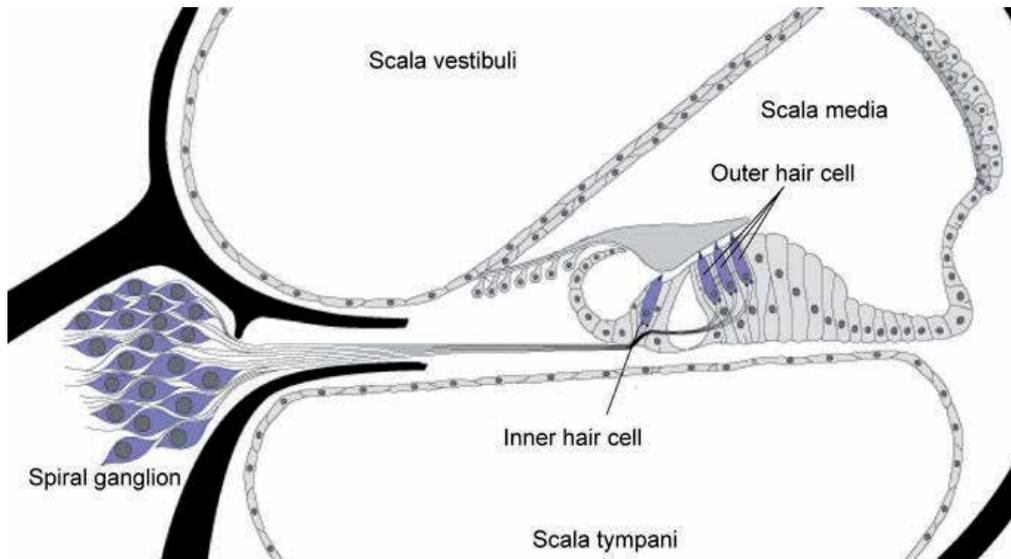


Figure 5. Expression pattern of the KCNQ4 channel in the cochlea. Protein expression is indicated in purple.

We identified two disease-associated mutations of KCNQ4: in one, a tyrosine residue (Tyr270) is replaced with histidine (His); and in the other, there is a deletion, c.806_808delCCT, leading to a p.Ser260del located between S5 and the pore helix (PH) in the gene product of KCNQ4. We then generated a computational structural model of the KCNQ4 channel by referring to the crystal structure of the Shaker family K⁺ channel, Kv1.2 [23, 24]. In the following sections, we speculate about the molecular mechanism underlying hearing loss according to the basic quantum chemistry of a KCNQ4 channel formed with the Tyr270His (p.Y270H) and p.Ser260del (p.S269del) mutations and we discuss how two more severe mutations- p.Gly285Ser (p.G285S) [14] and p.Gly287Arg (p.G287R) [25]-might cause severe to profound hearing loss from a structural point of view.

3. Materials and methods

3.1. Genetic analysis

Initially, KCNQ4 was selected as a candidate gene involved in hearing loss on the basis of clinical features [26]. Prior to this study, the patient was confirmed to have neither *GJB2* mutations, the most common causative gene of hereditary hearing loss, nor the mitochondrial m.1555A > G and m.3243A > G mutations. Genomic DNA was extracted from blood samples via the Gentra Puregene Blood Kit (QIAGEN, Hamburg, Germany). PCR primers specific for KCNQ4 were designed in our laboratory [23].

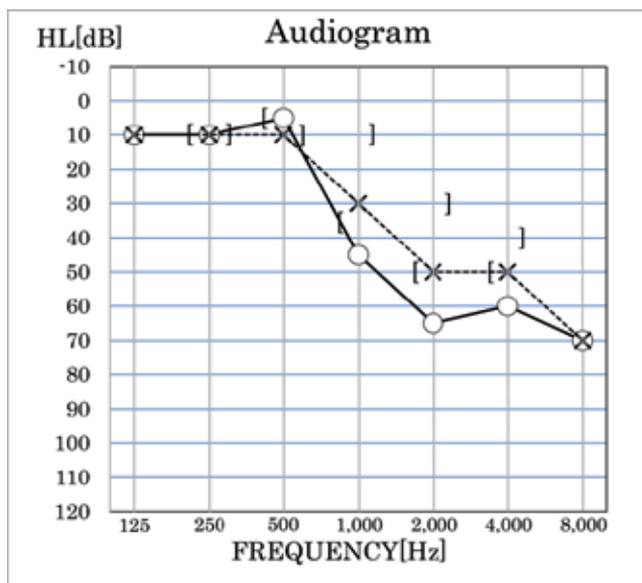


Figure 6. An audiogram from a patient with the KCNQ4 Tyr270His mutation. Open circles indicate the hearing levels of air conduction for the right ear. Crosses indicate the hearing levels of air conduction for the left ear. “[” and “]” indicate the hearing levels of bone conduction in the right and left ear, respectively. In conductive hearing loss, the bone conduction hearing thresholds are normal but there is a loss of hearing for air conduction sounds. This means that the cochlea is normal, but there is a blockage to sound in the middle or outer ear. This audiogram shows that the patient has sensorineural hearing loss (SNHL) because the hearing pattern of air conduction is close to that of the bone conduction.

3.2. Molecular modelling of KCNQ4

A series of molecular modelling was conducted by the following procedures.

- 1. PDBsum:** To find structural relatives of KCNQ4 for molecular modelling, we utilized PDBsum [27] and Gapped BLAST [28] in an attempt to search for the protein that is most homologous in amino acid sequence to the full length of KCNQ4 and that has a macro-molecular structure deposited in the Protein Data Bank.
- 2. Swiss-Model:** Among the channels with available three-dimensional structures, the sequence of Kv1.2 was most similar to that of KCNQ4 (27.5% identity for the transmembrane sequence Ser32 to Thr417). The transmembrane domains (S1–S6) including the voltage sensor and the pore region of KCNQ4 (Tyr80 to Gln329) and its Tyr270His and p.Ser260del mutants were modelled using the fully automated protein structure modelling server Swiss-Model [29-31] and the crystal structure of Kv1.2 (PDB ID: 3LUT, chain B) as a template [17]. The structural model of KCNQ4 was calculated by automatic modelling mode with default conditions.
- 3. Verify_3D Structure Evaluation Server:** The quality of the structural model was evaluated by Verify_3D [32-34] and was found to be trustworthy [23].

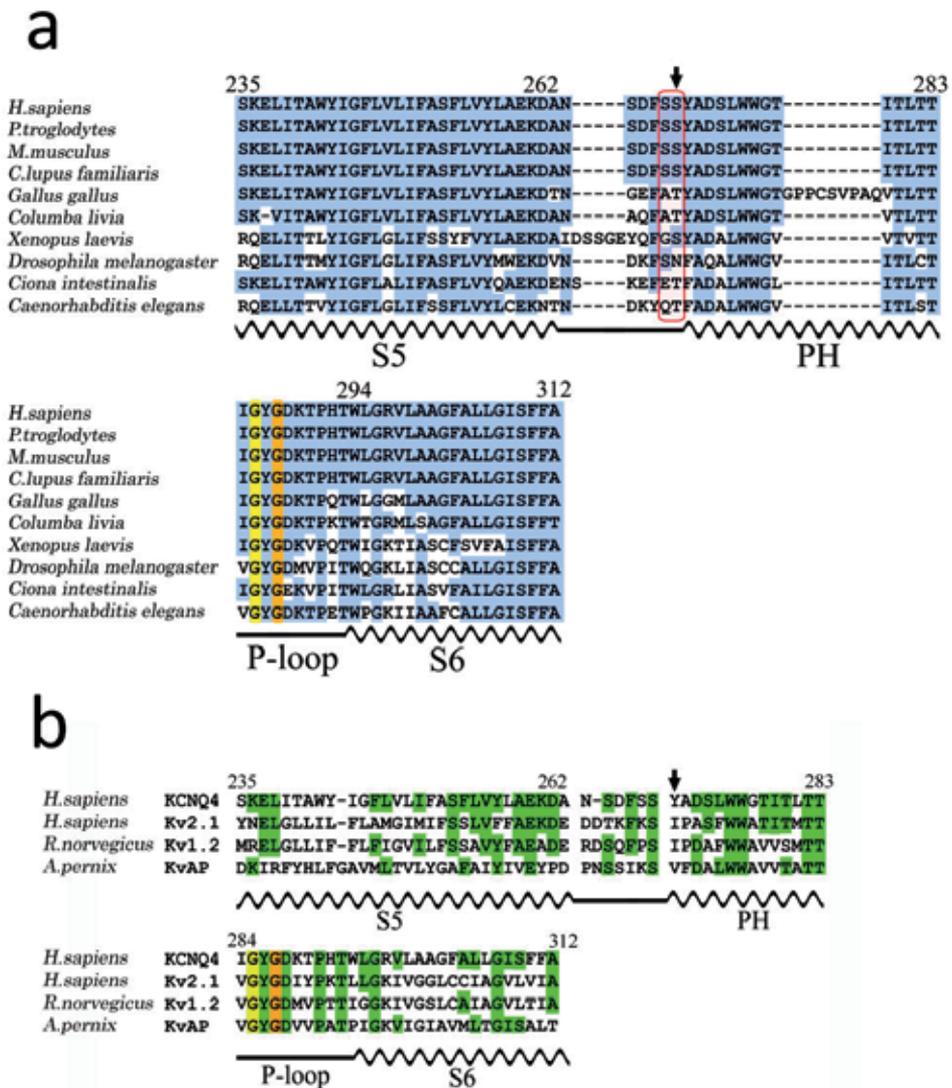


Figure 7. Sequence alignment of KCNQ4 proteins. (a) Sequence alignment of the orthologous KCNQ4 pore region. Positions highlighted in light blue indicate that the amino acid is identical to that in human KCNQ4. The position of Ser269 and Tyr270 is boxed in red and Tyr270 is indicated by an arrow. The positions of Gly285 and Gly287 are highlighted in yellow and orange, respectively. The positions of S5, PH, S6 (wavy lines) and the P-loop (straight line) are indicated below the sequences. (b) Alignment of the KCNQ4 pore-region sequence with those of Kv channels deposited in the PDB. Positions highlighted in green indicate that the amino acid is identical to that in human KCNQ4. The sequence of human KCNQ4 is indicated at the top of both alignments.

- UCSF Chimera:** The structural models of KCNQ4 and the Tyr270His, p.Ser260del, p.G285S and p.G287R mutants were fitted into the corresponding transmembrane domains of the Kv1.2 structure and UCSF Chimera [35] was used to visualize the putative structure by α -carbon frame or by ribbon model with electric surface potentials.

4. Results and discussion

To date, 21 missense mutations, a splice-site mutation and four small deletion mutations in KCNQ4 have been reported to be associated with hearing loss [36]. Eleven of the missense mutations are located in the pore region; thus, these mutations are considered to be pathogenic [14, 21, 23, 24, 37, 38]. As mentioned above, KCNQ4 contains six transmembrane α -helices, (S1–S6), a pore helix (PH), a pore-loop (P-loop), a short N-terminal region and a long C-terminal region (Figure 4). The pore regions of Kv channels are formed by contiguous S5, PH, P-loop and S6 regions. Functional and structural characterizations of Kv channels have demonstrated that the PH and P-loop are responsible for selective K⁺ transport, whereas S4, acting as a voltage sensor, regulates the open–closed state of the channel [17, 18]. Tyr270 and Ser269 are predicted to be the N-terminal residues of the PH.

Among the mammalian KCNQ4 sequences that are available, the pore-region sequences are identical and those of non-mammals (e.g., birds, fruit flies, tunicates and nematodes) are very similar to the mammalian sequences (Figure 7a). In addition, the PH and P-loop sequences of non-KCNQ4 Kv channels are somewhat conserved (e.g., Kv2.1 from *Homo sapiens* is identical in 14/24 amino acid residues, Kv1.2 from *Rattus norvegicus* in 11/24 residues and KvAP, a prokaryotic Kv channel from *Aeropyrum pernix*, in 13/24 residues). In addition, the positions of Gly285 and Gly287 on the P-loop are fully conserved across mammalian and non-mammalian KCNQ4 sequences. These commonly mutated residues are thought to be important for proper functioning of the channel (Figure 7a).

Tyr270, at the N-terminus of PH, is located at the same position as the corresponding Kv1.2 residue (Ile361) (Figure 7b). Gly285 and Gly287 are also fully conserved in Kv channels. We therefore assessed the electrostatic characteristics of structural models containing mutations of these residues to determine whether these residues might be responsible for the patient's hearing loss.

4.1. p.Y270H mutation

The ribbon model of wild-type KCNQ4 with the electrostatic surface potential superimposed suggests that the side chain of Tyr270 (coloured white in Figure 8a) should be electrostatically neutral. For Tyr270His, by contrast, the side chain of His270 (coloured blue in Figure 8b) is predicted to retain at least a partial positive charge, which reflects the standard pK_a value (6.5) for histidine.

Moreover, His270 is surrounded by the negatively charged residues Asp272, Asp266, Asp262 and Glu260, as well as the polar residues Ser269, Ser268 and Ser265, which are capable of hydrogen bonding (all of these residues are within 10 Å of His270, Figure 8c). In haemoglobin (PDB ID: 2hhb), the side-chain pK_a of His97, which is surrounded by two negatively-charged asparagine residues within five Å distance, is shifted to an abnormally high value ($pK_a=7.8$) [39]. For these reasons, the side chain of His270 may also have a pK value that is greater than the standard value; therefore, it is predicted that more than half of the histidine side chains at position 270 carry a positive charge at near physiological pH (Figure 8b).

Alteration of the electrostatic surface potential of a single helical residue in the pore region might affect the structural stability of the channel as a consequence of a change in the helix dipole moment. A substantial dipole moment with positive and negative unit charges at, respectively, the N- and C-terminus of an α -helix is often found (Hol, 1985). A comparison of three Kv channel structures suggests that the dipoles of the transmembrane helices orient the helices and are responsible, at least in part, for the structure of the pore [15, 40, 41]. The electrically neutral Tyr270, which, as noted above, is positioned at the N-terminus of the PH, is located within 9 Å of Ala263 at the C-terminus of S5 (Figure 8a, b). The presence of a positively-charged histidine residue adjacent to, or at the N-terminal of, an α -helix can destabilize the helix dipole [42]. Replacement of Tyr270 with histidine may therefore increase the dipole moment of the PH.

Alternatively, or in conjunction with the destabilization effect, His270 may impede K⁺ transport. The electrostatic repulsion between positively-charged His270 and K⁺ would be stronger than that between electrically-neutral Tyr270 and K⁺. In the p.Y270H model, the distance between His270 and the centre pore of the channel is ~20.5 Å (Figure 8d, e). This distance is small enough to affect the electrostatic interaction between two charged molecules in a non-polar environment (e.g., the interior of a membrane protein) because the electrostatic force is strong and affects charges separated by 500–1800 Å [43–45]. In addition, the dielectric constants in the interior of a protein and in a lipid membrane are assumed to be five and two, respectively, whereas that of bulk water is ~80 at room temperature [46], a value that can be elicited by basic Coulomb's law (see Formula). The electrostatic potential energy for two charges in protein or lipid membrane interiors is therefore between 16 and 40 times greater than that in water. Consequently, the long-range electrostatic repulsive force between positively charged His270 and a K⁺ ion might possibly impede passage of K⁺ through the channel [47], whereas the force on K⁺ would be smaller in the extracellular region.

Several mutations in the PH of KCNQ4 (e.g., Leu274His and Trp276Ser) have been correlated with hearing loss [21]. Interestingly, ectopic expression of both the Trp276Ser mutant and wild-type KCNQ4 in a cultured cell line caused a reduction in the channel current, whereas expression of the mutant alone caused impaired trafficking of the protein to the cellular membrane [48]. The similar clinical symptoms and locations of the mutations within KCNQ4 support the proposal that p.Y270H is a pathogenic mutation associated with progressive SNHL. Further physiological and three-dimensional structural characterization of the p.Y270H KCNQ4 protein may identify which of our working hypotheses—namely, structural distortion of the channel caused by a change in the dipole moment of the PH or electrostatic impediment of K⁺ transport (or both)—causes hearing loss in the patient and may provide insight into how to reverse hearing loss caused by KCNQ4 mutations.

Formula: (1) The electrostatic repulsive interactions in the pore region can be explained by classic Coulomb's law. Tyr270 in KCNQ4 is neutrally charged, whereas His270 in the Tyr270His mutation is predicted to be positively charged (at physiological pH 7.4). Thus, extra electrostatic repulsion force (q) is generated between His270 and K⁺, which is more intense than that between Tyr270 and K⁺.

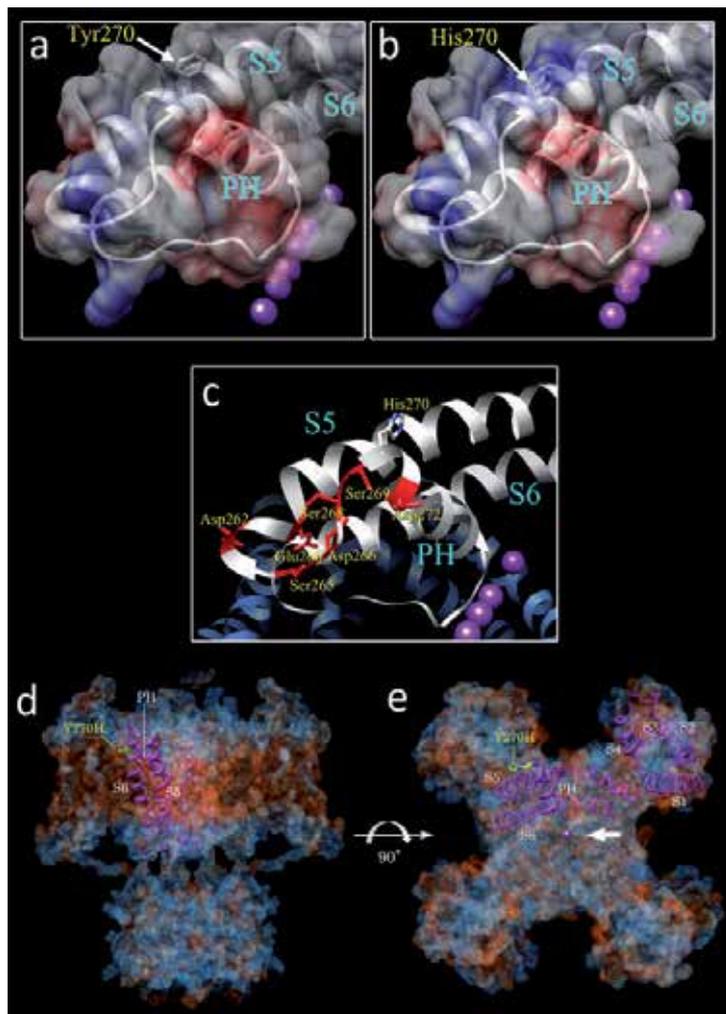


Figure 8. Structural models of KCNQ4 and the Tyr270His mutant. (a, b) Part of the wild-type KCNQ4 model (a) and the Tyr270His model (b) overlaid with their corresponding electrostatic surface potentials. Arrows indicate the side chains of Tyr270 (a) and His270 (b). Blue, red and white indicate positively-charged, negatively-charged and neutral atoms, respectively. (c) Ribbon model of the Tyr270His pore region. Residues surrounding His270 that are negatively-charged or that can form a hydrogen bond are shown in red. (d, e) Superimposition of the Tyr270His ribbon model onto a horizontal view of the plasma membrane (d) and an extracellular view (e) with the four rotational axes of the Kv1.2 crystal structure shown as a hydrophobicity surface. Hydrophobic and hydrophilic residues are indicated in red and blue, respectively, in the hydrophobicity surface representations. His270 is shown in yellow. K⁺ in the central pore is shown as a purple sphere in a–e and indicated by an arrow in e.

$$\begin{aligned}
 q_Y q_{K^+} &\approx 0 \\
 q_H q_{k^+} &> 0 \\
 q_H q_{K^+} &> q_Y q_{K^+}
 \end{aligned}
 \tag{1}$$

(2) The electrostatic potential energy (E) between His270 and K^+ is affected by the dielectric constant (ϵ).

$$E = \sum_{i < j} \frac{q_i q_j}{4\pi\epsilon r_{ij}} \quad (2)$$

(3) Assuming that the K^+ ion is within the pore region of the K^+ channel, the ϵ value is 5, whereas in water, the ϵ value is 80. Therefore, the electric repulsive force would be much more intense between His270 and K^+ within the pore region of the K^+ channel than in the extracellular space.

$$E_{pore} = \sum_{i < j} \frac{q_H q_{K^+}}{4\pi 5 r_{ij}} \gg E_{water} = \sum_{i < j} \frac{q_H q_{K^+}}{4\pi 80 r_{ij}} \quad (3)$$

(4) The Van der Waals potential energy (V) between His270 and K^+ can be ignored because it is extremely weak as compared with the electrostatic force. This energy decays exponentially with the internuclear distance (r). A and B represent the repulsive and attractive constant, respectively.

$$V_{(r)} = \frac{A}{r^{12}} - \frac{B}{r^6} \quad (4)$$

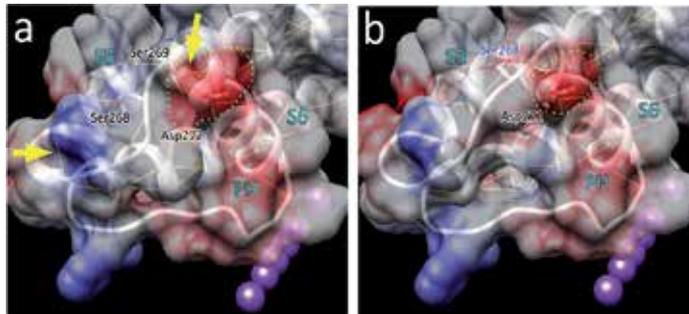


Figure 9. Partial structural model of KCNQ4 and the p.Ser269del mutation. (a, b) Ribbon models of the wild-type KCNQ4 subunit (a) and the KCNQ4 subunit with the p.Ser269del mutation (b) overlaid with their corresponding electrostatic surface potential. Red or blue areas indicate negatively-or positively-charged residues, respectively; yellow dots indicate the negatively-charged surface potential on the N-terminal region of the PH; pale yellow lines indicate hydrogen bonds and yellow arrows indicate hydrogen bonds within S5 and PH.

4.2. p.Ser269del mutation

The ribbon model of the wild-type KCNQ4 subunit overlaid with the corresponding electrostatic surface potential demonstrates that the surface of the N-terminal region of the PH is

negatively charged owing to the negatively-charged side chains of Ser269 and Asp272 (Figure 9a). The model of KCNQ4 with the p.Ser269del mutation demonstrates a reduction in the negatively-charged surface area in this region (Figure 9b). In case of the p.Y270H mutation, reducing the electrostatic surface potential in this area is predicted to impede K⁺transport owing to the long-range electrostatic force between the PH and K⁺(Formula).

In addition, hydrogen bonds at the C-terminus of S5 and the N-terminus of the PH of wild-type KCNQ4 (Figure 9a, yellow arrows) are absent in Ser269del KCNQ4 (Figure 9b). Loss of the hydrogen bonds around the N-terminus of the PH results in shortening of the PH and has been attributed to the destabilization of α -helix formation, resulting in a change in the helix dipole moment [39]. A change in dipole moment in this case might also destabilize the pore region structure, which is also likely to impede K⁺transport. Overall, the molecular impairment is likely to be a mild dominant-negative effect, resulting from the relatively small influence of the p.Ser269del mutation on the normal KCNQ4 channel subunit.

4.3. p.G285S and p.G287R mutations

Of particular importance in the P-loop are the three amino acids GYG, which are highly conserved in the Kv channel family (Figure 7b). The structure derived from molecular modelling reveals that the GYG residues (Gly285 and Gly287) bind directly to K⁺ and this electric static level is an integral part in the normal function of K⁺ penetration. In the p.G285S mutant structure, the electrostatic value of glycine is very similar to that of serine; however, the side chain of serine is polarized (Figure 10a, b: yellow arrow). This side chain protrusion of serine changes the normal structure of the central route of K⁺, providing decisive evidence of the impediment of K⁺ transport. In support of this, the current measured by electrophysiological examination in *Xenopus* oocytes was markedly reduced by the dominant-negative effect of p.G285S as compared with wild type [14].

The pK_a value for Arg (12.48) is positively charged in normal periplasmic environment and generates a strong repulsive force between Arg287 and K⁺ (Figure 10c). In addition, the side chain of Arg287 protrudes into the K⁺ position, resulting in complete blockage of the passage of K⁺. Thus, the effect of the p.G287R mutation is much stronger than that of the p.G287S mutation, from the structural analysis data. However, the hearing levels and patterns of patients with the p.G285S and p.G287R mutations are very similar [14, 25].

At the genetic level, KCNQ4 channels with missense mutations are predicted to act via a dominant-negative mechanism to induce progressive, predominantly high-frequency hearing loss [14]. In families with deletions that lead to frameshifts and stop codons, however, the phenotype is characterized by better low-frequency deterioration, but more rapid high-frequency deterioration [22, 49]. In addition, it is thought that truncating mutations such as p.Gln71SerfsX138 and p.Gln71fs on heterozygous alleles are probably not synthesized from these alleles and functional impairment is considered to be due to haploinsufficiency [24, 37, 50]. Missense mutations are usually translated into protein, whereas nonsense and large truncating mutations are not. For a heteromeric missense mutation of KCNQ4, incomplete tetramers might be generated; however, the functional structure of KCNQ4 is a tetramer of protein subunits. If one mutated subunit is integrated in the tetramer, the function of the K

*transportation will be lost, even though three subunits are still normal. Thus, a dominant-negative effect occurs. In the case of haploinsufficiency, a normal tetramer of KCNQ4 will be expressed, although the allelic expression is 50% of the normal value. These possibilities indicate that differences in hearing patterns and levels might occur between missense and nonsense (or large deletion) mutations.

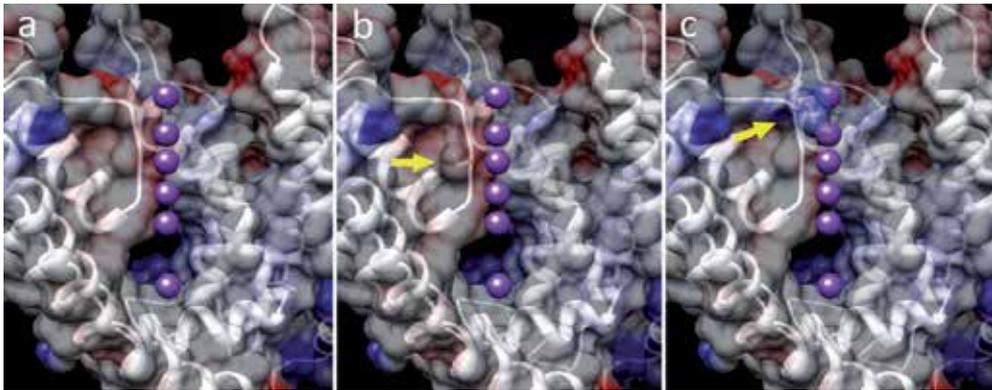


Figure 10. Structural model of the centre pore region of KCNQ4. The wild-type KCNQ4 (a), the p.G285S mutation (b) and the p.G287R mutation (c) models are overlaid with their corresponding electrostatic surface potentials. The side chains of Ser285 (b) and Arg287 (c) are indicated by arrows. The periplasmic region is at the top.

5. Conclusion

From our structural analysis, KCNQ4 is a transporter that has been refined during protein evolution. To develop molecular drugs targeted at, for example, the pore region of KCNQ4, it is necessary to take a special approach other than the normal screening of seed compounds as candidate inhibitors of a pathogenesis factor. Considering the molecular interpretation described in this chapter, it seems that there are two approaches to the development of drugs that might act on mutations in the P-loop in a target. The first is a drug that acts on the P-loop directly. However, it is presumed that the development of drugs directly targeting the P-loop would be somewhat difficult because the P-loop is hypersusceptible and there is no space in the centre pore for K^+ passage. The second and ideal approach is to screen for compounds that act on mutations on the P-loop at long range, taking into consideration our p.Y270H mutation model. It is hoped that such a drug that can control the penetration of ions from a remote position might be developed in future studies. In this chapter, we have described a case of structural impairment caused by KCNQ4 mutation. Elucidation of the impairment mechanism underlying missense mutations and the strategy of drug design will be applicable to the development of therapies for many genetic illnesses.

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Effect of Vitamins, Amino Acids and Phyto-Active Biomolecules on *Aspergillus flavus* in Poultry Production

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Additional information is available at the end of the chapter

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1. Introduction

Aspergillus is a genus of fungus consisting of several hundred mould species found in various climates world-wide. Taxonomically, they belong to kingdom Fungi, phylum Ascomycota, class Eurotiomycetes, order Eurotiales, family Trichocomaceae and genus *Aspergillus*. There are several hundreds of *Aspergillus* species, including *Aspergillus aculeatus*, *Aspergillus candidus*, *Aspergillus clavatus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus ustus*, and *Aspergillus tamari*. [1]

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as moulds on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates such as monosaccharides (e.g., glucose) and polysaccharides (e.g., amylose). *Aspergillus* species are common contaminants of starchy foods and grow in or on many plants and trees. In addition to their growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy, whereby they are capable of growing in nutrients-depleted environments or environments in which there is a complete lack of key nutrients. *A. niger* gives a prime example of this oligotrophic tendency, as it is found growing on damp walls as a major component of mildew. Species of *Aspergillus* are important medically and commercially. Some species are known to cause infection in humans and other animals [2].

Aspergillus flavus is a major food-borne pathogen that produces aflatoxin, a toxin that is carcinogenic [3]. It is a leading cause of aflatoxicosis in poultry. Aflatoxicosis results from ingestion of aflatoxin in contaminated feed. Effects of the aflatoxicosis include toxicosis and

immunosuppression [4]. The toxins released during *Aspergillus* infection depress production parameters and, specifically, cause impaired growth in poultry, while the immunosuppressive effect predisposes the animals to many secondary infections from other pathogens, such as fungi, bacteria and viruses. The consumption of a mycotoxin-contaminated diet by broilers has been reported to induce haematological, biochemical and liver changes. Other documented effects include growth depression, economic losses, increased mortality, decrease blood cell count, lower egg production, lower feed consumption, reduced resistance to infectious disease and vaccination efficiency, gross and microscopic changes in the liver and other organs, such as hepatomegaly, paleness, and hydropic degeneration. Fatty changes in the adipocytes, bile duct, hyperplasia and periportal fibrosis are other effects of aflatoxicosis [5]. Depletion of lymphoid organs such as the thymus and bursa of fabricius [6], kidney and spleen lesion [9] unfavourable reproductive changes [10], impairment of the humoral and cellular immune response [9] are common symptoms of aflatoxin ingestion in poultry and other livestock. Another possible effect of aspergillosis is the possible transmission of fungal mycotoxin residues to meat and eggs from infected chickens, which is potentially hazardous to public health.

Heterocyclic metabolites of the genera *Aspergillus* are aflatoxin and ochratoxin. It has been reported that both lower and higher doses of AFB₁ affect the haematological parameters of broiler chicks. This observation is closely linked to depressed cellular immunity due to suppression of the phagocytic activity of macrophages and decrease in T-lymphocyte activities [10]. Liver damage and temporary dysfunction may further predispose to deficiency of humoral immunity.

Aflatoxins have been known to be toxic and reported to cause immune suppression in birds [11]. These immune suppression effects of aflatoxins predispose the animal to many secondary infections due to other fungi bacteria and viruses [12]. Earlier reports [6] opined that contamination of broiler ration with aflatoxin resulted in a drastic reduction in performance both from a growth and a feed-efficiency standpoint. The aflatoxin-producing fungus, *Aspergillus flavus*, is a causal agent of pre-harvested contamination of food commodities, which can result in serious economic hardship for producers and an adverse health impact on both humans and domestic animals. The liver has been reported as the primary target organ of aflatoxin in most animals and humans, where Aflatoxins B₁ is metabolized to the toxic and carcinogenic aflatoxins B₁-epoxies are formed by cytochrome P450 enzymes [13]. The disease produces a hard nodular area in the lungs and infection of the air sacs. Sometimes the air sac lesions are similar to that produced by infectious sinusitis or CRD. In some birds, colonies of mould growth can be seen on the air sac membrane [14]. The mode of transmission involves contact with the organisms through contaminated feed, litter or premises (formites). The disease is not contagious and does not spread vertically. Most healthy birds can withstand repeated exposure to *Aspergillus*. Inhalation of large amounts of the infectious form of the mould leads to reduced disease resistance of the bird. In the acute form of aflatoxicosis in young birds, the main symptoms include gasping, sleepiness, loss of appetite and sometimes convulsions and death (brooder pneumonia). Occasionally, CNS signs may become apparent in the brain, causing paralysis or other forms of nervous symptoms. The more chronic form in older birds

usually results in loss of appetite, gasping or coughing, and a rapid loss of body weight. Mortality is usually low and only a few birds are affected at one time.

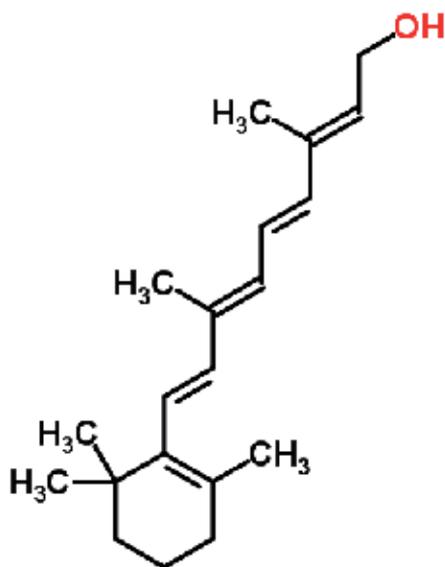
In broilers, a dose of 1.5 ppm of aflatoxin has been shown to impair bile salt availability, which causes a decrease in the absorption of fat soluble vitamins. In poultry, aflatoxicosis is characterized by restlessness, anorexia with decreased growth rate, poor nutrient utilization, decreased weight gain, decreased egg weight and production, increased susceptibility to environment and microbial stresses, and increased mortality. Post-mortem signs include yellowish caseous and hard nodular deposits in the infected air sacs. Sometimes the air sac lesions are similar to those produced by sinusitic or CRD infections. In some birds, colonies of mould growth can be seen on the air sac membrane (Figure 1).

Providing a diet containing high fat and high protein levels and augmenting the ration with vitamin supplements may be of value in mitigating the effects of *Aspergillus* infection [4]. In pigs, treatments with vitamins and protein supplementation have been shown to have some proactive effects [15]. Vitamins are an essential component of a well-balanced diet, and supplementation is aimed at optimizing the immune response in chickens [16]. Vitamin A is essential for the integrity of epithelial tissues, which represent a major defence against the entry of pathogens [16]. Vitamin A is a fat-soluble vitamin naturally occurring in plant and animal sources; these sources constitute the major forms in which the vitamin exists. In plants, the major form of vitamin A exists as a precursor (or provitamin) carotene, which can be converted to vitamin A during intraluminal absorption. The major sources of plant vitamin A are green vegetables, carrot, pawpaw, red palm oil, etc. In these sources, vitamin A exists as various types of carotene with varying vitamin A activities, e.g., all-trans- β -carotene, neo- β -carotene, γ -carotene and all-trans- α -carotene. Of all the feed ingredients used in animal production, only yellow maize has an ample amount of provitamin A. Vitamin A activity of carotenes is species dependent. In pigs, 5 μg β -carotene is equivalent to 1 μg retinol or vitamin A activity, while in poultry the ratio is 2:1 of β -carotene to retinol or vitamin A activity.

RE = 1 mg all-trans retinol
= 6 mg all-trans b-carotene
= 12 mg other biologically active carotenoids
= 3.33 IU retinol
= 10.0 IU carotene

Vitamin A in food is found as retinol or as carotenes. Retinol is found exclusively in animal foods including eggs, milk, and milk products [18]. With the exception of fowl, meat products, including beef and pork, do not contain significant quantities of preformed vitamin A.

Carotenoids are found primarily in plant foods, whereas meats, fats, and dairy products are reportedly low in carotenoid content [18]. The richest known sources of provitamin A are palm oils. Red palm oil, a common cooking product in West Africa, is usually cited as having the highest concentration of provitamin A activity [19]. Vitamin A in the form of retinoic acid



Retinol source: [17]

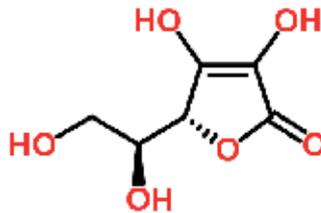
(tretinoin) has been reported to prevent acute promyelocytic leukaemia (APL) through the induction of terminal differentiation (anti-cancer), in which the leukaemic promyelocytes lose their ability to proliferate. It has also been reported to stabilize lysosomes, increase ribonucleic acid polymerase activity, increase prostaglandin F_2 cAMP, and cGMP levels, and increase the incorporation of thymidine into DNA [18].

In poultry, deficiency of vitamin A is manifested as impaired vision due to hyperkeratinization of the epithelial cells of the eye, drying of the cornea (xerosis) and irreversible drying of the cornea as a result of corneal hyperkeratinization and degeneration leading to blindness (keratomalacia). The impact of vitamin A deficiency on poultry productivity is linked to the use of sight for food seeking and consequently voluntary feed intake, as earlier itemized. Other deficiency symptoms are follicular keratosis observed in ruffled feathers, calcification of kidney lining, decreased bone growth, and central nervous syndrome (CNS) observed as paresis, unstable gait, etc. Deficiency of vitamin A also impacts negatively on poultry immunity by depressing cell-mediated immunity (CMI). In laying hens, early signs of deficiency are noticeable on epithelial tissues. Other consequences include eye conditions (xerophthalmia), predisposition to disease conditions, pale bird syndrome (PBS), renal dysfunction, ocular and nasal discharges, and reduction in egg production. In chicks, symptoms of vitamin A deficiency include post-hatch mortality, ataxia, poor growth and feathering. Vitamin A deficiency in neonate chicks may increase early embryonic mortality and failure to develop a neonate circulatory system [20].

Vitamin C (ascorbic acid) is a water-soluble vitamin, which is needed by the body to form collagen in bones, cartilage, muscle, and blood vessels, and which aids in the absorption of iron. Dietary sources of vitamin C include fruits and vegetables, particularly citrus fruits such

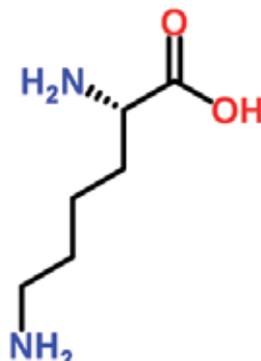
as oranges. Epidemiologic evidence suggests a role for vitamin C in hindering the development of cancer and heart disease, as well as a number of other diseases. Studies on CVD risk factors indicate that vitamin C may moderately decrease total serum cholesterol levels, increase HDL levels, and exert a hypotensive effect [21,22]. Chronic latent vitamin C deficiency leads to hypercholesterolaemia and the accumulation of cholesterol in certain tissues. Ascorbic acid supplementation of the diet of hypercholesterolaemic humans and animals generally results in a significant reduction in plasma cholesterol concentration [23]. Severe deficiency of vitamin C causes scurvy. Although rare, scurvy includes potentially severe consequences and can cause sudden death.

Vitamin C (ascorbic acid) has been reported as a non-essential nutrient for poultry, since birds are capable of synthesizing enough of the vitamin endogenously. This synthesis is attributed to the endogenous enzyme gulonolactone oxidase [24]. Studies have shown that exogenous ascorbic acid given in feed or drinking water or by injection improved performance of chickens during heat stress [25,26].

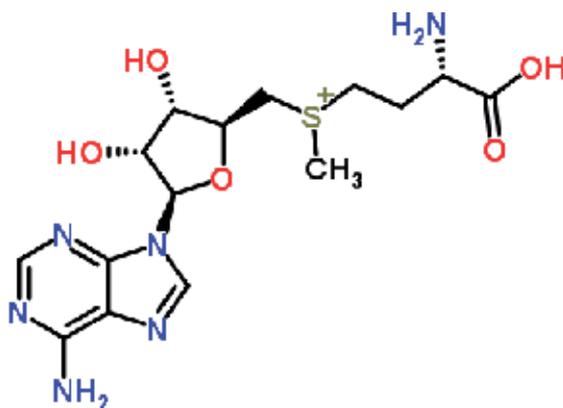


Vitamin C source: [17]

In *in vitro* testing, liquid methionine hydroxyl analogue has been observed to have an inhibiting effect on *Aspergillus flavus* [27]. According to [28], an approximate inclusion of 1.33% and 0.52% lysine and methionine, respectively, in diets of broilers subjected to aflatoxin-contaminated feed can give maximum performance.



Lysine source [17]



Methionine source: [17]

Feed refusal has also been reported to be a rapid and direct response to the presence of aflatoxin [29]. In an earlier report, contamination of broiler rations with aflatoxin resulted in a drastic reduction in performance both from a growth and a feed-efficiency standpoint [28]. Unfortunately, there is no treatment for aspergillosis once established in the flock. The common practice is to administer antibiotics to prevent secondary infections, while nutrient supplements are given *ad lib* to enhance tissue rejuvenation so as to hasten recovery. As a practical precautionary measure, sulpha drugs are administered on-farm to prevent *Aspergillus* infection [14].

Bark infusions of shea butter have medicinal and antimicrobial properties, e.g., against dysentery. They are applied in trado-medical practice as eyewash to counteract spitting-cobra venom. Shea butter is a suitable base for many medicines: its application relieves rheumatic and joint pains, and heals wounds, swellings, dermatitis, bruises and other skin problems. It is used traditionally to relieve inflammation of the nostrils. It is also administered to horses for the treatment of sores and galls. Extracts of the bark of *Vitellaria paradoxa* have been reported to possess antifungal properties against *Aspergillus niger*, *Aspergillus flavus*, *Epidermophyton floccosum*, *Microsporum audouinii* and *Trichophyton mentagrophytes* [31].

Gallic acid (trihydroxybenzoic acid or 3, 4, 5-trihydroxybenzoic acid) is a biologically active phenolic compound. It has been reported to show antioxidant and antimicrobial activities. It exists as free molecules or as part of tannin. Gallic acid is a trihydroxybenzoic acid, a type of organic acid. It is a colourless, crystalline organic powder. It is found in almost all plants. The chemical formula is $C_6H_2(OH)_3COOH$ or $C_7H_6O_5$, and the molecular weight is 170.12. Salts and esters of gallic acid are termed "Galletes". Despite its name, it does not contain gallium. Gallic acid is commonly used in the pharmaceutical industry. It is used in the synthesis of the psychedelic alkaloid mescaline as a starting material. It is used as a standard for determining the phenolic content of various analytes in the Folin-Ciocalteu assay; results are reported in gallic acid equivalents. It seems to have anti-fungal and anti-viral properties. Gallic acid acts as an antioxidant and it helps to protect human cells against oxidative damage. Gallic acid extracted from grape may also benefit diabetes patients by triggering the release of insulin by

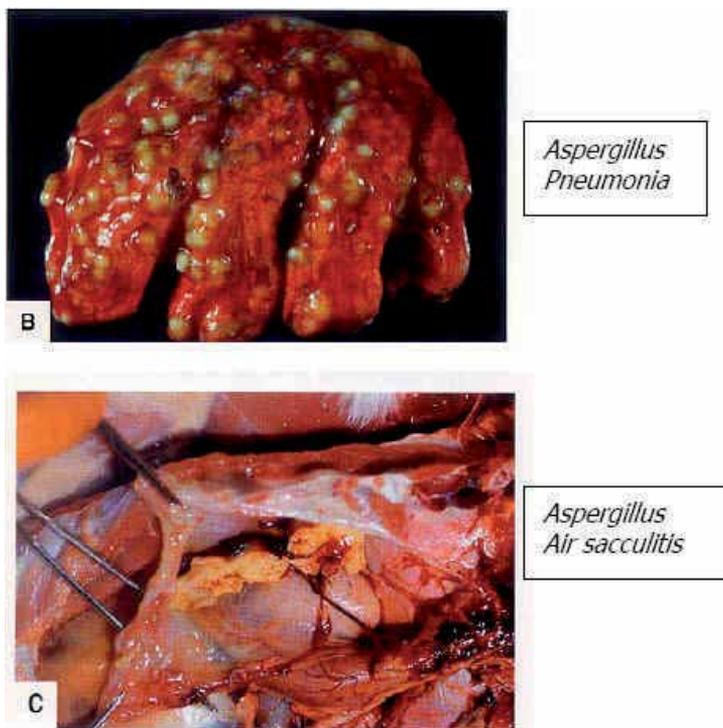
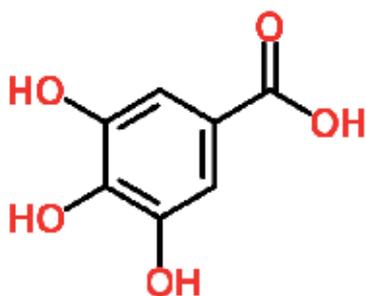


Figure 1. *Aspergillus* infection of the lung and air sacs [30]

the pancreatic cells. It exists in plant material in the form of free acids, esters, catechin derivatives and hydrolysable tannins. This ubiquitous chemical is one of the most biologically active phenolic compounds of plant origin. Antioxidant activity of gallic acid and its derivatives has been reported in several studies. Gallic acid has been shown to possess antimicrobial activity against human pathogens (*Staphylococcus aureus*, *Corynebacterium maccolans*) and plant pathogens (*Candida albicans*). The antifungal activity of gallic acid, isolated from *Oenothera biennis* roots, has been investigated [32]. Methylgallate has been demonstrated to show activity against a number of Gram-positive and Gram-negative bacteria and fungi. The cytotoxic effects of Triphala, an Indian herbal drug, on breast and prostate cancer cells have been attributed to gallic acid. Since gallic acid can act as a nucleophile, it can therefore scavenge electrophilic mutagens [33].

The poultry population of Nigeria is estimated at 140 million, the largest in Africa. The poultry industry is a major contributor to the economy. Production intensified steadily at a growth rate of 306.6% between 1999 and 2004 [34]. However, production within this sector is still below resource capacity. It is common to observe that, whereas broilers normally reach market weight at about six weeks in the West, in Nigeria market weights are rarely achieved before 10 weeks in intensive production. Mortality rates on a typical farm may also range between 10-15%, which further reduces farm profits [35]. Myriad factors have contributed to this underutilization of capacity in this sector, e.g., disease, lack of technological know-how, etc.



Gallic acid source [17]

Currently, the most common methods of suppressing pathogens in animals have been treatment with antibiotics as a therapeutic agent and use of growth promoters, because these are readily available. However, the use of antibiotics in the treatment of animal disease is currently a subject of public health concern, as the development of resistant strains (superbugs) is a potential danger to humans. Furthermore, some of these antibiotics have recently been found to exhibit neurotoxic effects, while some others cause severe liver damage and bone marrow depression. Antibiotics are used mainly to protect poultry from pathogenic organisms and to enhance their growth and health. However, the emergence of antibiotic resistance in pathogenic bacteria has led to international reconsideration of the use of antibiotics in livestock [36]. Recommendations to ban sub-therapeutic use of antibiotics in animal feeds have been documented. Antibiotic resistance has been displayed by *Escherichia coli* isolates from commercial turkey farms, including resistance to Enrofloxacin, one of the most recently approved antibiotics for use in poultry [37]. With the recent ban on the use of sub-therapeutic antibiotics in the production of livestock by the EU [38], research attention has shifted towards the development of positive alternatives.

The use of plants for medicinal purposes predates the introduction of antibiotics and other modern drugs, and there has been renewed interest in natural products from higher plants which contain active ingredients of medicinal value. Scarcity and sale of fake and adulterated pharmaceutical drugs, which has been on the increase especially in the developing world, has made ethnoveterinary approaches even more attractive. The studies presented here present some alternative strategies to manage aspergillosis in poultry.

2. Study 1

2.1. Vitamins and amino acids

Broiler chicks were challenged with *Aspergillus flavus* via drinking water at the age of two weeks. Yellowish caseous deposits in the lung were established as confirmatory lesions of aspergillosis in the challenged birds [39]. The experiment included positive (non-challenged birds) and negative (birds challenged without dietary supplementations) control groups.

Dietary interventions of *Aspergillus*-challenged birds included vitamins A and C (A+C), methionine and lysine (METH+LYS), and vitamins A and C, lysine and methionine (A+C+METH+YS), which were incorporated into the basal diet formulated to meet the nutrient requirement [24] for broilers (Table 1).

2.2. Materials and methods

Commercial broilers of 120 days of age were used in this study. The chicks were weighed and randomly allotted to five treatment groups with three replicates of 24 chicks each. Birds were housed in an electrically heated metabolic battery cage. Routine management and vaccination procedures were followed. Feed and water were administered *ad libitum* for the 56-day feeding trial. Feed intake and weight gain were recorded weekly and used to determine the feed-to-gain ratio. Nutrient retention was determined at four weeks of age. Proximate analysis of the diet and faecal samples were determined according to the method given in [40]. At the end of the experiment, nine birds were selected for the treatments, denied feed overnight and slaughtered by severing the jugular vein. Blood samples were collected and used for haematological and serological indices according to [41], using Wintrob's microhaemometer improved Neubauer counter. Data obtained from the experimental trial were analysed using the completely randomized design [42]. Significant differences were subjected to the Duncan Multiple Range Test [43] at 0.05 probability.

Daily feed intake and feed conversion efficiency were influenced by the treatments (Table 1). The highest feed intake was observed for *Aspergillus*-challenged birds supplemented with A+C+METH+LYS, which compared favourably with the positive control birds. The lowest feed intake was observed for the negative control birds. Daily weight gain varied in response to dietary interventions of the challenged birds. The trend also followed the observation for feed consumption. Feed conversion efficiency was poorest for the negative control birds. Dietary interventions may have positively reduced the deleterious effects of aspergillosis on the performance of broilers. This effect was particularly pronounced for the *Aspergillus*-challenged broilers fed A+C+METH+LYS. It is also noteworthy that the performance of birds fed the supplemental combination A+C+METH+LYS compared favourably with the positive control groups. This diet may have stimulated the immune response of the broilers and thus enhanced their performance. According to [15], vitamins and protein supplementation in pigs has some proactive effect on the incidence of aflatoxicosis. In the same vein, [2] reported that providing a diet containing high protein and augmenting the ration with vitamin supplementation may be of value in mitigating the effects of *Aspergillus* infection. In the same vein, [42] reported the inability of vitamin supplements alone to totally prevent the negative effect of mycotoxin in broiler chicks.

The results of this study suggest that dietary vitamins A and C together with an increase in lysine and methionine can enhance the feed intake weight gain and feed conversion efficiency of broiler chickens infected with *Aspergillus flavus*. Thus, a combination of vitamin and protein supplementation may be an attractive alternative to on-farm use of vaccines. It may also serve as a contribution to the effective management of aspergillosis in poultry, since curative drugs have not been found to be effective in the control of the disease.

Diets	Feed intake	Weight gain (g/ bird/day)	Feed conversion ficiency (%)	Protein retention (%)	Fat retention (g/bird/ day)
Control	42.48 ^a	20.14 ^a	2.11 ^b	63.30 ^a	73.5 ^a
Control*	38.67 ^b	12.48 ^c	3.09 ^a	51.4 ^b	63.9 ^c
Vit. A+C	40.97 ^b	15.88 ^b	2.58 ^b	60.6 ^a	68.9 ^b
LYS +METH	39.08 ^b	15.14 ^b	2.58 ^b	53.9 ^b	66.6 ^b
Vit. A+C+					
LYS + METH	42.81 ^a	18.37 ^a	2.33 ^b	61.1 ^a	72.0 ^a

a,b,c, values in the same column are similar ($p>0.05$)

* challenged broilers without dietary intervention

Table 1. Dietary intervention and performance of *Aspergillus*-challenged broiler chicks

3. Study 2

3.1. *Vitellaria paradoxa*

V. paradoxa bark was collected and air dried for a period of two weeks. The extracts were pre-crushed in a mortar and later pulverized into fine powder. Extraction was done using cold water as the extraction liquid. Extraction was done in a rotary orbital shaker at 60 rpm for 24 hours. The mixture was further filtered through a sterile 0.45 µm Millipore filter. The filtrates were evaporated to semi-solid mass and subsequently dried to give a dark brown resinous mass. The dry extracts were later concentrated using a rotary evaporator. These dried extracts were reconstituted for antimicrobial activity evaluation.

The spore was established by growing a plate of *Aspergillus flavus* on a culture medium, which was left for three to seven days to sporogate. The spores were later scraped off the surface of the culture plate. The treatments were: Group 1, control; Group 2, infected and treated with antibiotics; Group 3, infected but no treatment (negative control); Group 4, infected and treated with 5 mg/ml of extract; Group 5, infected and treated with 10 mg/ml of extract.

3.2. Materials and methods

3.2.1. Plant collection

The bark of the shea butter tree (*Vitellaria paradoxa*) was collected from a permanent site at the University of Ilorin. Ilorin is located at latitude 08 29'N and longitude 004 35'E. The elevation is 305 m 1001'. The annual temperature range is 22-34°C and the annual precipitation is 80-12 mm [44]. The plant was identified by experts from the University's Herbarium Unit. The bark was collected daily at 08:00 and air-dried to a constant weight. The samples were pre-crushed in a mortar, and then blended in an electric blender (Moulinex, Philips) to a fine particle (0.5 mm). 100 g of the sample was soaked in 500 ml of cold water for extraction. The mixture was

fitted to a rotary shaker and agitated at 60 rpm for four hours. The mixture was further filtered through a sterile 0.45 µm Millipore filter. The filtrates were evaporated to semi-solid mass and subsequently dried in a beaker on a water bath to give a dark brown resinous mass. The dry extracts were later concentrated using a rotatory evaporator (Model 349/2, Corning Limited) for the antimicrobial activity evaluation [45]. The extract was reconstituted to 5 mg/ml and 10 mg/ml and administered through drinking water.

3.2.2. Source of *A. flavus*

A. flavus spores were collected from the Department of Microbiology, University of Ilorin and grown on a plate on a culture of Potato Dextrose Agar (PDA), and incubated at 28°C for five to seven days. The spores were later scraped off the surface of the culture plate for inoculation.

3.3. Management of birds

Mixed-sex Hubbard broilers of 100 days of age were purchased from a commercial hatchery in Ilorin, Nigeria. The birds were brooded in an electrically heated metabolic cage and thereafter allotted randomly to five different treatments (Table 2). Each treatment was replicated in four pens containing five birds per replicate. The birds were given a basal diet (Table 1) and water *ad libitum* during the trial period. Routine vaccinations and medications were administered.

Ingredients	% Inclusion
Maize	37.0
Corn bran	6.0
Groundnut cake	24.0
Soyabean meal	24.0
Fishmeal	2.6
Bone meal	2.5
Oyster shell	1.5
Salt	0.2
*Vitamin/mineral premix	0.2
Total	100.0

Nutrient composition

Protein: 23.5%; Energy: 2700 kcal/kg;

*Vitamin mineral premix contains antioxidant 125 mg, biotin 80 mg, choline chloride 500 mg, cobalt 240 g, copper 6 mg, folic acid 1000 mg, iodine 1.4 mg, selenium 240 mg, vitamin A 15,000 IU, vitamin B₁ 200 mg, vitamin B₂ 600 mg, vitamin B₆ 400 mg, vitamin D₃ 3000 IU, vitamin E 3000 IU, vitamin K 250 mg, zinc 60 mg, vitamin B₁₂ 20 mg.

Table 2. Composition of experimental diet (%DM)

Treatment	Infected with <i>A. flavus</i>	Supplemented with <i>V. paradoxa</i>	Supplemented with antifungal (furaprol)	Remark
1	-	-	-	Positive control
2	+	-	-	Negative control
3	+	-	+	Antifungal
4	+	+	-	5 mg/ml
5	+	+	-	10 mg/ml

Table 3. Composition of experimental treatments

3.4. Inoculation of chick feed with *A. flavus* spores

At the second week, bird feeds (except the positive control) were inoculated with the spores of *Aspergillus flavus*. The infected birds were placed under close observation for three to seven days, within which they would have manifested infections. Confirmation of infection was established by caseous yellow deposits in the lung.

3.5. Data collection

The experiment was conducted over six weeks. Body weights of broilers were determined weekly. Feed consumption and weight gain were recorded and feed conversion ratio (feed intake/weight gain) was calculated. Mortality was recorded daily. During the third week of the eight-week study, protein and fat nutrient retention were carried out for 72 hours.

Nutrient retention was calculated as follows:

$$\text{Nutrient retention} = \frac{(\text{Nutrient consumed} - \text{Nutrient voided in faeces}) \times 100}{\text{Nutrient consumed}}$$

Items	Treatment groups				
	1	2	3	4	5
Av. Body weight gain (g/bird)	216.10 ^a	200.50 ^b	154.90 ^c	198.40 ^b	200.70 ^b
Feed intake (g/bird)	504.40 ^a	473.00 ^b	321.80 ^c	463.80 ^b	473.90 ^b
Feed:gain ratio	2.3 ^a	2.3 ^a	2.0 ^b	2.3 ^a	2.3 ^a

a,b,c values in the same column are similar (p>0.05)

Table 4. Effects of *Vitellaria paradoxa* on the performance of broiler chicks

The production parameters average body weight gain, feed intake and feed:gain ratio were enhanced at the various levels of *V. paradoxa* interventions (Table 2). The negative groups showed the least performance. It was observed that the 10 mg extract (T5) was the most effective dose against *A. flavus*. At this level, histological architecture of specific organs (ileum, liver and breast muscle) was preserved (Figure 2).

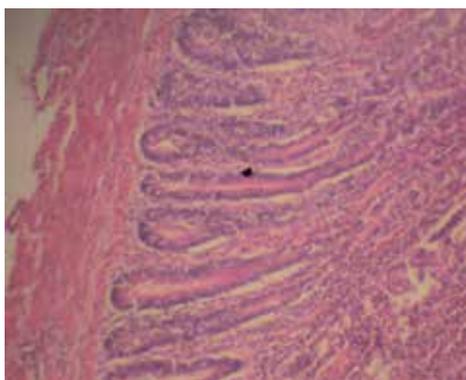


Figure 2. Micrographs of birds fed 10 mg extract of *V. paradoxa* showing normal ileal sections (X60)



Figure 3. Micrographs of birds fed the negative control showing deranged ileal section (X60)

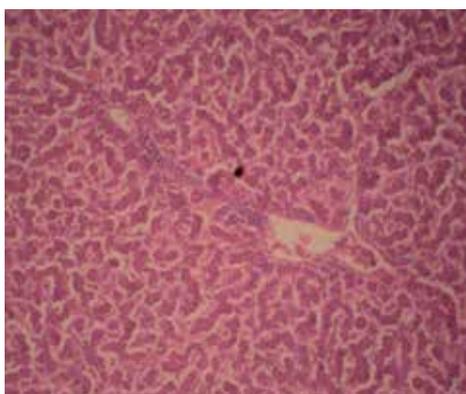


Figure 4. Micrographs of birds fed 10 mg extract of *V. paradoxa* showing normal liver sections(X40)



Figure 5. Micrographs of birds fed 10 mg extract of *V. paradoxa* showing abnormal liver sections (X60)

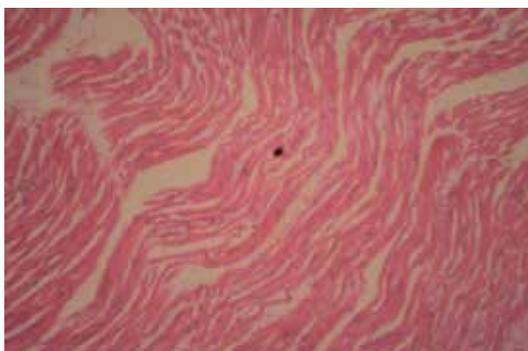


Figure 6. Micrographs of birds fed 10 mg extract of *V. paradoxa* showing normal breast muscles (X40)



Figure 7. Micrographs of birds fed 10 mg extract of *V. paradoxa* showing abnormal breast muscle sections (X60)

4. Study 3

4.1. Chloroform and butanol extracts of *Vitis vitifera* peel

The peeled rinds of *Vitis vitifera* were ground and mixed at 140 g/litre of water. Methanol was added and the mixture was decanted after 72 h and distilled. The distillates were further extracted using either chloroform or butanol. Gallic acid content of the extracts was separated with HPLC and concentrated. The concentration of gallic acid in the extract was determined. Pure gallic acid was dried out in a rotary evaporator. The extract was reconstituted and amended with the test extracts. The test fungus *Aspergillus fumigatus* was aseptically introduced into the growth medium and incubated at ambient temperature for seven days. The fungal growth was thereafter measured. Two controls (positive and negative) also constituted treatments. Chloroform and butanol extracts were administered to the inhibition test at 0, 50, 100, 150, 200 and 250 mg/ml

4.2. Materials and methods

4.2.1. Source of Vine Grape

The vine grape used for the experiment is found growing naturally around the Tanke area in Ilorin, Kwara State, Nigeria. The fruits were picked carefully, selecting grapes free of physical damage and microbial attack.

4.2.2. Preparation of crude extract of gallic acid

The fruits were peeled to remove the rind from the juicy part. The peels were ground manually using mortar and pestle. About 700 g of the ground peels was weighed into a 5 l container using an electronic balance. Two and half litres of methanol were added to the ground peel and left for 72 h to ferment. The sample was decanted into a flat-bottomed flask and distilled after the third day of fermentation using a water bath. Anti-bombing agent was added to the mixture during distillation to prevent bombing. The concentrate obtained was weighed.

For butanol extraction, a quantity of the concentrate obtained above (about 200 g) was mixed with 250 ml of butanol in a 500 ml conical flask. The mixture was shaken thoroughly manually for one hour and later allowed to settle in a separating funnel. The two liquids' layers were separated by gently running them off from the separating funnel. The butanol was distilled using a heater. This procedure was repeated for the chloroform extract but distillation was carried out using a water bath as the heating source. Phytochemical screening was carried out on the extracts to determine the presence of tannins, flavonoids and gallic acid.

4.2.3. Test of anti-fungal property of the extracts

The fungal culture used (*Aspergillus fumigatus*) was obtained from the Department of Microbiology's laboratory at the University of Ilorin. It was routinely sub-cultured for purity during storage on a Potato Dextrose Agar (PDA) slant, and stored at 4°C until required for use.

4.2.4. Reconstitution of the extract

The crude extracts were diluted with 12 ml of butanol and chloroform to obtain varying concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml, and 250 mg/ml. These were refrigerated until required for use.

4.2.5. Preparation of PDA and its amendment with the extracts

Thirty-nine [39] grams of PDA powder was dissolved in 1000 ml of sterile distilled water in a conical flask. The suspension was heated to homogenize it and the flask was plugged with cotton wool, wrapped with aluminium foil and autoclaved at 121°C for 15 minutes. The medium was amended with the extracts at the designated concentrations, i.e., 50 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml and 250 mg/ml. The control treatment was PDA only, without the extract.

4.2.6. Determination of the growth of the test fungus

The test fungus was aseptically introduced into the growth medium in petri dishes (9 cm diameter). The dishes were incubated at ambient temperature for seven days, after which growth was determined by measuring the diameter of the fungus following two perpendicular lines passing through the centre of the dish.

4.2.7. Results

The chloroform extract of *V. vitifera* peel had partial (26%) percentage inhibition on *A. fumigatus* (Table 5). The butanol extract of gallic acid was observed to completely suppress *A. fumigatus* growth, i.e., 100% inhibition (Figures 3-7) Gallic acid acts as an antioxidant and helps to protect human cells against oxidative damage. Gallic acid extracted from grapes may also benefit diabetes patients by triggering the release of insulin by the pancreatic cells. It exists in plant material in the form of free acids, esters, catechin derivatives and hydrolysable tannins.

Extract	Concentration mg/ml						% inhibition
	0	50	100	150	200	250	
Butanol	0.00	0.00	0.00	0.00	0.00	0.00	100
Chloroform	10.75	8.26	6.84	16.43	14.07	8.01	26

Table 5. Inhibition of *A. fumigatus* growth by the different gallic extracts



Plate 1. Control treatment



Plate 2. Negative control



Plate 3. Butanol extract (200 mg/ml) showing *Aspergillus* inhibition



Plate 4. Butanol extract (250 mg/ml) showing 100% inhibition



Plate 5. Chloroform extract (200 mg/ml) showing partial inhibition

5. Conclusion

Herbs and spices are known to exert antimicrobial actions *in vitro* against important pathogens including fungi [46]. The active substances are largely the same as those mentioned previously for antioxidative properties, with phenolic compounds being the principle active components [47].

The antimicrobial mode of action is considered to arise mainly from the potential of the hydrophobic essential oils to intrude into the bacterial cell membrane, disintegrate membrane structures, and cause ion leakage. High antibacterial activities are reported also from a variety of non-phenolic substances, for example, limonene and compounds from *Sanguinaria canadensis* [47]. Microbiological analysis of minimum inhibitory concentrations (MIC) of plant extracts from spices and herbs, as well as of pure active substances, revealed levels that considerably exceeded the dietary doses when used as phytogenic feed additives (Burt et al., 2004). This may indicate that antimicrobial action of phytogenics should not contribute significantly to the overall efficacy of this class of feed additives. On the other hand, some studies with broilers have demonstrated *in vivo* antimicrobial efficacy of essential oils against *E. coli* and *Clostridium perfringens* [48].

With the current emphasis on the use of alternatives to chemicals and antibiotics in the treatment of livestock diseases, potent materials of natural origin are becoming attractive. The strategies documented in this paper lend credence to the fact that livestock diseases can be

managed in a more robust manner than with non-biodegradable chemicals that are potentially a danger to public health. Generally, vitamins A and C combined with lysine and methionine act as an immune modulator, which can be adapted as an alternative to on-farm use of vaccines in poultry in the management of aspergillosis. Botanicals such as extracts of *Vitellaria paradoxa* and *Vitis vinifera* can be incorporated for robust control of aspergillosis in poultry production. Further studies on potential pharmacological, biochemical and physiological effects, for example in relation to safety limits, target organs of the active substances, side effects (especially on non-target organs), and so on, is imperative if the benefits of these botanicals are to be successfully harnessed.

Author details

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Immunopharmacology

Pharmacological and Immunological Properties of Wasp Venom

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Rakesh Kumar Shrestha and Gopi Aryal

Additional information is available at the end of the chapter

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1. Introduction

Animal toxin envenomations have medical as well as ecological significance. Toxin-producing animals are categorized under either venomous group or poisonous group. Venomous animals are capable of producing and delivering the toxin during a biting or stinging act whereas poisonous animals are those whose tissues, either in whole or in part, are toxic. [1] About 75% of the world's animal species are arthropods—a few of which have appreciable interaction with humans and is capable of causing significant medical problems. [2] Hymenopterous insects, snakes and spiders are the three animal groups most often responsible for human deaths attributable to venomous animals. [1] However, the evolution of venom in these animals has its own purpose of balancing the ecosystem and maintaining its position in the food chain. Hymenoptera is an insect order under phylum arthropoda. It is the third largest of all insect order, and perhaps the most beneficial to humans. The order Hymenoptera comprises approximately 115,000 described species which includes wasps, bees, ants, ichneumonids, calchids, sawflies etc. Collectively, the Hymenoptera are most important to humans as pollinators of wild and cultivated flowering plants, as parasites of destructive insects and as makers of honey and beeswax. Nonetheless, the order poses significant public health concern as well. [3] The three medically important group of stinging insect of the order Hymenoptera belong to the families of Apidae (bees), Vespidae (paper wasps, hornets and yellow jackets, commonly referred as *wasps*) and Formicidae (ants). [4] The sting from these social wasp become clinically significant if the patient is allergic to Hymenoptera venom or if the patient is exposed to large quantity of the venom due to massive or multiple stings. Most deaths related to wasp stings are the result of immediate hypersensitivity reactions causing anaphylaxis. A single sting is sufficient to cause fatal anaphylaxis in hypersensitive patients. Massive enve-

nomation can, likewise, cause death in non-allergic individuals, probably due to the toxic effects of the venom. A wide range of clinical sequelae is observed during wasp stings—from simple allergic skin manifestations to severe systemic reactions and toxic reactions leading to death. [5] Wasps, being highly diverse insects, are solitary or social, parasitic or predatory, phytophagous or carnivorous or omnivorous.

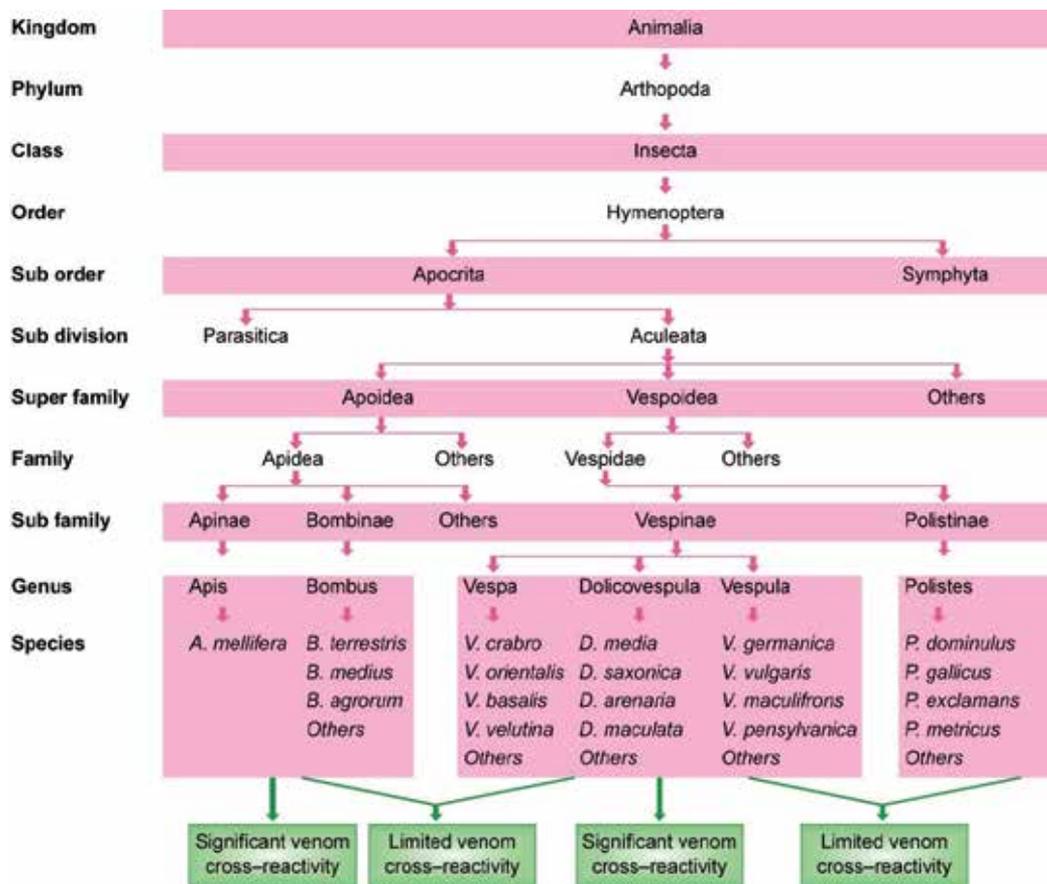


Figure 1. Taxonomy of Hymenoptera and cross reactivity of their venoms.

2. Epidemiology of stings

The insect order Hymenoptera is established on every continent except Antarctica. [6] In countries with predominantly moderate climate, they are present in the environment for a larger part of the year. [7] The season for wasp starts from spring and lasts till early fall. The stinging incidents are high during late summer and early fall with the numbers reaching a peak in August. [5] Various environmental factors such as temperature, humidity, solar radiation, rainfall and

wind speed influences the wasp activity. Activities occurred at all temperatures above 7°C and below 41°C with maximum activity occurring between 20–35°C. [8]

The epidemiology of hymenopteran stings occur often throughout the world; more prevalent in adult males involved in outdoor occupations or hobbies. Among the hymenopterans, the sting is more often from vespids, particularly paper wasps and yellow jackets (not hornets), than apids, such as bumblebees and honeybees. [9] Single stinging events usually occurs when large number of hungry wasps are attracted to the food of humans eating outdoor or if it is accidentally stepped on, swatted or otherwise disturbed. In contrast, mass stinging events occur when wasps respond to a human intruder as a threat to their colony, for example, when someone inadvertently stumbles into a colony or otherwise disturbs their hives by throwing rocks at or shooting at or chopping a tree containing the colony. [10] Many times the incident takes place when the adults set fire on the colony to collect the larva which is considered very nutritious. Such practice is very common in the countryside.

Limited and under-estimated data exist on the epidemiology of hymenopteran stings. Depending upon the climate, 56.5–94.5% of the general adult population remember receiving hymenoptera sting at least once in his life. The prevalence of sensitization, which is indicated by a positive skin test and/or detection of specific IgE in patients with no previous case history, is estimated between 9.3–28.7% in adults. The prevalence of large local reactions in the general population ranges from 2.4–26.4%, up to 38% in beekeepers. European epidemiological studies reports a prevalence of systemic reactions between 0.3–7.5% among the adults whereas in the USA, the prevalence ranges form 0.5–3.3%. [7,11]

3. Components present in the wasp venom: Classification, list, structure and function

Wasp venom components are generally categorized as: a) high molecular weight proteins that includes phospholipases, hyaluronidases, antigen 5 etc.; b) low molecular weight peptides that includes mastoparans, wasp kinins and chemotactic peptides, and c) bioactive molecules such as histamine, serotonin, catecholamines, acetylcholine, tyramine etc..

Vespid venom is more variable in their composition among the species, different to that of apid (bee) venom. They are complex mixture of powerful allergens and pharmacologically active compounds, primarily made up of proteins. The vespid venom contains three major proteins that act as allergens and a wide variety of vasoactive amines and peptides. The important allergens are antigen 5, phospholipases and hyaluronidase. Antigen 5 is the major allergen in all vespid venom and has been most thoroughly studied among the others. [4] Two additional proteins, Vmac 1 and Vmac 3 from *V. maculifrons*, with allergenic activity have been described, but are incompletely characterized. [4, 12] Similarly, serine-protease has been identified as an important allergen for vespid-allergic individuals in European *Polistes* [13, 14] venom and dipeptidylpeptidase IV [15] and vitellogenin [16] in *V. vulgaris* venom. The vasoactive amines in vespid venom includes serotonin, histamine, tyramine and catecholamines. Wasp kinins and mastoparans are the peptides unique to vespid venom.

3.1. Antigen 5

Animal tests have shown that antigen 5 is not a toxin. [17] It is a member of a conserved family of proteins found in eukaryotes, including yeasts and have sequence identity with other proteins of diverse origin and tissues, such as mammalian cysteine-rich secretory proteins in salivary and reproductive organs, secretory proteins of helminths produced during sexual maturation, human brain tumor proteins, reptile venom, pathogenesis-related proteins of plants and fire ant venom. [17, 18] The mature antigen 5 from yellow jacket and hornet have 201 and 205 amino acids respectively, with several highly conserved regions. Almost all of the sequence variations seen in hymenoptera antigen 5 were found on the surface. The highly cross reactive groups within the genera have few changes. The antigen 5 homolog from ants do not exhibit antigenic cross reactivity with those from vespid wasps due to the low degree of surface conservation and changes in loop lengths. [18] However, in hyperimmune sera, occasionally, antigenic cross reactivity has been observed between vespid antigen 5 and homologs from other animals. [19]

3.2. Phospholipases

The wasp venom phospholipase (PL) belongs to a different superfamily than those of bee venom phospholipase. Vespid wasp phospholipases have PLA₁B specificity and are members of GX class lipase, lipoprotein lipase superfamily, pancreatic lipase homologous family and RP2 sub-group of phospholipase. [18, 20] The PLs from vespid wasp venom usually do not contain carbohydrate and have highly homologous regions surrounding the active sites. The cross reactivities of the PLs generally follow the phylogeny: closely related species are highly cross reactive and those that are further removed are less cross reactive.

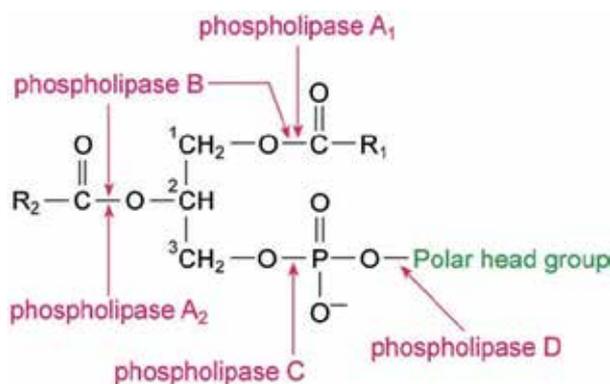


Figure 2. Site of Phospholipase action.

The characterized PLs in vespid venom are PLA₁, PLA₂ and PLB. The vespid venom PLs has an offensive as well as defensive role. The venom is not only used as toxins for preyed insects,

but also digests their cell wall components of diacylphospholipids such as phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine to fatty acids and lysophospholipids by the containing PLs. PLBs are more universally digestive enzymes than PLA₁ and PLA₂, which seems to be a kind of enzymatic adaptation of the carnivorous insects, i.e, vespids against vegetarian insects, i.e, apids that contain only PLA₂ in the venom. Besides the enzymatic activity, PLBs also possesses the haemolytic activity and cardiotoxicity. [21]

Phospholipase A₁ (PLA₁) is an enzyme that hydrolyzes ester bonds of phospholipids at the *sn*-1 position and produces 2-acyl-lysophospholipids and fatty acids. Vespid-venom PLA₁s belong to the pancreatic lipase family and exhibit PLA₁ activities but do not show any lipase activities. The tertiary structure of lipases have two surface loops, the lid and the β9 loop, which covers the active site and are implicated in substrate recognition. The amino acid sequence alignment of the pancreatic lipase family members (eg, phosphatidylserine-specific PLA₁ (PS-PLA₁), membrane-associated phosphatidic acid-selective PLA₁ (mPA- PLA₁), vespid PLA₁, hepatic lipase (HL), endothelial lipase (EL), pancreatic lipase, and pancreatic lipase-related protein 2 (PLRP2)) revealed two molecular characteristics of PLA₁s: first, lipase members exhibiting PLA₁ activity have short lids; and second, lipase members exhibiting only PLA₁ activity have short β9 loops. Thus, pancreatic lipase and LPL which exclusively exhibit triacylglycerol lipase activity have long lids and long β9 loops, while PS- PLA₁, mPA- PLA₁, vespid PLA₁ which only shows PLA₁ activity have both short lids and short β9 loops whereas EL and PLRP2 which exhibit both PLA₁ and triacylglycerol lipase activity have short lids but intact β9 loops [22, 23] PLA₁, thus, possess direct cytolytic effects, besides their role in allergic and inflammatory processes.

PLA₂ catalyzes the specific hydrolysis of ester bonds at the *sn*-2 position of 1,2-diacyl-3-*sn*-glycerophospholipids into their corresponding lyso compounds with release of free fatty acids. Thus, it is able to disrupt the phospholipid packings from several types of biological membranes leading to pore formation and/or cell lysis. [20, 24] Vespid PLA₂ has very potent cytolytic actions.

3.3. Hyaluronidase

Hyaluronidases (Hyal) are a widely distributed glycoside hydrolases that cleaves β-1,4-glycosidic bonds between N-acetylglucosamine and D-glucuronic acid of hyaluronic acid (HA) [14], one of the primary components of the extracellular matrix in all the vertebrates. They are also present in almost all venoms, acting as a “spreading factor” by facilitating the penetration of the other harmful venom components and enhancing their action in various tissues into the bloodstream. They are the “allergenic factors” in vespid and apid venom and are able to induce severe and fatal anaphylactic IgE-mediated reactions in humans. [25] They are the phylogenetically most strongly conserved Hymenoptera allergens. Sequence homologies between *Vespula* and *Dolichovespula* species hyaluronidases are 90% or greater, whereas those for antigen 5 and PLA₁ are only around 60% to 65%. In agreement with this, immunologic cross-reactivity between different vespid genera is strong with hyaluronidases but more restricted with antigen 5 and PLA₁. Vespid hyaluronidases are significantly similar with honey bee hyaluronidase which shows 50% sequence homology with vespid homologs Ves v 2, Ves

g 2 and Dol m 2. In accordance with this, hyaluronidases have been identified in inhibition studies using patients' sera as the most important cross reactive allergens in yellow jacket and honeybee venom. [14]

Hyaluronidase of wasp venom is an allergen. The asparagine-linked carbohydrate often appears to constitute the common IgE-binding determinant. Irrespective of the nature of the protein, protein-linked glycans can bind IgE, which turns many proteins, especially those of higher molecular mass, into apparent allergens. Hyaluronidase is the dominating glycoprotein in the wasp venom and contains α -1,3-fucose-containing N-glycan which is responsible for allergenicity. The cross-species survey performed by Kolarich et al shows that venom from six wasp species (*V. vulgaris*, *V. germanica*, *V. flavopilosa*, *V. maculifrons*, *V. pennsylvanica* and *V. squamosa*) contained the difucosylated paucimannosidic N-glycans MUF³F⁶ and MMF³F⁶ as the major glycan structures. [26] The allergic response is initiated by the epitope that cross-links the Fc-receptor-bound IgE antibodies on the surface of mast cells. This is followed by rupture of mast cell membrane and the release of stored mediators, such as histamine, which are responsible for the immediate type hypersensitivity reaction. [27]

Hyaluronidases, on the basis of mechanism of action, are classified into three classes: a) the group of endo- β -N-acetyl-D-hexosaminidases that hydrolyse the high molecular weight substrate (HA) to tetrasaccharide as the main end product, represented by the testicular enzymes; b) the β -endoglucuronidases group represented by hyaluronidases from leeches and hookworm, and; c) the group of lyases that act via β -elimination, yielding disaccharides as the main products represented by the bacterial hyaluronidases. The enzymes of the first class also catalyses transglycolation reactions, producing hexa-, di- and octa- saccharides during hydrolysis of HA. Hymenoptera venom hyaluronidases belong to the first class. Unlike the latter two classes of hyaluronidases, this first class acts not only on HA, but also on chondroitin 4-sulfate and chondroitin 6-sulfate. [25]

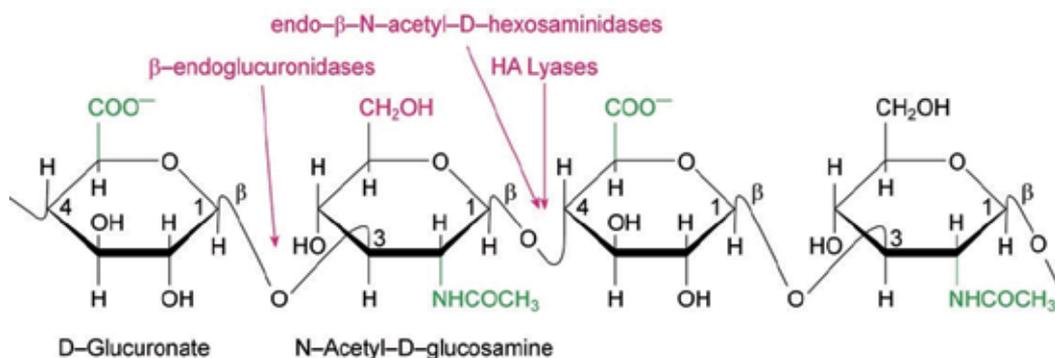


Figure 3. Hyaluronic Acid and Site of Action of Different Hyaluronidases.

3.4. Mastoparan

Mastoparans are low molecular weight peptides, generally tetradecapeptides, extracted from the venom sac of social wasps that act in the defense system of these insects. They are rich in hydrophobic and basic residues which are distributed in the peptide chain in such a way that, in adequate environment, they form amphipathic helical structures [28] which favours electrostatic interactions with the negatively charged phospholipid head groups of the biological membranes. This characteristic may lead to peptide insertion into the membrane bilayer and thus interact directly with G proteins on the cytoplasmic face attacking the transmembrane signalling [29] and sometimes to membrane destabilization with its consequent lysis. [30, 31] These peptides, thus, present important biological activities such as antimicrobial, mast cell degranulation, haemolytic activities, [28] activation of G-protein mediated mechanisms, stimulation of phospholipase A₂, C and D, mobilization of Ca²⁺ from mitochondria and sarcoplasmic reticulum, activation of ryanodine receptor, modulation of various enzymes, such as Na⁺-K⁺-ATPase of rat brain, induction of the mitochondrial permeability transition and cell death by necrosis and apoptosis. [32]

Mastoparans are discovered in wasp venom in a screening test for mast cell degranulating agents. They are also the potent stimulants of purified PLA₂ from different sources. They bind to phospholipids making them the better substrates. They facilitate the PLA₂ of both venom and victim, thereby promoting generation of arachidonic acid, the precursor of prostaglandins and leukotrienes which are mediators of adverse reactions associated with immediate type hypersensitivity. The high affinity binding of mastoparan to calmodulin has led to speculate the role of the peptides in inhibition of calmodulin-mediated reactions. [33] Mastoparan is a potent stimulator of exocytosis from diverse mammalian cells. It causes secretion of histamine from mast cells, serotonin from platelets, catecholamines from chromaffin cells and prolactin from the anterior pituitary. In case of histamine secretion, the effect of mastoparan is mediated by an increase in cytoplasmic Ca²⁺ that is itself caused by an increase in the intracellular second messenger inositol-1,4,5-triphosphate (IP₃). Such IP₃-mediated increase of intracellular Ca²⁺ is controlled at the level of phospholipase C (PLC) by one or more of a group of GTP-binding regulatory proteins, or G proteins. [29] Mastoparan induced apoptosis or oncosis is initiated by Ca²⁺ release from intracellular release from intracellular stores via PLC and IP₃, and the disruption of plasma membrane integrity occurs secondarily. [34] Mastoparan, an activator of G_i and mast cells, selectively stimulates an PLD₂, independently of G_v, ADP-ribosylation factor-1 (ARF-1), protein kinase C and calcium, in intact cells and in isolated preparations enriched in plasma membranes where PLD₂ is located. [35] PLD catalyses the hydrolysis of the major membrane phospholipid, phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. PLD is involved in the exocytosis of secretory granules from mast cells and neutrophils. One possible function of PLD that would rationalise its role in exocytosis may be related to the ability of PA to regulate PI(4)P 5-kinase. PI(4)P 5-kinase is one of two kinases required for the synthesis of PIP₂ (PI3). This unique lipid is essential for many membrane trafficking events including exocytosis. The product of PC hydrolysis by PLD is PA, which therefore has the potential to dynamically regulate the synthesis of PIP₂ in specific membrane compartments, ie, where PLD is active. [36]

Mastoparans: Vespinae		
Origin	Name	Amino acid sequence
<i>Paravespula lewisii</i>	Mastoparan	INLKALAALAKKIL-CO-NH ₂
<i>Vespa mandarina</i>	Mastoparan-M	INLKAIAALAKKLL-CO-NH ₂
<i>Vespa xanthoptera</i>	Mastoparan-X	INWLQIAAMAKKLL-CO-NH ₂
<i>Vespa analis</i>	Mastoparan-A	IKWKAILDAVKKVL-CO-NH ₂
<i>Vespa tropica</i>	Mastoparan-T	INLKAIAAFKLL-CO-NH ₂
<i>Vespa orientalis</i>	Mastoparan-II	INLKALAALVKKVL-CO-NH ₂
<i>Vespa basalis</i>	Mastoparan-B	LKLSIVSWAKKVL-CO-NH ₂
Mastoparans: Polistinae		
<i>Polistes jadwigae</i>	Polistes mastoparan	VDWKKIGQHILSVL-CO-NH ₂
<i>Parapolybia indica</i>		INWAKLGKLALEVI-CO-NH ₂
<i>Ropalidia sp.</i>		INWSKLLSMAKEVI-CO-NH ₂
<i>Protonectarina sylveirae</i>	Protonectarina mastoparan	INWKALLDAAKKVL-CO-NH ₂
<i>Agelaia pallipes pallipes</i>	Agelaia-MP	INWLKLGKAIIDAL-CO-NH ₂
<i>Polybia paulista</i>	Polybia-MPI	IDWKKLLDAAKQIL-CO-NH ₂
<i>Protopolybia exigua</i>	Protopolybia MPI	INWLKLGKKSAIL-CO-NH ₂
<i>Protopolybia exigua</i>	Protopolybia MP II	INWKAIEAAKQAL-CO-NH ₂
<i>Protopolybia exigua</i>	Protopolybia MP III	INWLKLGKAVIDAL-CO-NH ₂
<i>Polistes rothneyi iwatai</i>	Polistes-mastoparan-R 1 [Pm-R1]	INWLKLGKKILGAI-CO-NH ₂
<i>Polistes rothneyi iwatai</i>	Polistes-mastoparan-R 2 [Pm-R2]	LNFKALAALAKKIL-CO-NH ₂
<i>Polistes rothneyi iwatai</i>	Polistes-mastoparan-R 3 [Pm-R3]	INWLKLGKQILGAL-CO-NH ₂

Table 1. Amino Acid Sequence of Mastoparans in the Venom of Vespinae and Polistinae

3.5. Wasp kinins

Wasp kinins are of interest because two kinins- bradykinin (BK) and lysyl-bradykinin or kallidin occur in humans, produced by plasma kallikreins and tissue kallikreins respectively. The peptides are generated and act locally in humans but are stored in venom. They are important mediators of inflammatory responses, potent pain producers and increase vascular permeability and vasodilatation. [33, 37, 38] Bradykinin is a nonapeptide usually found in body secretions such as urine, saliva and sweat. They are also found in several tissues such as heart, vasculature, blood, kidney, colon and liver. [39]

Wasp kinins are polypeptides (9-18 amino acid residues) containing a bradykinin-like sequence at the C-terminal. In some cases the whole nonapeptide sequence of bradykinin is present within the wasp kinin sequence. The primary sequence of most kinin related peptides

from animal venom are longer with potent pharmacological actions and long lasting effects compared to bradykinin. Due to a greater taxonomic diversity, a series of different bradykinin-related peptides (wasp kinins) have been identified in the venom from different species of wasps. Neurotoxic kinins, such as threonine-bradykinin (Thr⁶-BK) and megascoliakinin (Thr⁶-BK-Lys-Ala) and glycosylated wasp kinins have been described. Wasp kinins are experimentally involved in constriction and relaxation of muscles, activation of leukocytes followed by a release of cytokines, prostaglandins, leukotrienes, reactive oxygen species and the blockage of the cholinergic transmission in the insect central nervous system. [38]

Peptides	Amino acid sequence
Bradykinin	RPPGFSPFR
Protopolybiakinin-I	DKNKKPIRVGGRR PPGFTPFR
Protopolybiakinin-II	DKNKKPIWMAGF PGFTPIR
[Thr ⁶] Bradykinin	RPPGFTPFR
Vespakinin-M	GRPXGFSPFR ID
Vespakinin-X	ARPPGFSPFR IV
Vespakinin-A	GRPPGFSPFR VI
Vespakinin-T	GRPXGFSPFR VV
Polisteskinin-3	pETNKKKLR GRPPGFSPFR
Polisteskinin-R	ARRPPGFTPFR
Polisteskinin-J	RRRPPGFSPFR
Polisteskinin-C	SKRPPGFSPFR

Table 2. Amino Acid Sequence Alignments of Bradykinin, Protopolybiakinins and Some Wasp Kinins

The general pharmacological effects of kinins have been attributed through two G protein coupled receptors—B₁ or B₂ receptors. Intact BK is the characteristic agonist for the B₂ receptors, whereas kinin metabolites such as Lys-des-Arg⁹-BK or des-Arg⁹-BK produced by neuronal endopeptidase action activate the B₁ receptor which is far less expressed in normal tissues. [40]

Wasp kinins impart its pharmacological effects via B₂ receptor activation. [41] When injected into the vertebrate predators by stinging, they produce severe pain, thus producing a significant role in their defense system. On the other hand, they are used to irreversibly paralyse the prey. [42]

3.6. Other bioactive molecules

There are many other bioactive molecules in the wasp venom such as histamine, 5-HT, acetylcholine, tyramine, catecholamines, and various peptides. Bioamines such as histamine, 5-HT, acetylcholine, tyramine, catecholamines etc are only a minor portion of the wasp venom, however their pronounced reaction is due to the endogenous release initiated in the victim by

other factors such as mastoparans, wasp kinins and phospholipases. [38] Peptides carry out important biological processes in venom. Their activities are very diverse and range from neurotoxic and inflammatory to antibacterial. Besides, mastoparans and wasp kinins discussed above, several other peptides includes protonectins, mandaratoxins and chemotactic peptides. Chemotactic peptides recruit macrophages and polymorphonuclear leukocytes near the site of stinging. Protonectins are the mast cell degranulating peptides responsible for histamine release. Several other peptides have been identified with antibacterial properties. [43]

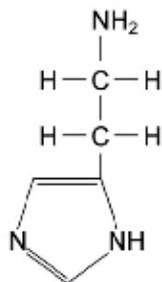


Figure 4. Histamine.

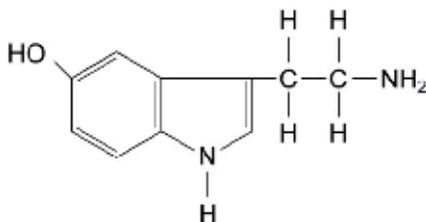


Figure 5. Serotonin.

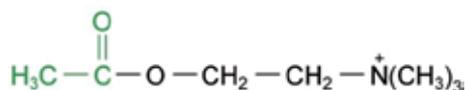


Figure 6. Acetylcholine.

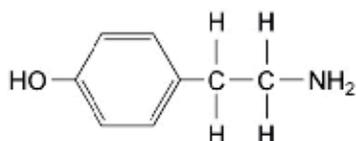


Figure 7. Tyramine.

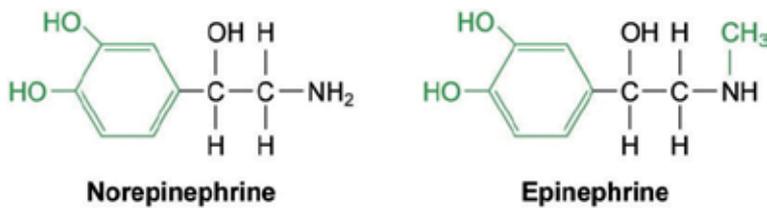


Figure 8. Catecholamines.

Protonectins: Polistinae		
Origin	Name	Amino acid sequence
<i>Protonectarina sylveirae</i>	Protonectin	INWLKLGKKILGAI-CO-NH ₂
<i>Polybia paulista</i>	Polybia-CP	LNFKALAALAKKIL-CO-NH ₂
<i>Polistes rotheyi iwatai</i>	Polistes-protonectin	INWLKKGKQILGAL-CO-NH ₂

Table 3. Amino Acid Sequence of Protonectins in the Venom of Polistinae

4. Venom allergens, hypersensitivity reactions and lethality

Antigens that elicit clinical allergic reactions are typically referred to as allergens. [44] The major allergens identified in the vespid venom are phospholipase A₁, hyaluronidase and antigen 5. The minor allergens described are Vmac 1 and Vmac 3 from *V. maculifrons*, [18] serine protease from *Polistes spp.* [44] Vmac 3, the band between hyaluronidase and phospholipase on SDS-PAGE, appears to be a variant form of hyaluronidase on protein sequencing results, however the high molecular weight fraction Vmac 1 which is yet not well characterized contains significant amounts of carbohydrate. [18] Mastoparan, a tetradecapeptide, also elicit some immunological properties. Immunochemical studies with the vespid venoms have elucidated that the major immunogens or allergens are those of large molecular weight venom components such as hyaluronidases, phospholipases and antigen 5. Small peptides are generally poor immunogens. [45] Both carbohydrate-based epitopes (glycotopes) and protein-based epitopes are able to inflict immune responses. [46] Phospholipases and hyaluronidases are glycoprotein where cross-reactive carbohydrate determinants (CCDs) play major role in inflicting immune responses whereas antigen 5 does not contain carbohydrates and inflicts immune responses via its peptide-epitopes. Epitopes are a particular portion of an allergen that specifically binds serum IgE. Structurally, epitopes can either be linear or conformational. Linear (continuous) epitopes are defined by the primary amino acid sequence of a particular region of a protein. The sequence that interact with the antibody are situated next to each other sequentially on the protein. Conformational epitopes consist of amino acids which are in close proximity while the protein is folded correctly. They may be continuous or discontinuous, i.e,

the amino acids may be situated next to each other or are found on multiple regions in the primary structure. Most allergenic epitopes are conformational. [44]

There are four types of hypersensitivity reactions, viz:

- a. Type I hypersensitivity reaction (IgE mediated),
- b. Type II hypersensitivity reaction (IgG mediated),
- c. Type III hypersensitivity reaction (Immune complex mediated), and
- d. Type IV hypersensitivity reaction (Cell mediated)

Hymenoptera sting reactions are classified into a) normal local reactions, b) large local reactions, c) graded systemic reactions, d) systemic toxic reactions and e) unusual delayed reactions. [47, 48] Hypersensitivity reactions of type I, III and IV have been found to involve in sting reactions. Local reactions are type IV hypersensitivity IgG-mediated reactions and are either focal or large local. Systemic reactions are IgE mediated type I hypersensitivity reactions and are graded from I to IV depending upon the presence of one of the following respectively: urticaria, angioedema, airway obstruction, or anaphylaxis. Unusual delayed reactions are IgG- and IgM-mediated type III reactions which include serum sickness, vasculitides, central nervous system signs and symptoms, haemolytic events, myocardial infarction, disseminated intravascular coagulation, and acute kidney injury. Immune complex mediated type III reactions are the indirect cause of systemic toxic reactions in wasp envenomation. [48, 49]

Wasp stings are usually not fatal. The commonest manifestations are related to allergy and low grade systemic reactions characterized by pain, swelling, urticaria and redness at the sting site that usually lasts for 1-2 days. [50] Large local reactions are late phase manifestations that may involve a large area and persist up to a week. They are not life threatening unless they involve the airway. They may result in considerable morbidity because of temporary loss of function, such as occurs when the sting involves a foot or hand or is near eye. [51] These local reactions generally resolves without any treatment. [52] Death from wasp envenomation is a rare event – most often it is caused by IgE mediated type I anaphylaxis attributed even to a single sting in venom allergic individuals. Hence, these local reactions and anaphylaxis are not dose dependent or related to number of stings. Less commonly, in non-allergic individuals, death occurs from the toxic effects of massive envenomation involving hundreds to thousands of stings. Such toxic reactions are venom volume dependent. However, it is noteworthy that wasp venom toxicity varies among and even within the species which is due to the variation in the composition and the quantity of the venom released. *Vespula*, *Dolichovespula* and *Polistes* stings release 1.7 – 3.1 µg, 2.4 – 5.0 µg and 4.2 – 17 µg of venom proteins, respectively. [47] Mammalian toxicity tests on mice has revealed that wasp venom has more deleterious effect than that of bee venom despite of the fact that wasps inject less venom per sting than bee (50 µg – 140 µg). Organ dysfunction, eg, renal failure, and death may occur in the range of ~20 – 200 wasp stings and ~150 – 1000+ bee stings. [10]

Classification of reactions	Hypersensitivity reactons	Onest times	Reacting Igs	Clinical manifestations
Local	IV	4-48 h	Cell-mediated IgG	Painful, pruritic, and edematous sting lesions, 2.5-10cm in diameter, lasting <24 h
Large local	IV	4-48 h	Cell-mediated IgG	Painful, pruritic, and edematous sting lesions, >10cm in diameter, lasting >24h
Systemic grade I: urticarial	I	10-20 min up to 72 h	IgE	Anxiety, malaise, generalized urticaria, itching
Systemic grade II: angioedema	I	10-20 min up to 72 h	IgE	Any grade I signs above, plus ≥ 2 of the following: angioedema (grade II if alone), dizziness, vomiting, diarrhea, chest tightness, abdominal pain.
Systemic grade III: airway obstruction	I	10-20 min up to 72 h	IgE	Any grade II signs above, plus ≥ 2 of the following: stridor, dyspnea, wheezing (grade III if any of these alone), hoarseness, dysarthria, dysphagia, weakness, confusion
Systemic grade IV: anaphylactic	I	10-20 min up to 72 h	IgE	Any grade III signs above, plus ≥ 2 of the following: unconsciousness, hypotension, cardiovascular collapse, cyanosis, urine and /or fecal incontinence
Unusual delayed reactions	III	2-14 day	IgM, IgG	Serum sickness, generalized vasculitis, rhabdomyolysis, acute tubular necrosis, Central nervous system involvement (seizures, neuritis, peripheral neuropathy or radiculopathy, cerebrovascular accident), hemolysis, thrombotic thrombocytopenic purpura, Disseminated intravascular coagulation, Myocardial infarction

Table 4. Classification of Hymenoptera Sting Reactions [48]

4.1. Venom allergens and their systematic nomenclature

The venom allergens have been assigned official names by the International Union of Immunological Studies, Allergen Nomenclature Subcommittee. Vespid venom proteins are assigned

1 to phospholipase A₁B, 2 to hyaluronidase, 3 to dipeptidylpeptidase IV, [15, 53] 4 to venom protease, 5 to allergen 5, and 6 to vitellogenin. [16, 18, 54] Allergens are designated according to the accepted taxonomic name of their source as follows: the first three letters of the genus, space, the first letter of the species, space and an Arabic number. Numbers are assigned to allergens in the order of their identification, and the same number is generally used to designate homologous allergens of related species. [55] For example, Ves v 1 refers to the first venom allergen identified from *Vespula vulgaris*.

4.2. Cross reactivity among vespid (wasp) venom

The allergic manifestation is the commonest feature of wasp sting. It includes the typical dermatologic expression incorporating edema, erythema, pruritus, urticaria and pain at and around the sting site, usually. These clinical features generally subside within a few days to a week without any treatment or simple treatment such as ice-packs and analgesics. Such allergic manifestations are not fatal and majority of the populations have such simple allergy to wasp venom. These allergic reactions become serious when the individual is hypersensitive to the wasp venom and produces the venom specific IgE. Such specific IgE binds for a particular portion(s) of an allergen, referred to as an epitope(s). Cross-reactivity is the recognition of similar or identical epitopes on proteins from different sources. The presence of the cross-reactive epitopes can make an individual appear allergic to an insect venom protein which they have not encountered. This is merely due to the structural homology between the venom proteins. Treatment of insect allergies requires the identification of the sensitizing species and the cross-reactive epitopes make the identification of the sensitizing species difficult. This error in identification of the sensitizing species could lead to incomplete or partial protection of the patient during the treatment. [44] The allergenic cross-reactivity can, both, be due to protein-based epitopes or carbohydrate-based epitopes (glycotopes). Cross-reactive carbohydrate determinants (CCDs) are the key carbohydrate molecule attached to the glycoprotein. They share significant structural homologies and are thus prone to extensive cross-reactivity between the products from various sources. [56] Generally, the N-glycans found on most hymenoptera venom proteins possess a number of non-mammalian features rendering them potentially immunogenic. However, the hallmark of CCDs on insect venom allergens comprise carbohydrates carrying α -1,3-linked core fucose residues. IgE with specificity for such CCDs are key players in allergen cross-reactivity and represents a major concern for diagnostic and therapeutic approaches, however, the role of CCDs for occurrence of allergic symptoms is still controversial. [53] These CCDs are the reason for multiple sensitivity observed in the hypersensitive individuals.

Cross-reactivity generally follows phylogeny: closely related/homologous species are highly cross-reactive and those that are further removed are less cross-reactive. For example, the yellow jacket antigen 5 has 69% and 60% sequence identity with the white-faced hornet and *Polistes* wasp, respectively. Similarly, yellow jacket hyaluronidase and phospholipase show 92% and 67% sequence identity, respectively with their homologs of white-faced hornets. This sequence homology confers considerable cross-reactivity with an order of cross-reaction of the three vespid allergen as hyaluronidase > antigen 5 > phospholipase. The cross-reactivity among

the yellow jacket and white-faced hornet allergen is greater than the cross-reactivity among yellow jacket or white-faced hornet and *Polistes* wasps. [57]

4.3. Physiological manifestation of stings (single sting versus multiple sting)

Stinging events involving wasp are rare and the death due to wasp sting is infrequent. However, in wasp venom hypersensitive individual, a single sting is sufficient enough to cause the clinically significant case or death, which often is caused by IgE-mediated type I anaphylaxis. These single stings occur when a single insect is disturbed while searching for food. Besides, mass stinging events by wasps also occur when someone inadvertently stumbles into their colony or otherwise disturbed their hive by throwing stones at, shooting at, or chopping trees containing their colony. In such case, hundreds or thousands of wasps may sting resulting in massive envenomation to a person leading to the venom intoxication. In case of non-allergic individuals, the toxic effects of venom due to high venom load can result into physiological changes developing in clinical sequelae. [10]

Wasp toxin anaphylaxis is a typical immediate-type allergic reaction. Specific IgE antibodies directed against components of the toxin mediates the activation of mast cells and basophilic granulocytes, leading to the release of mediators that cause acute manifestations of disease. In the great majority of cases, a single sting is the cause. The reaction usually arises 10 to 30 minutes after the sting, although the latency may be shorter or longer. The severity of anaphylaxis is graded on the basis of clinical manifestations. Most patients recover without any permanent sequelae. The main causes of death due to anaphylaxis are airway obstruction and cardiovascular failure; rarer causes are disseminated intravascular coagulation (DIC), acute kidney injury and epinephrine overdose. Myocardial infarction, stroke and thrombotic events can cause permanent morbidity. [58, 59] Besides, the immune-mediated reactions upon single or multiple stings, direct toxic effects of venom are also observed which generally are the features of mass envenomation. Such direct toxic effects are venom-volume dependent and the venom components pose for the cytolytic or cytotoxic effects. The clinical manifestations for such toxic effects include intravascular Haemolysis, rhabdomyolysis, pigment nephropathy, renal impairment, acute kidney injury, liver impairment, disseminated intravascular coagulopathy, central nervous system damage and direct toxicity to multiple organ system. [5, 10, 60]

5. Diagnostic method for sting allergy

Hymenoptera venoms are known to cause life-threatening IgE-mediated anaphylactic reactions in allergic individuals. Proper diagnosis of hymenoptera venom allergy is severely affected by molecular cross-reactivities resulting into double or multiple positive tests. [61] Double or even multiple positive tests can be caused by true double sensitisation indicating potential systemic allergic reactions to the next sting by either insect species, if not treated by immunotherapy with both venoms; or by cross-reactive IgE antibodies which recognize either peptide based epitopes of venoms or carbohydrate-containing epitopes (CCDs) in glycoprotein allergens. [62]

It is important to distinguish between cross-reactivity and true double sensitization for the choice of venom(s) for immunotherapy. [47] Since, many patients fail to identify the stinging insect, skin testing and *in vitro* detection of venom specific IgE antibodies are the only tools to detect the culprit insect involved in the allergic reaction and are used to select the appropriate venom immunotherapy depending upon the severity of clinical symptoms. [63] Cross-reactivity within the vespid venoms is strong due to similarities of venom composition and structure of single allergens. The hyaluronidase enzyme of the honeybee and wasps show 50 % sequence identity and hence has been identified as the major cross-reactive component. [47]

The sera of 20% – 50% of patients with hymenoptera venom allergy show *in vitro* reactivity with both honeybee and wasp venom. [62] This IgE positivity to both hymenoptera (honey bee and wasp) venoms is referred to as true double sensitization leading to immunotherapy against both venoms. However, there are other reasons for IgE-double positivity: a) true independent sensitization (co-sensitization) to different allergens, which is a very rare phenomenon; b) immunochemical cross-reactivity due to sequence homologies between allergens from different sources; c) cross-reactive carbohydrate determinants (CCDs) in the glycoproteins from various sources; and d) non-specific absorption of IgE to the allergosorbent, a phenomenon that is particularly relevant when total serum IgE is extremely elevated. CCDs are important antigen targets for specific IgE (sIgE) binding providing at least two different IgE-binding sites. [63] 5% of non-allergic individuals and 10% of non-pollen allergic subjects have CCD-sIgE antibodies. [64] 10%-15% patients with grass pollen allergy have CCD-sIgE antibodies which increases up to more than 60% with concomitant sensitization to pollen from trees, grasses and weeds, [63] 23% patients with honeybee venom allergy and 11% patients with yellow jacket venom allergy have CCD-sIgE antibodies. [65] IgE inhibition studies by various methods can help distinguish true double sensitization from cross-reactivity. [62] Recent advancement on diagnostic procedure incorporates the recombinant allergen based IgE testing that effectively distinguishes true double sensitization from cross-reactivity. [66]

5.1. Diagnostic strategy

5.1.1. History

Informations regarding number, date and site of stings and its reactions, sort and severity of symptoms, interval between sting and the onset of symptoms, emergency treatment, risk factors of a particular severe reactions, risk factors for repeated re-stings, tolerated stings after the first systemic reactions and other allergies, if any, should be collected. [47]

5.1.2. Skin test

Skin tests are performed by skin prick or intradermal injection of venom. It is recommended at least 2 weeks after the reaction to a sting to avoid the possibility of false negative results during a refractory period. Stepwise incremental venom skin tests are recommended. The test is stopped when the patient has a conclusive reaction at a set venom concentration. Venom concentrations of 0.01 – 100 µg/ml are generally used for skin prick test, and 0.02 ml of venom concentration ranging from 0.001 – 1 µg/ml is intradermally injected into the volar surface of

the forearm for intradermal testing. Skin prick tests have lower sensitivity than intradermal tests even at 100 µg/ml concentration; hence the patients with negative skin prick tests should be confirmed by intradermal tests. The sensitivity of intradermal test is estimated at about 90% or higher for 1 µg/ml concentration. [47]

5.1.3. *In vitro* tests

In vitro tests specifically diagnoses or assists in diagnosis of venom allergy, depending upon the tests performed. Number of variables can be checked *in vitro* such as allergen-specific IgE, allergen-specific IgG, baseline serum tryptase, basophil activation test, basophil histamine release test, leukotriene release test and immunoblotting.

a. *Allergen-specific IgE Assay*

Venom-specific IgE (sIgE) usually increases within days or weeks after a sting. Following this initial phase specific IgE declines slowly with a large individual variation. The test should be repeated after a few weeks in patients with no detectable specific IgE to the presumptive relevant venom. *In vitro* radioallergosorbent test (RAST) and various derived methods are used to assay the allergen-specific IgE. [47] A sIgE value of ≥ 0.35 kU/L is regarded as positive. [67] Venom sIgE tests are, however, less sensitive and specific than intradermal skin test in the patients with a history of systemic sting reactions, especially after the first year following a reaction. [47] Skin test reactivity and levels of sIgE also do not correlate with clinical reactivity and hence must be interpreted in conjunction with clinical history. [68] Similarly, double positive or multiple positive tests are conferred either by true double sensitization or by cross-reactivity of venom sIgE with certain carbohydrate ligand. The RAST inhibition test is helpful in distinguishing between true double sensitization and cross-reactivity. The sIgE assay is thus modified by including an initial inhibition phase. [47] Contrast to sIgE, serum total IgE is generally regarded as a non-specific and global marker for atopy. [69] Recently, the component-resolved analysis using recombinant species-species major allergens (rSSMA) are incorporated to improve the differentiation between true double sensitization and cross-reactivity. [70]

b. *Allergen-specific IgG Assay*

The serum level of specific IgG (sIgG) primarily reflects the exposure to allergen. Venom-sIgG increases after a sting and does not correlate with the presence or absence of an allergic sting reaction. Venom immunotherapy is accompanied by an increase in allergen-specific IgG, however neither concentration of sIgE and sIgG nor sIgE/sIgG ratio closely correlates with the clinical improvement to immunotherapy. [47]

c. *Baseline Serum Tryptase*

Tryptase is a predominant protease of human mast cells that exist in three forms: α -tryptase, pro- β -tryptase and β -tryptase. α -tryptase and pro- β -tryptase are enzymatically inactive and released continuously. A persistent rise, therefore, in serum α -tryptase is an indicator of an elevated number of mast cells and thus may indicate for mastocytosis. β -tryptase is the enzymatically active tetramer stored in mast cell granula and released during acute allergic

reactions resulting into extensive mast cell degranulation. The serum concentration of β -tryptase is thus a measure for mast cell activation. If an elevated level of tryptase is observed outside an acute allergic reaction, it indicates an increased total body mast cell load, while within the context of an allergic reaction suggests mast cell activation. [67, 71] A significant proportion of patients presenting with anaphylaxis to hymenoptera sting have an elevated ($>11.4 \mu\text{g/L}$) baseline tryptase. Such patients fall into the spectrum of “mastocytosis” and further investigations including bone marrow examination to exclude systemic mastocytosis or monoclonal mast cell activation syndrome may be necessary. It has been reported that patients with elevated baseline tryptase with or without systemic mastocytosis develop significantly more severe reactions, especially cardiovascular anaphylactic reactions, as opposed to those with normal baseline tryptase. [68]

d. Basophil Activation Test (BAT)

Basophils are a rare population of peripheral leukocytes which play an important role as effector cells in allergic disease. CD63 and CD203c are the two markers utilized in assessing the basophil activation via flow cytometry. [72] This investigative tool is known as basophil activation test and hold promise as an alternative confirmatory assay for monitoring sensitization to wasp and other hymenoptera venoms. It can aid in clarifying history positive cases that have negative skin and serological tests for venom-specific IgE. [73] BAT correlates well with serum-specific IgE and have comparable sensitivity and specificity to skin tests and serum-specific IgE. [68]

5.1.4. Sting challenge test

Sting challenge test is performed in venom-allergic patients using live insects. As a matter of fact, some patients well-tolerate venom immunotherapy, but still have systemic reactions to a sting from the same insect. In such case, challenge tests with subcutaneously or intracutaneously administered venom are not reliable. Sting challenges are, thus, recommended in patients on maintenance VIT to identify those who are not yet protected. This test has the utility to assess the effectiveness of VIT in those patients under the increased risk of re-sting. The test has been tried for its prognostic value by using in untreated patients with or without a history of anaphylactic reactions in order to identify those who need immunotherapy, but was not successful enough to make the predictions. [47]

6. Risk factors for allergic reactions

Contrast to the higher prevalence of IgE sensitization in adults with no previous case history of an allergic reaction, systemic reactions occur in a small percentage for the reasons not known well. [74] There are, besides, various factors that determines the severity of reaction to wasp sting such as older age, male sex (male:female ratio, 2:1), insect type (honey bee stings are more dangerous than vespid stings), sting site (stings in the head, neck or throat are more severe than stings at extremities), atopy, pre-existing cardiovascular and respiratory disease and use of some medications such as β -blockers and angiotensin-converting enzyme inhibitors. [7]

Similarly, time interval between stings and number of stings also influences the natural history of the reaction. A short interval between stings increases the risk of systemic reaction to the later one. With increasing interval, the risk generally declines but remains in the range of 20 – 30 % even after 10 years. On the contrary, being stung very frequently appears to induce the tolerance, for eg., 45 % of beekeepers who are stung < 25 times a year had a history of systemic sting reactions when compared to those with > 200 stings per year. [47] However, patients who have had multiple stings at one time may have experienced true anaphylaxis and not a toxic response. [74]

Mastocytosis, even in non-allergic patients, may predispose subjects to a severe reaction after an insect. A high baseline tryptase, a marker enzyme of mast cells, represents a risk factor for an anaphylactic reaction after a sting in allergic patients. [7] Mast cell product, β -tryptase, degrades the allergens and IgE antibodies protecting the patients from venom toxicity. However, this theoretically beneficial effects of mast cells on downregulating allergic immediate type reactions are insufficient to protect patients with mastocytosis from severe anaphylaxis. [75] Mastocytosis has also been observed as a risk factor for venom allergy, side effects of VIT and VIT treatment failure. [76]

Geography, climate, temperature, insect behaviour and certain outdoor occupations, hobbies and activities influences the risk of receiving a sting. Beehives and wasp nests located in the near vicinity of residences and work places are also accounted as the risk factors. [47]

7. Current treatment and management strategies

There are, in general, four treatment and management strategies for hymenoptera venom allergic patients, viz., a) avoidance, b) pharmacotherapy, c) immunotherapy, and d) anti-IgE therapy. [73] Undoubtedly, venom immunotherapy (VIT) is a highly effective and the only specific treatment which aims to maintain a low risk of a systemic reaction during and after treatment, prevent morbidity and mortality and improve health-related quality of life. The treatment for systemic allergic reactions to wasp venom consists of emergency treatment and specific allergen immunotherapy. [77, 78] Anaphylaxis is a life-threatening emergency needing immediate treatment in wasp stings. The first steps of treatment are cardiopulmonary resuscitation (severity grade IV), epinephrine administration (grade II or above; usually given intramuscularly on the scene), and as soon as possible, shock positioning and the placement of intravenous catheter (all grades). Further component of basic treatment are:

- Oxygen administration (grade II or above)
- Intravenous glucocorticoid administration, and
- The administration of H₁ blocker (all grades).

Depending upon the clinical manifestations, fluid administration, renal replacement therapy and treatment of airway obstruction may be indicated. [58]

Venom immunotherapy, specific or native, should be recommended for adults with systemic reactions and for children with moderate to severe reactions, but there is no need to prescribe it for children who only present skin reactions after an insect sting, especially if the exposure is very sporadic. The recommendations differ by country. Importantly, the risk-benefit relationship should be assessed in each case. [77] In the case of cross-reactions as a cause for double positivity, the treatment with the venom of the primarily responsible insect alone would be sufficient. However, in case of true double sensitization, immunotherapy with both venoms is indicated. [62]

7.1. Venom immunotherapy as a treatment tool—Its indication and contraindication

Venom immunotherapy, being the only specific treatment for wasp sting allergy, is indicated based on clinical history of systemic reaction, positive diagnostic test and knowledge of the history and risk factors for a severe reaction. [78] The immunotherapy is administered with all of the venoms or allergens that tested positive. VIT begins with a very low dose of venom or allergen (0.05 µg) with incremental doses every week until a plateau or maintenance dose of 100 µg (300 µg if mixed vespoid venom) is achieved. Maintenance injections are then given monthly in their 1st year and then perhaps every 6 – 8 weeks for subsequent 3 years or until the skin test becomes negative. [55, 79]

Several mechanisms have been proposed to explain the beneficial effects of immunotherapy, such as, the induction of allergen-blocking IgG (IgG1 and IgG4) antibodies, reduction in specific IgE over the long term, reduced recruitment of effector cells, altered T cell cytokine balance (shift from Th2 to Th1), T cell anergy and the induction of regulatory T cells. Though the mechanism is not well clarified, the effects of immunotherapy on allergen-specific T-cell response are well affected. Following successful immunotherapy, there is reduction in allergen-specific T-cell and eosinophil recruitment in response to allergen challenge. Parallel to it, there is a shift in the balance of expression of Th1 cytokines (eg, interferon γ) and Th2 cytokines (eg, IL-4 and 13). Since cytokines formed by the Th2 subset governs the production of IgE antibodies, altered cytokine balance from Th2 to Th1 reduces the production of specific IgE and thus, may contribute to the treatment of allergic symptoms. Venom immunotherapy also induces the T regulatory 1 (Tr1) cells that produce IL-10 which is regarded as an immunosuppressive or regulatory cytokine. [55] Tr1 cells are able to produce high levels of IL-10 and TGF- β . TGF- β is involved in increasing tolerance to aeroallergens. IL-10 is responsible for VIT-induced IL-4 decrease. Tr1 cells are generated *in vivo* in humans during the early course of immunotherapy, suggesting that high and increasing doses of allergen induce Tr1 cells in humans. [80] Increased IL-10 is also responsible for specific T cell anergy. The anergic T cells do not secrete the cytokines required for the priming, survival and activity of the effector cells. [81] Specific immunotherapy is frequently associated with a rise in allergen-specific IgG antibodies and a modest reduction in specific IgE titres. Immunotherapy induced IgG antibodies have IgE blocking activities. However, the change does not always correlate with the clinical improvements. The induction of IgG antibodies with blocking activities can inhibit allergen-induced IgE-mediated release of inflammatory mediators from mast cells and basophils, thus preventing immediate symptoms. They can also inhibit IgE-mediated allergen

presentation to T cells which might reduce Th2 activation and the subsequent release of Th2 cytokines, hence providing protection against the development of the late-phase response. Besides, allergen specific IgG antibodies may also directly affect IgE production by memory B cells, either through competition with IgE for allergen binding or through the crosslinking of inhibitory IgG receptors on the surface. [82] The allergen-specific immunotherapy, thus, with following underlying mechanisms shows various effects. Very early effects are related to mast cell and basophil desensitization, intermediate effects are related to changes in allergen-specific T cells and late effects are related to B cells and IgE as well as mast cells, basophils and eosinophils. [83]

VIT study analyses have shown that the protection rate is better with vespid than honeybee extract and is better in children than in adults. Factors that influence the risk of incomplete protection after VIT include honeybee venom allergy, venom dose which sometimes needs to be increased to up to 200 µg, elevated baseline serum tryptase concentration, mastocytosis, or repeated side effects during VIT. [78]

7.1.1. Indications and contraindications

Venom immunotherapy is indicated both in children and adults with a history of severe systemic reactions including respiratory and cardiovascular symptoms and documented sensitization to the respective insect with either skin tests and/or specific serum IgE tests. It is not indicated when neither skin testing nor specific IgE antibodies showed venom sensitivity, or for unusual reactions such as vasculitis, thrombocytopenia, nephrosis etc. It is not recommended for large local reactions in either children or adults. For systemic non-life threatening reactions such as urticaria, erythema, pruritus etc., other factors may influence the decision to initiate venom immunotherapy such as risk of high exposure to culprit insects, concomitant cardiovascular disease, mastocytosis etc.. Contraindications are, in general, with the patients using medications such as β -blockers and ACE inhibitors. β -blockers can aggravate anaphylactic side reactions and delay the recovery. Patients who are receiving β -adrenergic blocking medications might be at increased risk if they experience a systemic reaction to an allergen immunotherapy injection. Hence, their use should be adopted cautiously. This is rarely a problem in immunotherapy for respiratory allergies in young patients who are seldom on β -blockers. Life threatening and potentially fatal reactions from insect venom allergy are most often observed in older patients who are frequently suffering from cardiovascular disease and therefore are on β -blockers. Though there is a good theoretical basis for contraindication of β -blockers during immunotherapy, the clinical evidence is modest. No severe reactions to immunotherapy or sting re-exposure were observed in patients receiving β -blockers and hence concluded that combination of β -blockers with venom immunotherapy may be indicated in venom-allergic patients with severe cardiovascular disease because the risk of the stinging insect hypersensitivity is greater than the risk of an immunotherapy-related systemic reactions. [78, 84-86] However, if the β -blockers are taken off for venom immunotherapy, it should be done under careful supervision, including monitoring of blood pressure and electrocardiogram during the dose-increase phase. [81]

Similarly, life-threatening anaphylaxis is being observed in venom-allergic patients who were on ACE inhibitors and receiving venom immunotherapy or had a sting provocation. It is prudent that venom allergic patients should avoid taking ACE inhibitors unless absolutely necessary and in patients in whom venom immunotherapy is indicated, temporary discontinuation of the ACE inhibitors prior (24 hrs before) to each venom injection may prevent subsequent adverse reactions. [86, 87]

8. Parasitic wasp—A biological pest controller

Hymenoptera sub-order Apocrita is sub-divided into Aculeata and Parasitica. Parasitica is essentially all of the parasitic wasps that have an ovipositor with the sole purpose of egg laying, either near the host, on the host (ectoparasitism) or inside the host (endoparasitism). Parasitic wasps are important natural enemies to a vast array of insects that are considered pests in the agricultural system. Hence, they are the unique bioindicators of the diversity of the hosts they attack. They are sensitive to ecological perturbations, especially pesticides and hence are ideal candidates for conservation studies. [3, 88] These parasitoids produce a wide range of venoms that could serve as models for developing new classes of synthetic chemical insecticides. [89]

In order to successfully parasitize the host, parasitoid wasps generate and release gene products at oviposition that alter the physiology of the host. The females internally store poisonous venom in their ovaries and secretory organs. [89] In endoparasitoid species, various stages of the host can be parasitized (egg, egg-larval, larval, pupal and adult) depending upon the species. However, in ectoparasitoid species, venoms often induce paralysis and/or regulate host development, metabolism and immune responses which benefit the externally developing parasite. Venom proteins from endoparasitic wasps are predominantly involved in regulation of host physiology and immune responses alone or in combination with other factors of maternal origin such as polydnavirus (PDVs) or virus-like particles present in the venom itself or produced in the ovaries or ovarian fluids. [90] Parasitoid venom has evolved to produce both immunosuppressive and stimulatory properties to create the optimal host environment for parasitoid offspring. Female parasitoid regulates the host without totally suppressing the host's physiology and creating an unregulated host environment in order to maximize progeny production. The parasitoid's progeny is subjected to unregulated microbial attack and invasion if the host become immuno-compromised. Thus, the female wasp evades the host immune response without compromising the host's immune system by injecting the venomous mixture that includes virus-like particles such as polydnavirus, in the host body at the time of oviposition. [89]

8.1. Mechanism of actions

8.1.1. Immune suppression

Immune system plays a major role in physiological interactions between the hosts and the parasites. Hosts will react to the invasion of foreign agents by producing antimicrobial

peptides and reactive oxygen species by contact epithelia, fat body and hemocytes. Phagocytosis, encapsulation and nodule formation by specialized hemocytes are more directly involved in the defence mechanism. The female wasp avoids this host immune response by introducing a venomous mixture, often together with virus-like particles and/or polydnaviruses, into the host body just before the oviposition. Such venom injection suppresses the host immune system by targeting two major host defense cascades, viz. a) the phenoloxidase cascade and b) the coagulation cascade. [88]

a. Phenoloxidase Cascade

Melanization of pathogens and damaged tissues forms a major innate defence system in invertebrates which is controlled by a multicopper oxidase enzyme, phenoloxidase. The enzyme results in the deposition of melanin around the damaged tissues or intruding object. This physical shield around the intruder prevents or retards its growth. More importantly, during melanin formation, highly reactive and toxic quinone intermediates are also formed which are involved in production of cytotoxic molecules such as superoxides and hydroxyl radicals that could aid in the killing of intruders. Reduction of phenoloxidase activity is, thus, a well-known strategy of parasitoid wasp, although it has so far been reported in braconid and ichneumonid species. [88] Various venom proteins from different parasitoid species are rendered possible inhibitory function on the phenoloxidase pathway, viz. serine protease such as serpin-1J [91], serpin-3 and serpin-6 [92]; cysteine-rich protein such as Cvp1 [93], LMPI-1 and LMPI-2; [94] and Egf1.0. [95]

b. Coagulation Cascade

The clotting reaction of insect hemolymph is a part of insect immunity. Female wasps make a feeding tube after injecting the venom into the host in order to connect the interior of the pupa with the exterior of the puparium. Inhibition of coagulation of host hemolymph is thus crucial since the wasp feeds on the host fluid drawn up through the feeding tube. Besides, the young parasitoid larva also feeds on the host's body fluids by grabbing and puncturing the host's integument with its mandibles. [88] Besides the action of maternal venom, larval secretions are also known to inhibit clotting system. Various contributory proteins identified for the inhibition of coagulation cascade of the hosts are reprodysin-type zinc metalloproteinase, [96] calreticulin, [97] and serine protease inhibitors. [98]

8.1.2. Immune stimulation

Parasitic wasps suppress the immune system of its hosts rather than completely shut down. A complete shut down of host's immune system would be potentially deleterious to the parasitoid's developing progeny, which conceivably would be forced to compete with microorganisms for host nutrients, could directly infect/attack the wasp offspring and/or contaminate their nutritional source. This is why the parasitoid's venom selectively suppresses the host's immunity and allows or even stimulates certain antimicrobial defences. Few venom proteins that contribute to immune stimulation in parasitized hosts are chitin-binding like proteins with antibacterial and wound healing properties; β -1,3-Glucan recognition protein (β GRP) whose interaction with β -1,3-Glucan initiates the activation of prophenoloxidase

cascade; dipeptidyl peptidase IV (DPP IV) that could possibly function as a stimulator of venom related processes, including immunity; and, angiotensin I-converting enzyme, a processing enzyme involved in the synthesis of antibacterial peptide. [99]

8.1.3. *Developmental arrest*

Insect host are modulated in various ways to provide favourable environment for the development of the wasp parasitoid's progeny. Typically, the host undergoes a developmental arrest and later on dies away after the parasitoid has become independent of its host. Teratocytes inhibits growth, alter development and affect the related physiological parameters. Likewise, polydnavirus coinjected with venom induces a variety of physiological changes in development and immunity through their gene expression. Other molecules such as EpMP3 metalloprotease, lysosomal arylsulphatase, and calreticulin-like venom proteins appears to be a critical agent in developmental arrest. [100]

8.1.4. *Increment of lipid levels*

Adult parasitoid wasps lack the capacity of lipogenesis. Growth and survival of parasitoid larvae is largely dependent on host. Thus, parasitoid ensures a suitable environment for their developing offspring by manipulating their host's physiology or development—either by influencing the feeding habit of host and its growth (koinobionts) or by arresting the host's development (idiobionts). Parasitism has been found to induce changes in the amount of amino acids, proteins, pyruvate and carbohydrates within the host in both endo- and ectoparasitoids. The female parasitoid wasp strategically creates the resource for its progeny's development which includes an increase in whole body lipid content, an enhanced metabolism of fat body triacylglycerols and a higher level of free fatty acids in the hemolymph. Larva in its early stages mainly consumes host hemolymph while in its later stages directly feeds the host's fat body. These changes in the nutritional content of the host are brought about by a variety of mechanisms such as teratocytes, wasp venom and associated mutualistic viruses. Teratocytes are cells derived from the dissociation of the embryonic membrane of parasitoid wasp which play an important role in nutritional exploitation by parasitoid larvae. They attach themselves to the host's fat body and contribute to its disruption (teratocyte-specific carboxylesterase involved in hydrolysis of host lipids, a fatty acid-binding protein involved in transport of host fatty acids to the developing parasitoid larva, two collagenases that may attack the collagen sheath surrounding the fat body to permit selective release of fat body cells). In addition to the effects of teratocytes, the parasitoid larva itself is capable of bringing about physiological changes, eg in hormone and lipid levels, aiding its own development. [101]

Maternal substances which are transferred along with the egg during oviposition provide an additional way of host exploitation. These include different types of viruses such as poly-DNAviruses, non-polyDNAviruses and virus-like particles as well as venom. Parasitoid venom contains various proteins that may disrupt the host's fat body such as matrix metalloproteinase [102] that causes lysis of cells and release of lipid particles from the fat body. Wasp venom triggers PLC activation resulting in IP₃ formation and subsequent Ca²⁺ release from

mitochondria which in turn activates PLA₂. The activated PLA₂ in turn increases the fatty acid level in host fat body. [30]

Hence, the multitude of mechanisms employed to manipulate host metabolism ensures an abundance of lipid resources during development, providing parasitoid larvae with a unique opportunity to consume host lipids instead of synthesizing them *de novo*. Manipulation and consumption of host lipids probably provides a selective advantage for parasitoid larvae, because *de novo* lipid synthesis is energetically expensive. [101]

8.1.5. Apoptosis

Venom for parasitoid wasps induces cellular injury and culminates in oncotic death. [88] Crude venom alone has been shown in *in vitro* assays to evoke disruption of plasma membrane integrity, blebbing, rounding, swelling and cell death thought to be linked to a G-protein dependent oncotic mechanism. [103] Various candidates in venom triggers the apoptotic cell death such as venom phenoloxidase, calreticulin, laccase, endonuclease G, and gamma-glutamyl transpeptidase-like venom protein. [88]

8.1.6. Nutritional functions

Nutritional and physiological milieu of the host is manipulated for the better nurturing of the parasitoid's offspring by the venom injected by the female wasp. For example, teratocytes of endoparasitoids have a secretory and nutritive function whereas venom of some ectoparasitoids changes the host metabolism to provide nutrients. [88] The discovery of trehalase in parasitoid wasp venom protein ensures the provision of glucose for developing wasp larvae from trehalose, which is the main reserve sugar in the hemolymph of flying insects. Several other digestive proteins in venom such as trypsin and other serine proteases, trypsin-like enzymes, lipase-like venom protein, and acid phosphatases are involved in assuring the optimal nutrition for its offspring. [88, 104, 105]

9. Conclusion

Wasp is a common name for any insect species of the order Hymenoptera and sub-order Apocrita, excluding bees and ants. A highly diverse group of insects, they are social or solitary, parasitic or predatory, phytophagous or carnivorous or omnivorous. The most primitive Hymenoptera possess ovipositors to insert eggs into plant tissues. In some parasitic groups, this structure and the glands associated with it have been modified to inject venom to paralyse other insects that they use for their developing larvae. These parasitic wasps are extremely beneficial to natural ecosystem and agriculture as biological pest controller. Their stings are not so painful to humans and are of none clinical significance.

Social wasps, however, are evolved with a venom system that is specialized as a defence weapon. The sting produces a range of clinical manifestations in humans—from simple skin allergic manifestation that do not require any medical treatment to fatal anaphylaxis and toxic

reactions where immediate medical intervention is utmost. Yellow jackets, hornets and paper wasps are three medically important stinging social wasps. Stinging social wasp venom comprises of various allergens, toxins and bioactive molecules that imparts physiological and pathological changes upon envenomation in humans. Immune-mediated and non-immune mediated mechanisms, both are involved.

Wasp stings are alkaline and are traditionally addressed by the application of vinegar or lemon juice to neutralize the venom. Besides, there are many local practices being observed, which might need scientific evidence to be proven, such as placing ice packs, freshly sliced cucumber, potato and onion on the sting sites and applying the aloe vera gel, garlic paste, ethanol and curds on the sting sites.

Wasp stings usually manifests allergic symptoms—normal local allergies to systemic anaphylaxis. Allergy, in general, is a public health threat of pandemic proportions today. Respiratory allergies, food and drug allergies and allergic reactions to insect venom are the commonly reported allergic incidents. Allergic patients not only suffer from the debilitating disease resulting in decreased quality of life, career progression and personal development but also constitute a significant burden on health economics and macroeconomics due to the days of lost productivity and underperformance. The symptomatic treatment for allergy are not sustainable which includes short-term symptom relieving or long-term anti-inflammatory drugs—the effect of which are suboptimal, relapse of the symptoms very shortly after ceasing daily use of medication even after years of a continuous and effective treatment, and the possible fear of adverse effect due to the long-term use of drugs. [106] These symptomatic medications as well imparts financial burden. Allergen-specific immunotherapy is an effective treatment used by allergists and immunologists for common allergic conditions, particularly allergic rhinitis/conjunctivitis, allergic asthma and stinging insect hypersensitivity [107] and can achieve substantial results for patients—improves the quality of life, reduces the long-term costs and burden of allergies and prevents the progression of allergic disease. Currently, it is the only curative treatment for Hymenoptera venom allergy. [106] However, various factors should be taken into consideration on a case-by-case basis, taking into account individual patient factors, before proceeding with the allergen specific immunotherapy such as the degree to which symptoms can be reduced by avoidance measures and pharmacological therapy, the amount and type of medication required to control symptoms, the adverse effects of pharmacological treatment and patient preferences. This form of therapy carries the risk of anaphylactic reactions, hence taking into consideration the indications and contraindications, it should be prescribed and practiced by physicians who are adequately trained in the treatment of allergy. Moreover, injections must be given under medical supervision in clinics that are equipped to manage anaphylaxis. [107] These requirements and preparations obviously make the allergen specific immunotherapy not so readily available and expensive but considering the beneficial effects of allergen specific immunotherapy and unsustainability of pharmacotherapy, allergen specific immunotherapy emerges as a reliable curative approach.

Parasitic wasps have their own significant space in this ecosystem and contribute as a biological pest controller. They are not only important in agricultural system, but as well have significant role in controlling the disease spreading, such as the toxicity of venom can inhibit the multiple

developmental stages of several mosquitoes and house flies, both of which are a major vector of human disease. The utility of their venom components provide a promising frontier in development of new classes of bioinsecticides.

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Pharmacology of Adenosine Receptors and Their Signaling Role in Immunity and Inflammation

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Additional information is available at the end of the chapter

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1. Introduction

Since the late 1920s, the importance of the physiological role of adenosine triphosphate (ATP) and its metabolites, mainly adenosine, has been clear. In 1970, strong evidence now suggests ATP as a neurotransmitter in nonadrenergic, noncholinergic (NANC) nerves supplying the gut. Initially, the hypothesis of purinergic transmission encountered some resistance, however, this concept is now widely accepted and purines are considered powerful extracellular messengers in peripheral and central nervous system and to non-neuronal cells, including immune, and inflammatory cells. Implicit in the purinergic hypothesis was the presence of purinoceptors. The first evidence for the existence of adenosine receptors, responsible for the physiological effects of adenosine, was only published in the beginning of the 1970s. Throughout the 1990s and 2000s adenosine receptors were cloned, and the mechanisms of signal transduction mediated by these receptors were described. Currently, it is well known that adenosine activates four G protein-coupled receptors named A_1 , A_{2A} , A_{2B} and A_3 . The A_1 and A_3 receptors preferentially interact with members of the $G_{i/o}$ family of G proteins, lowering the intracellular levels of cyclic adenosine monophosphate (cAMP), whereas the A_{2A} and A_{2B} receptors interact with members of the G_s family of G proteins, elevating of intracellular cAMP.

Throughout the 1980s and 1990s, several studies demonstrated that ATP metabolites, especially adenosine, are important signaling molecules, and adenosine receptors are important molecular targets in inflammation and immunity. During inflammation, the generation of the appropriate immune response can itself cause considerable damage and thus requires effective regulation. It has been suggested that this regulation of the immune system requires the

sensing of specific signals called “danger signals”. Thus, in the context of purinergic signaling, it has been proposed that the regulation of the immune system requires at least two ‘danger’ signals, the first (ATP) indicating the presence of danger from pathogens or other injurious events and leading to the activation of immune cells and defensive effector function and the second (adenosine) indicating the danger from overactive immune cells and triggering the downregulation of the proinflammatory activities of the immune system. These discoveries increased the interest in the purinergic signaling pathways, and there was an increase in publications studying the effects of adenosine and inosine in inflammation and immunity (Figure 1 A and B).

Substantial evidence demonstrates that adenosine receptors are expressed in most inflammatory cells and may therefore modulate different steps involved in inflammatory and immune responses. Moreover, several recent studies have indicated that inosine, a metabolite of adenosine, once believed to be an inert metabolite, can exert many immunomodulatory actions through adenosine receptors, mainly the A_2 and A_3 receptors. In this regard, adenosine receptors have become potential therapeutic targets for the treatment of several pathologies in which inflammatory modulation is a key component. In this regard, adenosine and inosine can be considered molecules with valuable therapeutic potential, performing the desired effect with minimal side effects, such as those present in therapy with adenosine (Adenocard®) for the treatment of paroxysmal supraventricular tachycardia (facial flushing, chest pressure, hyperventilation, dizziness, numbness and tingling). Moreover, no studies clearly demonstrate the side effects of inosine. However, it is important to warn that the purines can play deleterious effects not observed clinically, depending on the target tissue and the receptor that is activated according to figure 3.

Thus, this chapter was designed to highlight the importance of ATP metabolites, especially adenosine and inosine, and their modulatory effect on inflammation through the activation of adenosine receptors. In addition, we aimed to provide updated information about the pharmacology of adenosine receptors, especially about its proinflammatory versus anti-inflammatory effects. Furthermore, we are able to clarify the overall effect of adenosine and inosine in different inflammatory diseases. Finally, we intend to present a short overview concerning the advances in drug development targeting adenosine receptors.

2. The ATP metabolic pathways

Carbohydrates, lipids and proteins, also called “metabolic fuels” are constantly being oxidized to provide energy. Glucose is generally the primary energy source for cellular metabolism. It is catabolized by the following three main processes: glycolysis, the tricarboxylic acid (TCA or Krebs) cycle and oxidative phosphorylation, which lead to the production of ATP, the final energy-rich product that is used in many different active processes in an organism. Macromolecule synthesis, muscle contraction, active transport of ions and thermogenesis are some of the key processes that require energy. Since the mid-1920s, when ATP was discovered as a substrate used in muscle contraction, knowledge about this high-energy molecule has

constantly been expanded. The literature has shown that many aspects of cellular metabolism are directly linked to the production and consumption of ATP and has also emphasized its importance in purinergic signaling mechanisms. In this context, taking into account the importance of ATP in the maintenance of homeostasis and the evidence indicating the role of its metabolites in the control of immunity and inflammation, understanding ATP metabolism in the body has become more imperative [1].

2.1. The degradation of ATP and formation of ADP, adenosine and inosine

In situations of high energy demand such as inflammation and hypoxia, ATP may be converted into adenosine monophosphate (AMP) in the intracellular environment through a reaction dependent on ATPase and adenylate kinase. AMP can be converted into adenosine by the intracellular enzyme 5-nucleotidase and thereafter can be transported to the extracellular environment via bidirectional nucleoside transporters. ATP in the extracellular environment can activate P2₋type receptors in the surroundings or generate adenosine via ecto-5-nucleotidase (NT5E), the primary enzyme responsible for ATP metabolism under physiological conditions [2]. In the extracellular space, ATP and ADP are converted to AMP through the ectonucleoside triphosphate diphosphohydrolase-1 (CD39). The second step for extracellular adenosine formation is the ecto-5'-nucleotidase (CD73) conversion of extracellular AMP into adenosine. During inflammation and hypoxia, an increase in the activity and expression of adenosine deaminase and in its binding partner, CD26, has also been demonstrated [3]. This increase promotes adenosine conversion into inosine within seconds, terminates adenosine signaling and can thus initiate inosine signaling. Inosine can be converted into hypoxanthines and uric acid (UA) by purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO), respectively [4, 5]. Current studies using mice lacking the CD39 and CD73 genes have revealed the importance of these enzymes in contributing to extracellular adenosine generation in different organs and situations [5]. In agreement with those studies, certain CD39 polymorphisms increase ATP and ADP, lowering extracellular adenosine levels, which can lead to increased susceptibility to inflammatory pathological conditions such as inflammatory bowel disease (IBD) and multiple sclerosis (MS) [6, 7]. Furthermore, the loss-of-function mutation of CD73 in humans is suggested to be the basis for the development of peripheral arterial calcifications, indicating that adenosine generation can be vasoprotective [8, 9]. Currently, it is known that after adenosine is released from cells or generated in extracellular space, it diffuses into the surroundings, where it binds to adenosine receptors (A₁, A_{2A}, A_{2B} and A₃) on adjacent cells. Finally, after adenosine generation and receptor activation, adenosine diffuses away from the receptor and is rapidly transported into the intracellular space mainly through equilibrate nucleoside transporters (ENT-1 and ENT-2) [5, 10, 11].

3. ATP metabolites as danger signals: the role of adenosine and inosine

During inflammation, infection or hypoxia, the generation of the appropriate immune response can itself cause considerable damage and thus requires effective regulation [12]. It has been suggested that this regulation of the immune system requires the sensing of specific

signals called “danger signals” [13-15]. Although the immune response can be activated by recognizing the signatures of foreign pathogens, collectively called pathogen-associated molecular patterns (PAMPs), it is also able to respond to endogenous host molecules to trigger inflammatory responses. Most of these are produced as a result of cell death or injury or by tumor cells; they include degradation products of the extracellular matrix (ECM), heat-shock proteins and high-mobility group box 1 (HMGB1) proteins, UA crystals, amyloid- β and oxidized LDL (Ox-LDL), which act as stimulators for pattern recognition receptors (PRRs) and have been referred to as danger-associated molecular patterns (DAMPs) [15, 16].

Extracellular ATP and UA are well-characterized dangers signals, likely released from cells as a consequence of cell damage or nonapoptotic cell death. The exposure of local cells to elevated extracellular ATP and monosodium urate crystals has been described as proinflammatory because it activates P2X7 receptors, NALP3 (a member of NOD-like receptors, also called cryopyrin) and caspase-1 [17-19]. This activation leads to the processing and release of interleukin 1 β (IL-1 β) and results in inflammation [14, 20]. Furthermore, the elevation of extracellular ATP has been demonstrated to guide circulating neutrophils to the inflammatory microenvironment and can function as a “find-me signal” to attract inflammatory cells (particularly phagocytes) and direct the inflammatory response [12, 21].

Recent studies have shown that in situations of inflammation, trauma or hypoxia when extracellular ATP concentrations are elevated, there is an increased expression of ectonucleotidases that rapidly convert ATP/ADP into adenosine, terminating the proinflammatory effects of ATP [12]. Thus, in the context of purinergic signaling, it has been proposed that the regulation of the immune system requires at least two ‘danger’ signals, the first (ATP) indicating the presence of danger from pathogens or other injurious events and leading to the activation of immune cells and defensive effector function and the second (adenosine) indicating the danger from overactive immune cells and triggering the downregulation of the proinflammatory activities of the immune system [13, 22].

The effects of adenosine in different tissues may depend upon the repertoire of adenosine receptors present on the cell surfaces [22]. In this context, the A_{2A} and A_{2B} receptors have been described as the receptors most involved in the control of immunity and inflammation [13, 22, 23]. By binding to A_{2A} and A_{2B} receptors, adenosine triggers cAMP elevation in T cells, which results in the activation of CREB/ATF (cAMP-responsive element (CRE)-binding protein/activating transcription factor), an immunosuppressive mechanism [12, 23, 24]. This activation has been shown to trigger Treg cell activation, the production of anti-inflammatory cytokines such as TGF- β and IL-10 and inhibit the functional response of TCR-activated T effector cells, reducing the secretion of IL-2 and IFN- γ [24]. Moreover, similar to adenosine, the metabolite inosine is also known to exert wide ranging anti-inflammatory effects, which include inhibition of proinflammatory cytokines, chemokine production and protection from septic shock, colitis and acute lung injury [25-27]. Some studies have shown that inosine can stimulate adenosine A_{2A} receptors and is protective in models of concanavalin A-induced liver damage, endotoxin-induced sepsis [28, 29] and TNBS-induced colitis [30]. In this context, although there is no description of inosine as a danger signal in the current literature, some studies have described inosine as tissue protective. Taking into account that it can activate the same sensors (receptors)

as adenosine, we speculate that inosine might be an additional danger signal that could work with adenosine to dampen inflammation.

In summary, the immunosuppressive effects of adenosine have been broadly described in the literature as a “retaliatory metabolite” [31] or an “engineer” of inflammation [22], indicating that adenosine can manipulate the intensity and the time course of inflammatory process in vivo and suggesting biochemical control of immunity. These events appear to be biologically coordinated and may constitute a homeostatic mechanism of tissue integrity. Therefore, the failure of this protective mechanism may contribute to beginning and perpetuating chronic inflammation response.

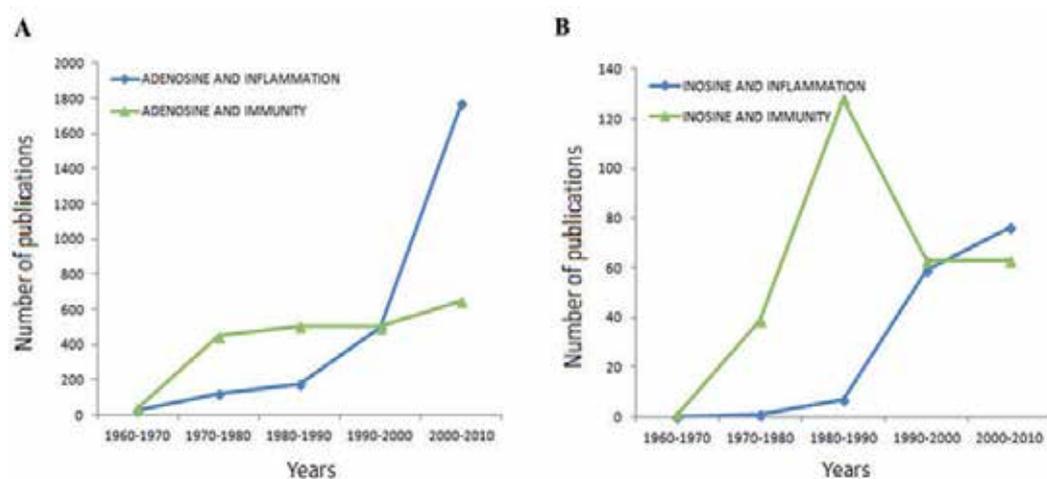


Figure 1. The number of publications regarding the investigation of adenosine and inosine effects in inflammation and immunity since the 1960s.

4. Adenosine receptors and inflammation: proinflammatory versus anti-inflammatory effects

4.1. A₁ adenosine receptors

The adenosine A₁ receptor is coupled to the G_{i/o} family of G proteins, lowering the intracellular levels of cAMP [32, 33]. Activation of A₁ receptors leads to increased intracellular Ca⁺² levels due to the stimulation of phospholipase C, which in turn promotes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (Figure 2). Moreover, the enhancement of intracellular calcium can activate certain enzymes, such as protein kinase C (PKC), phospholipase D (PLD), phospholipase A₂ (PLA₂) and others [10, 33].

The recently reported crystal structure confirms that adenosine receptors display the typical topology of GPCRs, a common central core domain consisting of seven transmembrane (TM) helices numbered from 1 to 7 that are composed of 20-27 amino acids and that are largely α -helical. The TM domains are also slightly bent and linked by three intracellular (IL-1, IL-2 and IL-3) and three extracellular (EL1, EL2, and EL3) loops [34]. The A_1 receptor amino acid sequence varies from 324 to 328 residues [35]. In 1992, Ijzerman and colleagues highlighted the role of two histidine residues in ligand binding, one located in TM6 and one in TM7 [36]. This result was in agreement with the first mutagenesis data on adenosine receptors, published in the same year, performed on bovine A_1 receptors, which demonstrated that residues His251 (6.52) and His278 (7.43) were important for ligand binding. The same study also revealed that the mutation of histidine residues to leucine led to a decrease in ligand affinity, especially His278 [37]. In 1994, two mutagenesis studies suggested a role of residue 270 (7.35, isoleucine or methionine in the bovine/canine receptor) in the binding of *N*6-adenine substituted compounds (A_1 receptor agonists) and the importance of residue 277 (7.42, threonine in human/bovine receptor) in the recognition and interaction of the adenosine ribose moiety [38, 39]. It has also been demonstrated that the Pro25Leu (1.48) mutation and substitution of Leu88 (3.33), Thr91 (3.36), and Gln92 (3.37) by alanine reduces the affinity for *N*6-unsubstituted adenosine derivatives [39]. In the same study, it was also demonstrated that the Gly14Thr (1.37) mutation increases the receptor's affinity for agonists, suggesting the constitutively active form of this mutant receptor [40]. Finally, analysis of the Thr277Ala (7.42) mutation by an allosteric enhancer suggested an allosteric role for this residue [41].

There is no consensus regarding the effects of A_1 receptors in the inflammatory response; some studies suggest proinflammatory effects, while others suggest anti-inflammatory effects. The A_1 receptors are expressed in leukocytes. At submicromolar adenosine concentrations, the activation of these receptors in human neutrophils produces a proinflammatory response by promoting chemotaxis and adherence to the endothelium [42, 43]. In lymphocytes, A_1 receptor antagonism contributes to adenosine's anti-inflammatory effects by reducing the expression of intracellular adhesion molecule-1 (ICAM-1), production of IL-12 and IFN- γ and lymphocyte proliferation [44]. In addition to these data, various studies with selective agonists and antagonists demonstrated the proinflammatory effects of A_1 receptors in different inflammatory models, some of which are summarized here. During acute pancreatitis induced with cerulein or taurocholate in rats, the selective A_1 receptors agonist CCPA (2-chloro-*N*6-cyclopentyladenosine) produced an increase in leukocyte infiltration and interstitial edema in pancreatic tissue, which was attenuated by FK-838 (6-oxo-3-(2-phenylpyrazolo[1,5- α]pyridin-3-yl)-1(6*H*)-pyridazinebutanoic acid), a selective A_1 receptor antagonist [45]. A_1 receptor antagonism was also protective in the lungs; treatment with DPCPX (8-cyclopentyl-1,3-dipropylxanthine), an A_1 receptor antagonist, prevented endothelial damage, neutrophil migration and alveolar injury in a model of ischemia reperfusion in the lungs [46]. In another interesting study, the A_1 receptor antagonist DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine) decreased the area of cardiac necrosis and improved ventricular function in a canine model of myocardial ischemia reperfusion, most likely due to inhibition of neutrophil chemoattraction [43]. Pretreatment with KW3902, a selective A_1 receptor antagonist, preserved hepatic architecture, decreasing the infiltration of neutrophils into hepatic tissue in a hepatic ischemia

reperfusion injury model in dogs [47]. Collectively, these findings demonstrate that the A₁ receptor is an important target in inflammation and that antagonists may be efficacious as anti-inflammatory drugs.

In opposition to such data, studies using pharmacological (selective A₁ receptor agonist or antagonists) and genetic tools (knockout animals) have shown that activation of the A₁ receptor can promote anti-inflammatory effects. CCPA, an A₁ receptor agonist, presented a protective effect in a mouse model of renal ischemia reperfusion, an effect reverted by DPCPX, a selective A₁ receptor [48]. Studies performed with knockout mice confirmed the renal protective effects of the A₁ receptor [49] and also revealed the protective effects of this receptor in other tissues and inflammatory conditions because the absence of the A₁ receptor promotes proinflammatory effects in the lungs, enhancing leukocyte migration and levels of cytokines, including IL-4 and IL-13 [50]. In the central nervous system (CNS), A₁ receptor knockout animals exhibited severe demyelination and axonal injury, involving the activation of macrophages and microglial cells [51]. In sepsis induced in mice, the A₁ receptor knockout animals had a higher degree of renal dysfunction induced by higher release of pro-inflammatory cytokines [52]. A previous study described the mechanism by which activation of A₁ receptor leads to anti-inflammatory effects, which involves phosphorylation of ERK, MAPK and Akt (Figure 2), all of which are involved in the upregulation of cytoprotective genes and also increase the phosphorylation of heat shock protein (HSP) 27, a molecular chaperone that prevents the denaturation and aggregation of cellular proteins, a cytoprotective effect [53]. In summary, the lack of consensus regarding the existence of the proinflammatory and anti-inflammatory effects of the adenosine A₁ receptor could be explained assuming that the A₁ receptor can activate intracellular signaling pathways that result in tissue injury or protection, through proinflammatory or anti-inflammatory effects, respectively, because the activated pathways depend on the following:

- the species/tissue/organ and the stage/progression of injury;
- Predominant inflammatory cell type as a function of species;
- Intracellular signaling and desensitization mechanisms as a function of species or cell/tissue/organ.

4.2. A_{2A} adenosine receptors

Most A_{2A} receptors are coupled to the G_s protein family. A subset, preferentially located in the striatum, is coupled to the G_{oif} protein family. It is well established that the biological effects triggered by A_{2A} receptors are due to enhancement of cAMP production followed by adenylyl cyclase activation. The increase of the cAMP level stimulates cAMP-dependent kinase (PKA) (Figure 2), which, in turn, activates several pathways through calcium channels, potassium channels, cAMP responsive element-binding (CREB), mitógeno-activated protein kinase (MAPK) and phospholipase C (PLC) activation [23, 54].

The A_{2A} receptor structure is very similar to other adenosine receptors. However, it differs in the four disulfide links observed at the extracellular level, which are critical for the packing and stabilization of the restricted conformation of the seven transmembrane helices. Another difference concerns the A_{2A} receptor length of the C-terminal region, which consists of

approximately 120 residues [55]. In 1995, Kim et al. published the results of site directed mutagenesis experiments on A_{2A} receptors [56], revealing the essential role of some residues for ligand interaction, particularly Phe182 (5.43), Asn253 (6.55), Ile274 (7.39), and Ser281 (7.46). The key role of His250 (6.52), Ser277 (7.42) and His278 (7.43) was also confirmed. After 1995, several mutagenesis studies revealed some of the amino acids residues involved in direct or indirect interaction with ligands or in allosteric regulation. It was observed that the conserved Glu 1.39 is critical for agonist but not antagonist binding [57, 58]. Glu13 (1.39) and His278 (7.43) were found to be critical for the allosteric regulation of A_{2A} receptors [59], and Gln89 (3.37) was suggested to play an indirect role in ligand binding, while Ser281 (7.46) mutation to asparagine improved agonist affinity [60]. Additional studies were carried out to analyze the role of loop residues of A_{2A} receptors, revealing the importance of Glu151 and Glu169 (EL2) for ligand binding [61]. Unlike A_1 receptors, there is a considerable consensus regarding the effects of A_{2A} receptors on the inflammatory response. A broad range of investigations using *in vivo* and/or *in vitro* approaches have provided evidence that A_{2A} receptor activation limits inflammation and tissue damage, therefore playing an anti-inflammatory role [13, 31]. The A_{2A} receptor signaling in suppressing inflammation is related to cAMP-increased levels, which also have a well-known immunosuppressive effect on immune cells. Corroborating these data, the fact that A_{2A} receptors are expressed on most cells of the immune system is crucial to its anti-inflammatory properties [62]. Thus, the anti-inflammatory properties of these receptors are due, at least in part, to the prevention of the activation of effector cells of the immune system. For example, they may prevent neutrophil migration and interfere in the activity of proteins on the surface of neutrophils and endothelial cells that are crucial to the adhesion and migration of the former into the inflammatory site. In these sense, A_{2A} receptor activation inhibits the adherence of *N*-formyl methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils to the endothelium [63] and downregulates Mac-1 [64], β_2 -integrin [65] and L-selectin [66]. Furthermore, activation of the A_{2A} receptors also downregulates the activity of other endothelial cell surface proteins, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) [67], alpha 4/beta 1 integrin (VLA4) [68] and platelet cell adhesion molecule [69]. On the other hand, the anti-inflammatory properties resulting from A_{2A} receptor activation are due to the reduction of the release of inflammatory mediators such as IL-12, $INF\gamma$, $TNF-\alpha$, and IL-4 from important immunomodulatory cells such as neutrophils, monocytes, dendritic cells and T lymphocytes [70, 71].

Many of the anti-inflammatory effects of A_{2A} receptors are mediated by adenosine itself. It is known that inflammatory tissue damage is accompanied by the accumulation of extracellular adenosine in inflamed sites due to its release from non-immune and immune cells. Because endogenous adenosine levels are elevated during an inflammatory process and endogenous adenosine can activate A_{2A} receptors to attenuate inflammation and tissue damage, strategies that aim to foment adenosine production and increase its availability to activate A_{2A} receptors present extraordinary anti-inflammatory potential. In this regard, an interesting study demonstrated that the immunosuppressive effects of activated Treg lymphocytes could be in part related to adenosine production in the extracellular environment through both CD39 (an ectonucleoside triphosphate diphosphohydrolase that converts ATP and ADP to AMP) and CD73 (an ectonucleoside that converts AMP to adenosine) expressed on the surface of these

cells. Moreover, the Treg immunosuppressive effects have been shown to be modulated by A_{2A} receptors [72]. In a model of ischemia reperfusion liver injury, treatment with ATL146e (4-(3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl)-cyclohexanecarboxylic acid methyl ester), an agonist of A_{2A} receptors, was associated with decreased inflammation and protection from liver damage. When A_{2A} receptor knockout mice were subjected to the same insult, the effectiveness of ATL146e was lost [73]. Later, similar approaches showed the protective effect of the A_{2A} receptor in models of myocardial infarction, acute lung injury and spinal cord compression injury [74] [75, 76].

In another study, the same A_{2A} receptor agonist, ATL146e, was associated with decreased leukocyte infiltration, inflammatory mediator production and necrosis in a model of inflammatory bowel disease [77]. In a model of LPS-induced lung injury, treatment with the A_{2A} receptor agonist ATL202 was associated with decreased recruitment of neutrophils to the lung together with reduced cytokine levels and pulmonary edema, whereas A_{2A} receptor knockout mice treated with LPS showed an increase in neutrophil recruitment [75]. In a mouse model of allergic lung inflammation, treatment with an A_{2A} receptor (CGS-21680) agonist resulted in diminished pulmonary inflammation [78]. On the other hand, A_{2A} receptor knockout mice have been shown to have higher lung inflammation compared to wild-type mice [79].

4.3. A_{2B} adenosine receptors

The A_{2B} receptor couples to Gi-type G proteins, leading to the inhibition of adenylate cyclase upon receptor activation [80]. In some cell systems, such as HEK-293 and HMC-1 mast cells, A_{2B} receptors are also coupled to phospholipase C via the action of Gq proteins in increasing intracellular Ca^{2+} levels [81-83]. The A_{2B} receptor has also been described to be involved in the ERK1/2 [80, 83, 84] and p38 MAPK pathways in mast cells (Figure 2) [85]. Furthermore, a link between A_{2B} receptor signaling and the arachidonic acid signal transduction pathway leading to vasoconstriction has been described [83, 86]. Among the four adenosine receptors, A_{2B} is the least well characterized receptor, mainly due to the lack of suitably specific ligands [87].

The A_{2B} receptor has low affinity for most agonists, so only some agonists are useful, including the non-selective and related adenosine derivative NECA [88] and the highly selective A_{2B} agonist BAY60-6583 [89]. Conversely, highly selective A_{2B} antagonists have been developed, such CVT-6883 and an A_{2B} -specific antagonist radioligand, [3H]PSB-603, which has high potency and specificity across species, including rodents and humans [90]. The A_{2B} receptors have a closely related structure to A_{2A} receptors, and sequence analysis of the human A_{2A} and A_{2B} receptors show an overall identity of 58% and a similarity of 73%. The most conserved residues are found within the seven transmembrane domains. [83]. In contrast to the A_{2A} receptor, the A_{2B} receptor possesses the longest extracellular loop 2 (ECL2) of all four adenosine receptor subtypes, with four cysteine residues – the highest number found in any GPCR – of which three (C154, C167, C171) are homologous to the three (C146, C159, C166) found in the A_{2A} receptor [83]. These cysteine residues are involved in disulfide bonds formed between the ECL and transmembrane domains (TM) of GPCRs and have been reported to play an important role in ligand binding affinity and receptor stability and function.

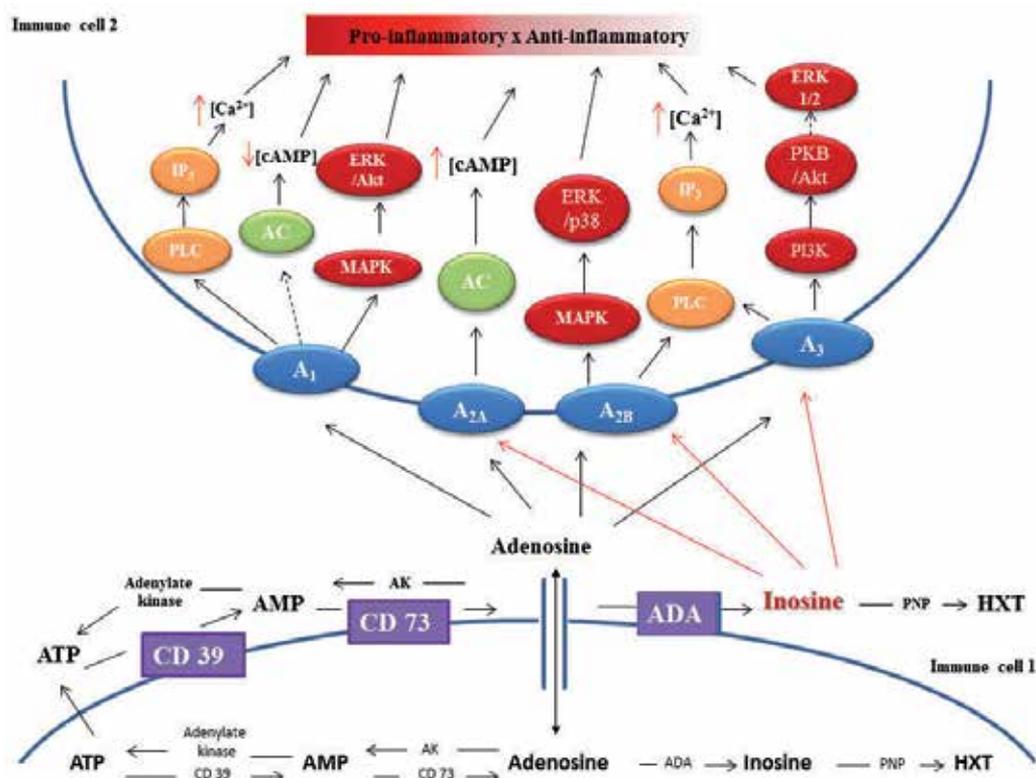


Figure 2. A brief summary of the role of the purinergic system in inflammation. A₁, adenosine A₁ receptor; A_{2A}, adenosine A_{2A} receptor; A_{2B}, adenosine A_{2B} receptor; A₃, adenosine A₃ receptor; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; ERK1/2, extracellular signal-regulated kinases 1 and 2; IP₃, inositol triphosphate; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; PLC, phospholipase C; PI3K, Phosphatidylinositol 3-kinase; ATP, adenosine triphosphate; AMP, adenosine monophosphate; HXT, hypoxanthine; AK, adenosine kinase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; ENTs, equilibrate nucleoside transporters; CD 39, ectonucleoside triphosphate diphosphohydrolase; Inhibition → ; Activation - - →

Studies of mutagenesis, X-ray receptor analysis and radioligand binding have shown that C78 in transmembrane domain 3 (TMD3) and C171 in ECL2 form an important disulfide bond related to ligand-binding affinity and receptor expression levels [83, 91, 92]. In agreement with these data, in a mutagenesis model in which the complete ECL2 of human A_{2B} was exchanged for the ECL2 of the A_{2A} receptor, the mutant A_{2B} (ECL2-A_{2A}) receptor mice had increased affinity and selectivity for A_{2B} agonists and antagonists as well as receptor-mediated cAMP accumulation, indicating that ECL2 is a ligand binding site [93]. ECL2 also seems to contribute to the low affinity of ligands to A_{2B} receptors because it has been shown to have between 4 and 10 more amino acids than A_{2A} receptors. The longer ECL2 may, in some cases, partially block the entrance of ligands into the binding pocket and explain why A_{2B} receptors typically show lower affinity for adenosine and adenosine derivatives (agonists) than A_{2A} receptors, which have a shorter ECL2, which may facilitate the entrance of ligands to the binding pocket [83]. In addition, residues Thr42, Val54 (2.51) and Phe84 (3.31) are also involved in the binding site;

mutation to alanine, leucine or serine, respectively, decreased agonist binding [94]. As mentioned above, in many tissues, A_{2B} receptors are considered low-affinity receptors with mostly low expression levels, so adenosine needs to reach higher concentrations to activate them [83, 95].

During hypoxia, a critical role of HIF-1 in the transcriptional induction of the adenosine A_{2B} receptor has been suggested. In such conditions, as in $A_{2B}^{-/-}$ receptor mice, the presence of A_{2B} receptors attenuates hypoxia-induced increases in vascular leakage, mainly in the lungs, a protective effect [95]. Accordingly, Yang and colleagues reported that A_{2B} -null mice present augmentation of proinflammatory cytokine levels, such as TNF- α , upregulation of vascular adhesion proteins and leukocyte migration in response to LPS-induced acute inflammation. Conversely, in adenosine-deficient mice and bleomycin-induced lung inflammation, the antagonist CVT-6883 attenuated pulmonary inflammation [96]. Moreover, A_{2B} activation resulted in an increase in IL-8 [85], IL-1 β , IL-3, IL-4, IL-13 and IgE leading to mast cell and Th2 and B lymphocyte activation [97]. Mice treated with CVT-6883 and MRS-1754 or A_{2B} -null mice developed less severe experimental autoimmune encephalomyelitis (EAE) with reduced IL-6 release and Th17 cell differentiation [98].

In spite of the structural similarity, adenosine has been recognized as the natural ligand of A_{2B} receptors, but inosine was not [99, 100]. However, a recent study conducted by our group has shown evidence that inosine can reduce both acute pleural inflammation and allergic lung inflammation through a mechanism that involves both A_{2A} and A_{2B} receptors, suggesting a regulatory role of inosine and A_2 in these process [11, 101]. Taking into account the lack of information about the A_{2B} receptor and its involvement in several inflammatory diseases, it may be a candidate target for future therapeutic intervention

4.4. A_3 adenosine receptors

Studies have demonstrated that both adenosine and inosine can activate A_3 receptors in vitro and therefore can directly modulate its activation and biological effects [99, 100]. Similar to other adenosine receptors, the A_3 receptors is a GPCR with seven transmembrane domains (TM). It is coupled to classical second-messenger pathways such as inhibition of adenylyl cyclase, stimulation of PLC and calcium mobilization [33, 102-104].

In the heart, A_3 mediates cardioprotective effects through the activation of K_{ATP} channels that are coupled to RhoA-phospholipase D signaling, mediating the protection of cardiac myocytes from ischemia [104]. With regard to cardiac protection, signaling through cAMP response element-binding protein (CREB)-Bcl2 pathways after A_3 receptor activation has also been described [104]. In addition, like other adenosine receptors, A_3 receptors are coupled to MAPK and lead to stimulation of extracellular signal-regulated kinases (ERK1/2), which relates A_3 receptor activation to cell growth, survival, death and differentiation [33, 80].

A_3 receptor activation in melanoma cells stimulates PI3K-dependent phosphorylation of protein kinase B (PKB/Akt), leading to the reduction of basal levels of ERK1/2 phosphorylation, which in turn inhibits cell proliferation (Figure 2) [105]. Consistent with these data, treatment with IB-MECA induced inhibition of tumor growth [33, 104], although in the hypoxic condi-

tions that occur in solid tumors, A_3 activation mediates angiogenesis and cell survival through increased HIF-1 α and VEGF production [106]. Moreover, the A_3 receptor is involved in adenosine and inosine induced-mast cell degranulation [99, 107]; inhibition of endotoxin-induced neutrophil granulation and TNF- α production [108, 109]; reduction of T cell tumoricidal activity and enhancement of natural killer cell cytotoxicity [104]; reduction of neuropathic pain [110]; and augmentation of bone marrow cells proliferation favoring myeloprotection [111]. One of the characteristics of the A_3 receptor is the rapid (within a few minutes) desensitization through phosphorylation by G-protein-coupled receptor kinase 2 (GRK2) at the intracellular threonine residues within the C-terminal domain after exposure to an agonist, which can limit the agonist's effect [112-114].

A_3 receptors exhibit the lowest degree of identity among species compared with other adenosine receptor subtypes [115]. In a study that used a combination of mutagenesis, radioligand binding, functional activity and molecular modeling approaches, a mutation of TMD3 His95 (3.37), which is conserved in A_3 receptors in various species including humans, sheep and rats, resulted in a decreased affinity of agonists and antagonists and is therefore considered critical to ligand binding. The same was observed when His272 (7.43) and Asn250 (6.55) were mutated. Moreover, residues Tyr243 (6.48) and Lys152 (ECL2) were needed only for antagonist binding [115], and Trp243 (TM6) is involved in the functional activation of the A_3 receptor based on the impairment of the coupling of the receptor to the G protein in the Trp243 mutant [115, 116]. Furthermore, molecular modeling suggested that Trp243 is in the binding pocket and might occupy a strategic position as a switch in the TM6-mediated structural transition from the resting to the active state [115]. Given the involvement of A_3 receptors in important pathological process, as described above, several studies evaluating the structure-activity relationships of agonists and antagonists with A_3 receptors have been conducted to identify new potential drugs for the treatment of deleterious diseases.

5. Adenosine and inosine regulation on leukocyte function

The action of adenosine and inosine on the immune system is determined by their bioavailability and adenosine receptor expression in immune cells. The rapid release of adenosine in response to tissue-disturbing stimuli such as hypoxia, ischemia, inflammation or trauma and the rapid conversion of adenosine into inosine have been reported to modulate the function of leukocytes, a basic constituent of immune system [117]. In this section, we discuss adenosine and inosine modulation of immune cells and the corresponding involvement of adenosine receptors.

Neutrophils are the first immune cells recruited to inflamed sites by a combination of chemo-attractant cytokines and adhesive interactions between leukocytes and the vascular endothelium [118, 119]. Adenosine mainly acts on A_{2A} receptors, signaling through cAMP-PKA-dependent pathways. It decreases neutrophil activation, neutrophil-mediated injury to endothelial cells, production of reactive oxygen species, PAF, and leukotriene B_4 , secretion of cytokines such as TNF- α and chemokines such as MIP-1 α /CCL3, MIP-1 β /CCL4,

MIP-2 α /CXCL2 and MIP-3 α /CCL20 and expression of adhesion molecules such as selectins and integrins [31, 120-128]. Similar to adenosine, inosine also interferes with neutrophil activation by blocking formyl-Met-Leu-Phe-induced superoxide generation [31, 100], neutrophil migration and release of the proinflammatory cytokines TNF- α and IL-1 β during acute inflammation, acting through A_{2A} and A_{2B} receptors [11]. A₃ and A_{2A} receptors are involved in the reduction of superoxide anion generation [127, 129]. A₃ receptors can also direct neutrophil migration [130]. Interestingly, the adenosine interaction with A₁ and A₃ receptors induces G-CSF production, which leads to a stimulatory effect on bone marrow cells, suggesting that adenosine is a chemoprotective agent that could restore the number of leukocytes and neutrophils to normal levels after chemotherapy [131].

Macrophages and dendritic cells are phagocytes that are widely dispersed throughout the body at portals of microorganism entry [31]. They initiate an effective innate immune response against microbes by recognizing pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) [119, 132]. This response involves pathogen processing and is regulated by the secretion of several cytokines and activation of lymphocytes and other immune cells.

In this context, studies have demonstrated that adenosine inhibited TNF- α , IL-6 and IL-8 release from macrophages stimulated with thioglycollate or LPS via A_{2A} receptors, although the A_{2B} receptors seems to play an underlying inhibitory role that may contribute to anti-inflammatory action [133, 134]. Some studies have demonstrated that adenosine can increase IL-10 production and release through a mechanism involving adenosine A_{2A} receptor-CEBP β axis activation [135]. The augmentation of the production of IL-10 and the decrease in systemic endotoxin-induced levels of TNF- α , IL-12, MIP-1 α and IFN- γ have also been ascribed to inosine [136]. Current data have shown that activation of both A_{2A} and A₃ receptors inhibited IFN- γ and IL-12 release after TLR-4 stimulation by LPS [137-139]. Adenosine and inosine were described to decrease M1 activation and the release of mediators, reducing Th1 response, by interfering with TLR-4 activation. Moreover, there is growing evidence that A_{2A} receptor activation also reduces the TLR-2, 3, 7, and 9 responses in M1 macrophages, upregulating VEGF and IL-10 expression and therefore polarizing macrophages into an M2-like phenotype, called M2d, which favors an angiogenic switch and plays a protective role in ischemia [70, 140].

Adenosine also interferes with mature dendritic cell stimulation by producing a dose-dependent inhibition of TNF- α and IL-12 release, whereas it enhanced the secretion of IL-10, preventing tissue injury mediated by innate immune mediators during overwhelming immune response [141]. Furthermore, dendritic cells matured in the presence of adenosine had a reduced capacity to induce T helper 1 (Th1) polarization of naive CD4⁺ T lymphocytes, evidence that adenosine diminishes the capacity of dendritic cells (DCs) to initiate and amplify Th1 immune responses [141].

Mast cells are resident in all normal tissues, where they are believed to play an important role in tissue homeostasis, wound healing and host defense, particularly in terms of bacterial infection. When activated, they secrete the autacoid mediators histamine, prostaglandin (PG)D₂ and leukotriene (LT)C₄, which contribute to the pathophysiology of many diverse diseases including rhinitis and asthma [142-144].

Rodent and human mast cells express the A_{2A} , A_{2B} and A_3 receptors [145-149]. It has been reported that adenosine and inosine binding to A_3 receptors expressed in mast cell membranes induces degranulation and release of vasoactive mediators [99, 107]. Accordingly, the release of reactive mediators following A_3 activation in mast cells is directly related to the bronchoconstrictor effects observed after topical administration of adenosine in the airways of patients with asthma and chronic obstructive pulmonary disease [109, 150]. In contrast, inosine has no effect on airway caliber, indicating that bronchoconstriction is a specific response to adenosine [151]. Engagement of the A_3 receptors on rodent mast cells mediates degranulation and cell migration through a mechanism that involves phosphoinositide 3-kinase (PI3K) or protein C kinase (PKC) activation and an increase in intracellular Ca^{2+} [146, 147]. Moreover, A_1 and A_{2B} receptor activation has been related to the release of histamine, IL-8, IL-4 and IL-13 by mouse and human mast cells [97, 152-154]. In contrast to the A_1 and A_{2B} receptors, A_{2A} activation results in the suppression of histamine and tryptase release from human mast cells [155]. A_{2A} and A_{2B} receptors provide a balanced control mechanism for mast cell activation. It is possible that at low concentrations of adenosine, only the 'off' signal provided by the engagement of the higher affinity A_{2A} receptors prevails, thus downregulating mast-cell mediator release. Conversely, in situations in which high concentrations of adenosine are reached, such as in asthma and COPD [156], the low-affinity A_{2B} receptor becomes activated, resulting in significant mast cell degranulation [108, 109, 157]. Although the effects of adenosine regarding mast cell activation are well described, there is a lack of information regarding the effects of inosine on the activation of the A_3 receptor. Recently, a study from our group suggested that inosine can activate A_{2A} , A_{2B} and A_3 receptors and decrease mast cell migration during allergic pulmonary inflammation, suggesting that inosine can modulate allergic inflammation [101].

Lymphocytes cells play a vital role in the induction of adaptive immune responses and in steering them toward particular effector phenotypes [119]. Several pieces of evidence suggest that adenosine and inosine generated in the site of inflammation can modulate lymphocyte function [23, 24, 28]. Experimental data demonstrated that in ConA-induced liver injury (an *in vivo* model mediated by T cells), inosine inhibited hepatocyte apoptosis and reduced the accumulation of proinflammatory cytokines (e.g., TNF- α) and alanine transaminase in $A_3^{+/+}$ but not $A_3^{-/-}$ or $A_2^{-/-}$ mice, suggesting the endogenous inosine can influence inflammatory responses and indicating the importance of A_3 receptors in controlling liver injury [28].

The extracellular adenosine generated in inflammatory or hypoxic environments affects regulatory T cell lymphocytes (Treg) through the activation of A_{2A} receptors. Treg cells are a specialized population of $CD4^+$ T cells implicated in the regulation of immune responses, maintenance of immunological self-tolerance and protection from excessive inflammatory damage [24, 158]. A_{2A} receptor stimulation expanded the Treg population [159] coordinated by coexpressed CD39 ecto-ATPase/ADPase and CD73 ecto-5'-nucleotidase and generating adenosine pericellularly [72, 160]. The CD39- and CD73-mediated generation of extracellular adenosine might provide Treg cells with the capacity to directly inhibit DC and T effector cells by activating their respective cAMP-elevating A_{2A} receptors [160]. Consistent with these data, the A_{2A} receptor has been described to suppress the development of T-cell receptor (TCR) - stimulated naive T cells into both Th1 and Th2 cells [135], interfering with early development

as well as the late effector stages of Th1- and Th2-cell responses [135]. The activation of A_{2B} receptors seems to indirectly inhibit Th17 activation. A recent study using EAE in mice indicated that blocking A_{2B} receptors with specific antagonists, such as CVT-6883 and MRS-1754, alleviated the clinical symptoms of EAE and protected the CNS from immune system-mediated damage. Confirming this hypothesis, the deletion or blockade of A_{2B} receptors inhibited Th17 cell differentiation by blocking IL-6 production from APCs such as dendritic cells. The activation of phospholipase C β -protein kinase C and p38 MAPK pathways was found to be involved in A_{2B}-mediated IL-6 production, suggesting A_{2B} as a target for the development of anti-multiple sclerosis drugs and indicating that adenosine might participate in regulation of this pathology [98].

6. Overall effect of adenosine and inosine on inflammatory diseases

6.1. Asthma and COPD

A great deal of evidence suggests that adenosine plays a detrimental role in asthma and COPD and perhaps other chronic airway disorders [161]. The potential role of adenosine triphosphate and adenosine in the pathogenesis of asthma and COPD has been supported by the bronchoconstrictor effects observed after their topical administration in the airways of patients with these diseases and the lack of reaction in healthy subjects [150, 162-164]. However, there is some controversy about the role of adenosine during allergic reactions in the airways, such as asthma. When administrated topically, as mentioned above, it seems to trigger airway hyperactivity, but the administration of non-selective and selective agonists of different adenosine receptors can suppress (such as adenosine A_{2A} receptors) [165-167] or contribute with airway allergic inflammation, as described below.

Treatment with CGS21860, a selective A_{2A} receptor agonist, reduced the number of leukocytes in bronchoalveolar lavage fluid, protein content and eosinophil peroxidase activity in Brown Norway rats immunized and challenged with OVA, a compound similar to the glucocorticosteroid budesonide [165]. Moreover, treatment with NECA, a non-selective adenosine A₂ receptor agonist, reduced the total leukocyte infiltration and eosinophilia in a model of allergic airway inflammation [166]. Interestingly, inosine but not adenosine was described to reduce leukocyte infiltration into the lungs, Th2 pro-inflammatory cytokine levels and improve pulmonary mechanics in OVA-induced airway inflammation through a mechanism involving the A_{2A} and A₃ receptors [101]. Consistent with these data, inosine and its stable analogue INO-2002 were described to reduce LPS-induced airway inflammation [26, 168]. Several studies have hypothesized that the bronchial response to adenosine observed in asthma and COPD in humans can be attributed to an indirect mechanism involving mast cell activation, likely via A_{2B} or A₃ receptors and the release of mediators such as histamine and leukotriene (LT)C₄ [109, 150, 169]. These are low-affinity receptors that can modulate the deleterious effect of high concentrations of adenosine in chronic airways diseases [108, 109, 157]. Likely, adenosine signaling through the A_{2B} receptor also plays a role in asthma development, promoting the upregulation of pro-inflammatory cytokines, leukocyte migration and airway

remodeling (Figure 3) [170, 171]. Other studies have described a pro-fibrotic role for A_{2B} receptor signaling, which results in the differentiation of human pulmonary fibroblasts into collagen-producing myofibroblasts, increasing the production of the pro-fibrotic molecule fibronectin in alveolar epithelial cells [147, 161, 172]. These data demonstrate the potential involvement of A_{2B} receptors in the remodeling and fibrosis observed in asthma and COPD. Although the A_3 receptors appear to reduce inflammation and eosinophil activation in humans, in mice, A_3 activation induces mast cell degranulation and increases inflammation, activating eosinophils and mucus production [161, 173].

The literature data describes differences in mast cell expression between rodents and humans and maybe it can explain the different effects of adenosine in modulating these cells during asthma and allergy. The adenosine A_3 receptor that was expressed in mast cells in rodents [174], have recently been described to be expressed in human lung mast cells [175]. While the expression of adenosine A_{2A} and A_{2B} receptors in rats was not described; in mice, both were expressed in bone marrow derived mast cells. The A_{2A} receptors were also described in cardiac mast cells, in mice [174]. In humans, both receptors have been described in lung mast cells and in human mast cells line HMC-1 [174, 176]. The adenosine A_1 receptor were not described to be expressed in mice and humans but recent data have shown that agonists of adenosine A_1 receptor can potentiate human cultured mast cell activation, suggesting a modulatory effect [152, 177, 178].

The A_1 receptor is also described to have pro or anti-inflammatory effects on airway inflammation. Treatment with an A_1 receptor antagonist [179] or with antisense oligodeoxynucleotides targeting this receptor reduced the bronchoconstrictor responses in an allergic rabbit model [180]. Conversely, in knockout $A_1^{-/-}$ mice, an increase in transmigration of polymorphonuclear cells and microvascular permeability in comparison to wild type mice was observed in a model of LPS-induced lung injury, suggesting a possible protective effect of the A_1 receptor in airways [181]. The engagement of adenosine receptors on inflammatory and pulmonary cells appears to play an important role in regulating chronic lung disorders such as asthma and COPD, so the complete and full characterization of adenosine receptor subtype distribution in the airways and their specific role in the response to adenosine and inosine in health and disease is important for the development of new therapies to treat asthma and COPD [161].

6.2. Skin inflammation and wound healing

The skin is a highly specific immune defense organ. Physical, chemical or immune-specific insults rapidly evoke cellular responses, characterized by the increased expression of a wide range of pro-inflammatory mediators [182]. Controlling the extent of an immune response is thus a major challenge for maintaining skin integrity, which is of paramount importance for host survival [183]. In this context, several lines of evidence have shown that adenosine and adenosine receptors contribute to the regulation of skin inflammation.

A recent study showed that activated Treg cells can produce adenosine in a CD39-dependent manner and abrogated the ear-swelling reaction induced by 2,4,6-trinitro-1-chlorobenzene (TNCB), indicating a role of adenosine in the Treg cell-induced suppression of contact

hypersensitivity responses. Moreover, the same study demonstrated that adenosine's effects involve the impairment of effector T cell adhesion to inflamed endothelium and downregulation of E- and P-selectin in the vascular endothelium [184]. A complementary study of IL-10-deficient (IL-10^{-/-}) Tregs showed impaired adenosine production, which contributes with their inability to suppress contact hypersensitivity responses, indicating that the reduced suppressive effects observed may not be exclusively attributable to the lack of IL-10 production [185]. Several lines of evidence indicated that adenosine's effects on skin are mediated by the activation of adenosine receptors.

The activation of A₁ receptors has been demonstrated to decrease the numbers of circulating neutrophil granulocytes and ear swelling in a model of stress-induced contact hypersensitivity response [186]. In addition to receptor A₁ activation, activation of receptor A_{2A} was described to reduce leukocyte activation and to prevent ischemia reperfusion wound formation in a rat model of a pressure ulcer [187]. Evidence from experiments performed on A_{2A} receptor knockout mice and with CGS21680 demonstrated that the A_{2A} receptor is the main adenosine receptor subtype involved in wound healing [149, 188]. In addition, histological analysis of mice treated with the same agonist showed faster re-epithelialization and increased matrix deposition, fibroblast density and vascularity in the granulation tissue of the agonist treated wounds as soon as 3 days after injury [188]. A study from the same group revealed that treatment of human microvascular endothelial cells (HMVEC) with the selective A_{2A} receptor agonists CGS21680 and MRE0094 (Sonedenoson) favors vascular tube formation by cultured HMVEC and downregulated the antiangiogenic matrix protein thrombospondin 1 (TSP1) secretion by these cells, indicating that A_{2A} activation induces angiogenesis [189]. Furthermore, treatment with MRE0094 increased the rate of wound closure in comparison to recombinant human platelet-derived growth factor (Becaplermin gel), an agent currently used to promote the healing of diabetic ulcers, indicating the importance of A_{2A} receptors in wound healing [190]. Moreover, the A_{2A} and A_{2B} receptors were described to contribute to tissue formation because their activation leads to enhanced fibroblast and endothelial cell migration [191].

Adenosine seems to have a fibrogenic role in the skin. A study using ADA-deficient mice reported a direct fibrogenic effect of adenosine on the skin, and pharmacological treatment with the A_{2A} receptor antagonist ZM-241385 prevented the development of dermal fibrosis by reducing dermal collagen content and the expression of profibrotic cytokines and growth factors (Figure 3) [192]. The data mentioned above are interesting and strongly suggest that adenosine and A_{2A} receptors have important modulatory effects in skin homeostasis. Although several studies have shown a role for adenosine in the skin, nothing has been found regarding a role for inosine. Additional studies addressing the real role of the purinergic system in the skin would be extremely useful to improving wound management and care, as well as controlling chronic inflammatory diseases in skin.

6.3. Arthritis

Arthritis is the term used to designate a particular pathological condition that encompasses a constellation of more than 100 diseases, among which osteoarthritis (OA) and rheumatoid arthritis (RA) stand out. OA is the most common adult joint disease and is increasing in

frequency and severity, with an estimated US prevalence of more than 25 million affected adults [193]. It is characterized by gradual loss of articular cartilage and is therefore being considered a slowly progressing degenerative disease. The etiology of arthritis involves biochemical and genetic factors as well as repetitive mechanical injury, which has been proposed as the critical mechanisms contributing to alterations in the normal functional activities of chondrocytes, the main cellular component of hyaline cartilage, disrupting chondrocyte–matrix associations and culminating in the initiation and progression of OA [194].

In early OA, a transient proliferative chondrocytes response (clonal growth) occurs along with increased synthesis of the cartilage matrix as an early repair attempt and increased synthesis of catabolic cytokines (such as IL-1, TNF- α and IL-18) and matrix-degrading enzymes (such as metalloproteinases, especially collagenases, and aggrecanases). Fibroblast- and macrophage-like cells in the synovia also generated catabolic cytokines in response to breakdown products from the damaged cartilage. All these events contribute greatly to the local loss of proteoglycans and cleavage of type II collagen, which initially occurs at the cartilage surface, contributing to water content increases and loss of tensile strength in the cartilage matrix as the lesion progresses [195, 196]. It is characterized by several inflammatory cascades, which all lead towards a final common pathway in which persistent synovial inflammation and associated damage to articular cartilage and underlying bone are present [197].

One inflammatory cascade that deserves attention in the pathogenesis of RA is the overproduction and overexpression of TNF- α . Interactions between T and B lymphocytes, synovial-like fibroblasts and macrophages are likely to be involved in TNF- α and IL-6 overproduction, contributing to both synovial inflammation and joint destruction [198]. In addition to inflammatory cytokines, rheumatoid factors are key pathogenic markers of classic RA. In this case, the immunoglobulins IgM and IgA are directed against the Fc fragment of immunoglobulin IgG, resulting in the formation of immune complexes, which are able to activate the complement system, initiating an immune response [199]. Adenosine has a known therapeutic potential against inflammatory joint diseases; studies have demonstrated its ability to limit synoviocyte [200] and chondrocyte [201] inflammatory responses and to minimize articular damage in adjuvant-induced models of arthritis in rats [202].

In an interesting study, Tesch and colleagues demonstrated that endogenously produced adenosine regulates articular cartilage matrix homeostasis *in vitro*. In this study, authors showed that the depletion of endogenous adenosine through exposure to adenosine deaminase (ADA) in cartilage explants resulted in cartilage matrix degradation, involving matrix metalloproteinases-3 and -13 (MMP-3, MMP-13), prostaglandin E₂ (PGE₂), and nitric oxide (NO) release. In addition to these data, this study suggested that endogenously released adenosine can regulate chondrocyte production of matrix-degrading enzymes and matrix loss, an effect believed to be in part mediated via A_{2A} receptors, because N⁶-[2-(3,5-dimethoxyphenyl)-ethyl]adenosine (DPMA, an A_{2A} receptor selective agonist) was able to prevent the release of PGE₂, NO and glycosaminoglycan (GAG) (Figure 3) [203]. Another *in vitro* study demonstrated that adenosine, N⁶-methyladenosine (a substituted adenosine derivative that is resistant to breakdown by adenosine deaminase), DPMA and 5'-N-ethylcarboxamidoadeno-

sine (NECA, a non-selective adenosine receptor agonist) suppressed NO production by LPS-stimulated equine chondrocytes [201]. The addition of exogenous adenosine and erythro-9-(2-Hydroxy-3-nonyl) adenine hydrochloride (EHNA, an adenosine deaminase inhibitor) further suppressed NO production by LPS-stimulated chondrocytes [201]. These two studies clearly demonstrate the protective effect of adenosine against degradation of articular cartilage *in vitro*, suggesting its potential to prevent joint damage and hence its effectiveness in the prevention of joint diseases such as osteoarthritis.

In the second half of the 1980s, remarkable attention was focused on investigating the role played by ADA in RA pathophysiology, which provided the first evidence of the role of adenosine in RA. These studies showed increased levels of ADA activity in the synovial fluid from patients with seropositive rheumatoid arthritis, suggesting a local release of this catabolic enzyme by cells within joints [204]. Subsequent investigations were aimed at characterizing the activities of different ADA isoforms in tissues, cell homogenates and serum samples obtained from patients with rheumatic disorders. The highest level of enzyme activity was found in lymphocytes and monocytes from patients with rheumatoid arthritis, and ADA-2 was the isoform specifically expressed in monocytes [205]. Iwaki-Egawa and colleagues observed a significant positive correlation between high activity of ADA isoforms in the synovial fluid of rheumatic patients and metalloproteinase-9 (MMP-9), an enzyme critical to the regulation of the cell matrix composition [206]. These results suggest that the high activity of ADA in the synovial fluid of patients with RA and consequently the reduction in local levels of adenosine are directly related to the development and maintenance of RA. In support of this hypothesis, Forrest and colleagues demonstrated that adenosine is able to suppress the elevated levels of proinflammatory cytokines such as TNF- α and IL-1 β in RA patients and that this effect appears to be mediated by adenosine receptors [207].

Recent studies have shown adenosine A₃ receptor upregulation in RA patients, suggesting the potential of this receptor as a therapeutic target. In agreement with these results, after oral treatment with CF101, a selective A₃ receptor agonist, a marked decrease in RA clinical manifestations including inflammation, pannus formation, cartilage destruction and bone reabsorption and lysis was observed [208].

6.4. Ischemia

Ischemia is defined as a lack of blood supply to an organ or tissue, resulting in cellular oxygen deprivation. Although ischemia itself is a serious condition, the phenomenon that seems to be the definitive treatment for ischemia, reperfusion of the ischemic tissue, can promote further tissue injury, especially after prolonged ischemia [209]. Thus, the tissue can be injured by both ischemic and reperfusion processes and can be defined as ischemia reperfusion (IR) injury. IR injury involves a complex cascade of events including oxidative stress, inflammation and interactions among many cell types [209]. Furthermore, it has widespread clinical relevance and is encountered in a variety of surgical settings (e.g., transplantation, cardiopulmonary bypass and aneurysm repair) as well as non-surgical settings (e.g., myocardial infarction, stroke, hemorrhage, trauma and shock).

One of the potentially most striking features of IR injury involves the generation of reactive oxygen species (ROS) (e.g., superoxide, peroxynitrite, hydrogen peroxide, and hydroxyl radical) [210, 211], especially during reperfusion of ischemic tissue, which, in turn, initiates an inflammatory cascade resulting in direct oxidative injury to cells and stimulation of pro-inflammatory mediators such as cytokines, chemokines and cell-adhesion molecules. Briefly, circulating and resident leukocytes, such as neutrophils, lymphocytes and macrophages as well as tissue resident cells, such as dendritic cells, contribute to the immune response to IR injury, particularly infiltrating neutrophils, which can impose significant tissue injury through ROS generation and further release of cytokines and proteases [212].

Strong evidence has indicated that cellular responses to hypoxia include robust increases in extracellular adenosine and signaling events through adenosine receptors. The hypoxic adenosine response in acute injury settings is able to promote tissue adaptation during hypoxia, including restoration of normal oxygen levels, enhancing metabolic ischemia tolerance and dampening inflammation [213]. Preclinical studies have shown that adenosine signaling is beneficial in ischemic acute injury in the lung [214, 215], kidney [216], heart [217], gastrointestinal track [218] and liver [73]. Some studies have demonstrated that chronic elevations of adenosine can contribute to tissue fibrosis in different organs including the lungs [170, 219], liver [220], skin [221], kidney [222] and following transplants [223]. Studies using CD39 and CD73 knockout mice, which lack both the enzyme that converts ATP to ADP/AMP and the enzyme that converts AMP to adenosine, and inhibitors of these enzymes demonstrated enhanced inflammation and tissue injury in models of hypoxia and ischemic injury [6, 224] concurrent with reduced production of adenosine. These results suggest that elevated extracellular levels of adenosine may display a protective effect in models of hypoxia and ischemia injury. An interesting strategy used to enhance extracellular levels of adenosine is the prevention of adenosine uptake by equilibrate nucleoside transporters (ENTs) [225].

Recently, Grenz and colleagues demonstrated that treatment with dipyridamole, an inhibitor of ENTs, led to increased adenosine levels in association with tissue protection in a mouse model of ischemic acute kidney injury [226]. Furthermore, genetic deletion of ENTs resulted in selective protection in ENT1^{-/-} mice. A more detailed analysis using adenosine receptor-knockout mice exposed to acute kidney injury showed that renal protection promoted by ENT inhibitors involves the A_{2B} adenosine receptor. In addition, Eltzschig and colleagues demonstrated that the A_{2B} receptor serves anti-inflammatory and tissue-protective roles in various acute injury tissue models associated with hypoxic or ischemic injury including the heart [217], lung [227], intestine [218] and kidney [226] using genetic (A_{2B} receptor knockout mice) and pharmacological (A_{2B} receptor agonists) approaches. In summary, adenosine receptor signaling in hypoxic or ischemia-reperfusion injury varies depending on the receptor activation and cell/tissue type. In general, A₁ and A_{2A} receptor activation has been shown to be protective in the lung, kidney, heart and liver, whereas the role of the A_{2B} and A₃ receptors remains obscure. A considerable number of studies both *in vitro* and *in vivo* have demonstrated the potential of inosine in preventing injury in different models of hypoxia or ischemia.

In the early 1980s, it was demonstrated that infusion of 4 mM of inosine presented a protective effect in fetal mouse heart organ cultures deprived of oxygen, a model of ischemic-like injury

[228]. At the CNS level, purine catabolite concentrations were monitored for up to 15 h in the auditory and somatosensory cortices of cats using microdialysis/HPLC and hydrogen clearance following middle cerebral artery occlusion (MCAo). MCAo led to the release of inosine and its metabolite hypoxanthine from the ischemic cortex in stroke animals, which reached maximum levels 1-2 h after the onset of ischemia [229]. Cerebral infarct (stroke) often causes devastating and irreversible losses of function, in part because of the brain's limited capacity for anatomical reorganization. In an animal cerebral ischemia model, which results from infarction in the right dorsolateral cerebral cortex and underlying striatum, continuous infusion of inosine 50 mM into the cisterna magna using osmotic minipumps (0.25 μ L/h) stimulated neurons on the undamaged side of the brain to extend new projections to denervated areas of the midbrain and spinal cord and consequently improved performance on several behavioral measures in adult rats [230].

Taken together, these data suggest that inosine promotes neuroregeneration after stroke. An interesting study recently published Shen and colleagues demonstrated that intracerebroventricular administration of inosine (25 nmol/L in 25 μ L) before middle cerebral artery occlusion in rats resulted in a higher level of locomotor activity (lasting up to 2 weeks after stroke) and less cerebral infarction [231]. In addition, they indicated that coadministration of a selective A_3 receptor antagonist, MRS1191, significantly attenuated inosine-mediated protection. Moreover, in the electrophysiological study, inosine antagonized glutamate-induced excitation in cerebral cortical neurons. In summary, the authors proposed that inosine may inhibit glutamate postsynaptic responses and reduce cerebral infarction via the activation of the A_3 receptor, presenting a neuroprotective action (Figure 3) [231].

6.5. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a common and lifelong disabling gastrointestinal disease that includes Crohn's disease (CD) and ulcerative colitis (UC) [232]. Worldwide UC incidence varies greatly, ranging from 0.5-24.5 /100,000 inhabitants, and CD ranges from 0.1-16/100.000 inhabitants, with the highest incidence in developed countries [233]. Genetic, environmental, and immunological factors interplay in a complex manner to contribute to the genesis of IBD. Generally, the presence of one or more genetic factors triggers an over-reaction of the host mucosal immune system to normal constituents of the mucosal microflora. This over-reaction involves either a Th1-type T cell-mediated inflammation in the case of Crohn's disease or a Th2-type T cell-mediated inflammation in ulcerative colitis. This inflammatory process leads to the release of multiple cytokines, including interferon (INF)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-1,, IL-6, IL-8, IL-12, IL-13,IL-17, and monocyte chemotactic protein (MCP)-1 [234]. These cytokines are responsible for the attraction and activation of neutrophils, eosinophils, mast cells and macrophages, which, in turn, produce large amounts of unstable chemical species such as reactive oxygen species (ROS) or oxyradicals (i.e., superoxide anions, hydrogen peroxide, hydroxyl radicals, and peroxyxynitrite), mediators that contribute greatly to the tissue injury seen in IBDs [235, 236].

In early stages of immune or inflammatory response, significantly elevated extracellular concentrations of adenosine are anti-inflammatory and tissue protective. Thus, one of the most

widely used strategies to study the effects of adenosine on different inflammatory processes involves inhibition of adenosine catabolism, mainly through inhibition of adenosine deaminase (increasing adenosine levels) or direct activation of adenosine receptors. Antonioli and colleagues have demonstrated, using a model of 2,4-dinitrobenzenesulfonic acid (DNBS)-induced colitis, that treatment with 4-amino-2-(2-hydroxy-1-decyl) pyrazole[3,4-d]pyrimidine (APP; novel adenosine deaminase inhibitor) or erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; standard adenosine deaminase inhibitor) for up to 7 days was able to increase food intake and weight gain and ameliorate macroscopic and microscopic inflammatory colonic alterations with a concomitant reduction of mucosal and plasmatic pro-inflammatory mediators such as TNF- α and interleukin-6 [237]. In the same vein, Siegmund and colleagues showed that treatment with {4-amino-1-(5-amino-5-deoxy-1- β -d-ribofuranosyl)-3-bromo-pyrazol[3,4-*d*] pyrimidine} (GP515), an inhibitor of adenosine kinase, the enzyme responsible for the conversion of adenosine to AMP, resulted in a significant improvement of mucosal morphology and clinical score (weight loss, stool consistency, and bleeding) as well as decreased IFN- γ concentration in the colonic tissue in dextran sulfate sodium (DSS)-induced colitis [238]. These results clearly demonstrate that increased levels of adenosine can attenuate mucosal inflammation in experimental colitis. The activation of adenosine A_{2A} receptors seems to be one of the main mechanisms involved in the effects of adenosine (Figure 3).

Odashima and colleagues studied the anti-inflammatory effects of ATL-146e in acute and chronic rabbit formalin-immune complex models of colitis and the SAMP1/YitFc mouse model of spontaneous ileitis. ATL-146e (20 and 40 μ g/kg, i.p.) significantly reduced the acute and chronic inflammatory index and tissue necrosis and prevented mortality. Furthermore, TNF- α , IFN- γ and IL-4 concentrations were significantly suppressed with ATL-146e treatment in supernatants from cultures of mesenteric lymph node cells of SAMP1/YitFc mice. Thus, these results support important anti-inflammatory actions of ATL-146e in the intestine, including the suppression of lymphocyte-derived cytokine-mediated pro-inflammatory responses, suggesting that the activation of A_{2A} receptor-mediated signaling through selective agonists may be a novel therapeutic approach for patients with IBD [77]. To this end, Cavalcante and colleagues evaluated the effects of a new selective A_{2A} receptor agonist (ATL 313) on *Clostridium difficile* toxin A-induced injury in murine ileal loops. ATL 313 treatment directly into ileal loops significantly reduced toxin A-induced secretion and edema, prevented mucosal disruption, neutrophil infiltration, TNF- α production, adenosine deaminase activity and prevented toxin A-induced cell death. Based on these findings, the adenosine system may represent a promising target for therapies for inflammatory intestinal disorders, either by manipulating its metabolism or through direct activation of its receptors, especially A_{2A} receptors [239].

6.6. Multiple sclerosis

MS is an autoimmune disease mediated by T cells that is characterized by CNS demyelination and neurodegeneration [98]. There are many other diseases that are associated with inflammation of the CNS, including meningitis, encephalitis, among others. However, one of the most common is MS. It affects more than 2.5 million people around the world and is characterized by the loss of neurological function, which occurs due to axonal demyelination and

represents the main symptom of the disease. Recurrences, commonly associated with increased lymphocyte infiltration of the CNS, make the patient increasingly weak over time [240-242].

Studies in mice using an EAE model demonstrated that adenosine signaling modulates the development of EAE. A_{2B} receptor blockade with CVT-6883 and MRS-1754 (both specific antagonists of A_{2B}) promoted a reduction in the symptoms of EAE, thus protecting against CNS immune response damage. Moreover, both deletion and A_{2B} receptor blockade promoted inhibition of the differentiation of Th17 cells due to the reduction of IL-6 production by APCs (dendritic cells), suggesting that the A_{2B} receptor is a potential new target for "anti-multiple sclerosis" drug action [98]. Post-mortem analysis of brain tissue from patients diagnosed with MS showed the presence of cells with high expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine in characteristic lesions of the disease [243, 244].

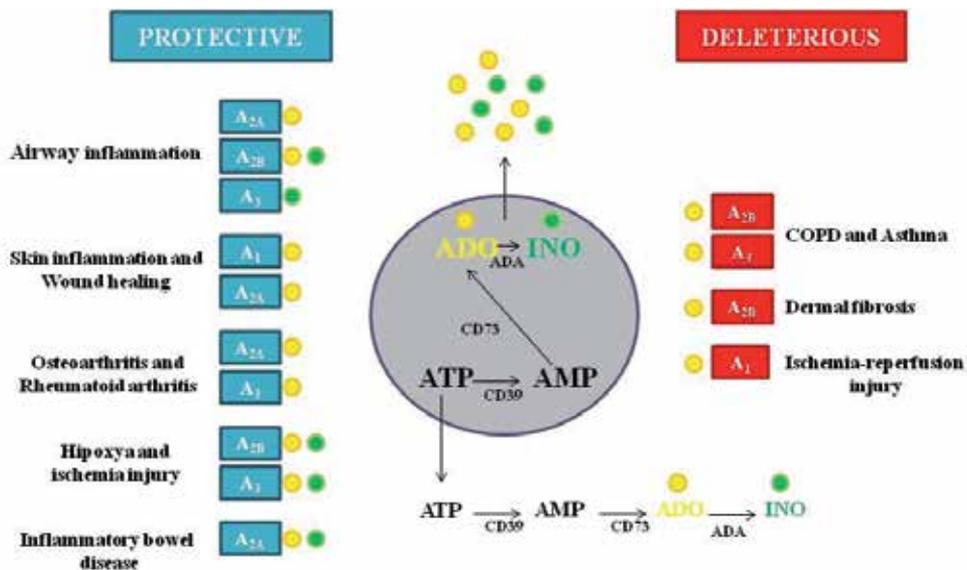


Figure 3. Adenosine deleterious and protective effects in inflammation and immunity. A_1 , adenosine A_1 receptor; A_{2A} , adenosine A_{2A} receptor; A_{2B} , adenosine A_{2B} receptor; A_3 , adenosine A_3 receptor; ATP, adenosine triphosphate; AMP, adenosine monophosphate; ADO, adenosine; CD 39, ectonucleoside triphosphate diphosphohydrolase.

Peroxynitrite, the end product of iNOS activity, which leads to the formation of NO and then to superoxide reaction, is highly reactive, causing a variety of toxic chemical changes in nerve tissue, including the nitration of tyrosine residues [245]. It has been previously described that UA, which is the final product of purine metabolism in humans, is a peroxynitrite scavenger [246], and for this reason UA therapy has become a potential method to alleviate the neuronal damage induced by peroxynitrite in MS treatment. Furthermore, inosine, an endogenous precursor of UA, seems to be an attractive candidate for the treatment of MS, given that patients who received inosine showed some evidence of clinical improvement and no sign of disease progression [247]. In addition, recent studies using adenosine receptor antagonists as well as

mice that were not capable of hydrolyzing adenosine from extracellular AMP (CD73^{-/-}), have suggested that blockade of adenosine receptors or CD73 deletion protected mice from EAE, decreasing lymphocyte infiltration in the CNS [248]. One very interesting note about the involvement of the adenosinergic system during EAE is that both CD73 and A_{2A} receptor presented increased expression in the choroid plexus [248, 249] in comparison to other regions of the CNS. This result shows that choroid plexus is higher permeable to lymphocytes than other regions, therefore, it is speculated that ATP, released as result of damage, and its conversion to adenosine represent a signal that regulates the entry of lymphocytes into the CNS [250, 251].

7. Adenosine receptors as drug targets: future directions for new drug development

There is increasing interest in the therapeutic potential of adenosinergic compounds (including receptor agonists and antagonists, enzyme inhibitors and others), and many adenosine compounds have been evaluated for therapeutic use. For a long time, adenosine itself was the only adenosine agonist used in humans. It is widely used in the treatment of paroxysmal supraventricular tachycardia (Adenocard®) due to the activation of A₁ receptor and is also used as a diagnostic tool for myocardial perfusion imaging (Adenoscan®) as a consequence of its A_{2A} receptor-activating effects, resulting in vasodilation [87].

Spinal administration of adenosine and adenosine analogs in humans also exhibited an analgesic effect. A phase I clinical safety study in healthy volunteers demonstrated that 1000 µg of adenosine given intrathecally led to a significant decrease in mustard oil-induced inflammatory pain and tourniquet-induced ischemic pain and decreased areas of secondary allodynia after skin inflammation with low side effects [252]. Recently, some studies have demonstrated new approaches regarding the development of allosteric modulators that enhance the potency of endogenous agonists and aim to minimize the side effects [253]. Allosteric enhancers of the adenosine A₁ receptor have been linked to anti-arrhythmic and anti-lipolytic activity and also have therapeutic potential as analgesics. Oral administration of the A₁ receptor-selective allosteric enhancer T-62 was shown to reduce hypersensitivity in carrageenan-inflamed rats and was approved for phase I clinical trials for neuropathic pain treatment [254]. As was previously discussed, various studies of selective agonists and antagonists demonstrated pro-inflammatory effects of A₁ receptors in different inflammatory models [47]. One interesting study showed a decrease in leukocyte infiltration and reduced lung edema in rats treated with the A₁ receptor antagonist L-97-1 [255]. Furthermore, the selective A_{2A} receptor agonists, apadenoson, binodenoson and sonedenoson have been considered candidates for clinical use in cardiovascular disorders [189, 256, 257]. These agonists are of interest as vasodilator agents in cardiac imaging [258] and as inflammation suppressors. Moreover, as reported by Press and Fozard (2010), two clinical trial applications from Santen Pharmaceuticals claim that use of agonist of adenosine receptors such as regadenoson and sonedenoson [259] is useful in the treatment of glaucoma.

The A_{2A} receptor agonist BVT.115959 from Biovitrum completed clinical trials for diabetic neuropathic pain, and it was well tolerated but did not significantly improve pain symptoms [260]. The primary indication claimed for A_{2A} receptor antagonists is in Parkinson's disease [261] because animal studies indicated that adenosine A_{2A} receptors are localized with dopamine D₂ receptors in the striatum and provide an antagonistic interaction between adenosine and dopamine [262]. Vernalis plc and Biogen Idec are currently profiling BIIB014 (V2006) in Phase II clinical trials for Parkinson's disease [263]. Patents for A_{2B} receptor antagonists generally claim asthma and allergic diseases as the primary indications, in line with current views on the receptor's role in vivo [163]. CVT-6883, an A_{2B}-adenosine receptor antagonist, is in clinical development for the treatment of asthma, and this antagonist significantly inhibits in vivo growth of B-16 tumors compared to taxol, as described in a recent review [259].

A₃ receptor selective agonists are also currently in clinical trials and exhibit nanomolar affinity to the receptor. In this context, CF101 (Can-Fite Biopharma) and CI-IB-MECA (CF102) are in trials for autoimmune inflammatory disorders and liver cancer, respectively. Two other A₃ receptor selective agonists, CP-608,039 and its N6-(2,5-dichlorobenzyl) analog, CP-532,903, were previously under development for cardioprotection [264, 265]. Allosteric modulators of the A₃ receptor have also been developed, such as imidazoquinolines (LUF6000), which have been shown to inhibit adjuvant-induced paw joint swelling in an arthritis rat model [265].

8. Conclusion

This chapter presented a general and updated review of the therapeutic potential of adenosinergic system (including receptor agonists and antagonists, enzyme inhibitors and others) in the control of inflammatory and immune responses. Recently, it is becoming clear that both adenosine and inosine play primordial roles in regulating the inflammatory process, working together for example as danger signals, in order to constitute a homeostatic mechanism of tissue integrity. Furthermore, adenosine as well as inosine effects are mediated by adenosine receptors and depending on the tissue or cells where they are expressed, pro-inflammatory or anti-inflammatory effects are observed. In this regard, several preclinical studies are conducted to clarify the role of these purines during the inflammatory response and to better understand the adenosine receptors activation which has become an interesting target to control inflammation. Currently, adenosine is used in the clinical setting, especially in emergency units, to convert sinus rhythm of paroxysmal supraventricular tachycardia (PSVT) to normal sinus rhythm, with excellent cost/effectiveness. Besides the differences in receptor expression between rodents and humans, the use of experimental animal models that can mimic the main features of inflammatory and immune diseases, the improvement of biochemical, genetic and molecular techniques have helped us to better establish a translation between preclinical and clinical effects of adenosine and inosine, and to develop and test selective agonists and antagonists of adenosine receptors that can be used in the future treatment of chronic diseases with inflammatory and immune features.

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Environmental Pharmacology and Toxicology

Environmental Pharmacology – An Overview

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Additional information is available at the end of the chapter

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1. Introduction

Pharmacology is the science that studies the physiological and biological effects of exogenous substances which are not part of the internal milieu of a living organism whether natural or synthetic termed xenobiotics (drugs, chemicals) on the cells, tissues, or organs of the organisms. There are many specialized areas of pharmacology and these include clinical pharmacology; which deals with the application of pharmacological principles and methods in the medical clinic with the focus on patient care and outcomes, neuro-pharmacology; which deals with effects of medication on central and peripheral nervous system function, psychopharmacology; which observes the effect of medication on the changed behaviours of the mind and body and how molecular events are manifested in a measurable behaviourable form. Other areas are pharmacodynamics; the chemical effect on the body, pharmacogenetics; the genetic variation that gives rise to differing response to drug, and the related area of pharmacogenomics, which is the application of genome technologies and the influence of the whole human genome on drug response, toxicology; which studies the harmful effect of drugs or chemicals and their molecular targets and characterization, pharmacognosy; the study of medicinal substances of biological origin, pharmacokinetics; that deals with the effect of the body on chemical half life and lastly environmental pharmacology which can be defined as the effect of pharmaceuticals and house care products on the environment and the ecosystem (Halling-Sorensen et al 1998). Environmental pharmacology is a relatively new and emerging specialty of pharmacology. It involves the study of gene-environment interaction, drug-environment interaction and toxin-environment interaction. It is important to emphasize at this stage that the different terms used interchangeable with environmental pharmacology include ecopharmacology, and pharmacoenvironmentology, ecotoxicology. However, a close similarity exists between environmental pharmacology and ecotoxicology. Environmental pharmacology entails the study of environmental science, medicine, ecology, genetics and chemistry.

The impact of pharmaceuticals on the ecosystem has significance public health implications. The demand for more pharmaceuticals relative to world's population growth may place the public at risk through the destruction of species. The entry of chemicals and drugs into the aquatic ecosystem is of a serious concern and empirical evidences are making these concerns more compelling. In addition, the production of illegal drugs pollutes drinking waters supply by releasing possible carcinogens (Ruhoy and Daughton 2008). All these factors necessitate the need for substances that are more biodegradable for the manufacture of drugs by pharmaceutical companies. However, environmental contamination by drugs may or may not lead to toxic effect on the ecosystem but usually there can be modifying effects. (Daughton and Ternes 1999)

1.1. Historical perspectives

In recent years, human pharmaceuticals from numerous therapeutic classes have increasingly been detected in the environment, typically at ng/l to low µg/l in surface water (Holm et al 2008). Another notable observation was the traces of narcotics like cocaine in River Thames (Goswani and Orr 2005). In addition, illegal cultivation of marijuana in bushes close to surface water with potential effect on the ecosystem is also a source of concern. The observation in 1997 of the decline in the population of the Asian white-backed vulture (Gyp) and the Indian vulture nesting in Keoladeo Natural Park in North Western India prompted the Indian Government to ban the drug 'diclofenac'. The vulture population drastically reduced over the years from 150 in 1997 to 25 in 2010. It was observed that they die after feeding on cattle treated with diclofenac. Diclofenac sodium is a non steroidal anti-inflammatory (NSAID) pain killer used by veterinary doctors to treat cattle. Because of lack of proper detoxification pathway for diclofenac in vultures, its ingestion leads to visceral gout and subsequent renal failure and death when they feed on the carcasses of animals treated with the drug (Oaks et al 2004). An alarming decline in the number of vultures poses the threat of outbreak of epidemics because of increase in the population of undecayed carcasses and feral dogs which pose a range of disease threats such as rabies in India (Brakash et al 2003).

Effects of some drugs on aquatic organisms have been investigated in acute toxicity assays. However, the chronic toxicity and potential subtle effects are only marginally known (Fent et al 2006). Environmental contamination by pharmaceuticals is not restricted to developing countries but it is a worldwide phenomenon. This underscores the importance of this emerging field of pharmacology. A study in the United States by the Geological Survey Department found traces of many different drugs and personal care products including steroids, insect repellants and phthalates in the American water supply. Although the concentrations were in traces, the effect of chronic exposure can be unpredictable (Palla va Bagla 2004). The production of bulk drugs has also been recently identified as an important source for environmental pollution with active pharmaceutical ingredients in certain locations (Gunnarson et al 2009, Fick et al 2010). A growing number of pharmaceutical residues are also found in surface water worldwide, raising concerns about their effects on the aquatic organism and posing a major challenge to developing rational strategy for prioritizing drugs on which to focus the most extensive environmental research efforts (Fick et al 2010). Traces of drug residues may pose

risk to aquatic life as depicted by various studies (Daughton and Terres 1997, Cleavers 2003, Boxall et al 2004, Kidd et al 2007). There is equally concern to human health sequel to the exposure to contaminated drinking water (Daughton 2004, 2008).

2. Sources and fate of pharmaceutical and house care products in the environment

The potential routes of entry of pharmaceutical and house hold care products in the environment include :

- i.** patients' excretion either as a parent compound or metabolites, via the sewer system
- ii.** direct release into the waste water system from manufacturing, hospitals or disposed via toilets and sinks
- iii.** terrestrial depositions for example via sludge application to land, leaching from solid waste landfills or irrigation with treated and untreated waste water. It is generally accepted that excretion of pharmaceuticals after human and veterinary therapeutic use dominates the global input of pharmaceuticals into the environment. Manufacturing effluent discharges and the disposal of unused drugs make a relatively small contribution to the overall environmental drug load. In addition, localized increased concentrations of drugs can occur adjacent to discharges from hospitals.
- iv.** Non pharmaceutical industrial sources, for example plastic products manufacturers are potential sources for the release of bisphenol A; used in the manufacturing process and known to have pharmacological effects on man and aquatic animals. Another pollutant from many household products is phthalates.
- v.** Overflow of agricultural run off may contain herbicides, pesticides and fertilizers and pose a potential danger to the ecosystem.
- vi.** Aging infrastructure also promotes the release of pharmaceuticals to the environment. Even when waste water makes it to sewage treatment facilities, they are not equipped to remove pharmaceuticals. As a result, our streams and rivers are exposed to a cocktail of synthetic compounds from stimulants and antibiotics to analgesics and anti histamines.
- vii.** Another important source of pharmaceuticals in the environment are drugs destined for plant health. For example, plant parasitic nematodes cause global crop losses of about \$125billion annually. Current chemicals used to control these pests have been withdrawn due to their environmental toxicity. This necessitated companies like Bayer Crop Science to reinforce research in this respect in order to come up with a suitable and safer alternative product.
- viii.** There has been little consideration for herbal preparations and their interaction with the environment, since they possess pharmacological properties. A recent example

can be found in aristolochic acid, a carcinogen, mutagen and nephrotoxin commonly found in *Aristolochiaceae* family of plants including *Aristolochia* (Birthworth) and *Asarum* (wild ginger) which are commonly used in Chinese herbal medicine (Barceloux 2008, Henrich et al 2009, Gluhovschi et al 2011). Aristolochic acid I is the most abundant of the aristolochic acids and it is found in almost all aristolochia species. Aristolochic acids are often accompanied by arisolactams which has been implicated as the causative agent of Balkan endemic nephropathy, Chinese herbs nephropathy and urothelial cancer.

2.1. Pharmaceutical active compounds (PhACs)

Pharmaceutical active compounds (PhACs) are those pharmaceuticals that have by one route or another entered the environment as the parent compound or as pharmacologically active metabolites. For many years, PhACs was not given any meaningful attention due to the fact that environmental researchers concentrated on the well known environmentally dangerous chemicals that are largely used in agriculture and industry. PhACs have not until recently been seen as potentially toxic because regulations associated with pharmaceuticals are typically overseen by drug related organizations which have limited experience with environmental issues (Jones et al 2001).

The list of common PhACs found in the environment includes

- i. Analgesics with anti inflammatory and anti pyretic capabilities viz acetaminophine, acetylsalicylic acid, diclofenac, codein and Ibuprofen
- ii. Antibiotics: macrolide antibiotics, sulfonamide fluoroquinolones, chloramphenicol, tylosin, trimethoprin, erythromycin, lincomycin, sulfamethoxazole and trimethoprin.
- iii. Anticonvulsant: carbamazepine, primidone.
- iv. Beta – blockers: metoprolol, propranolol, betaxolol, bisoprolol, nadolol
- v. X ray media: 10 promide, 10 pamidol, 10 hexol and diatrizoate.
- vi. Steroid and hormonal preparation: 17 α ethinyl estradiol, mestrenol and 19-noresthisterone
- vii. Miscellaneous: household products, pesticides veterinary drugs and insecticides.

2.1.1. Fate in the environment

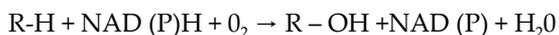
Once PhACs enters the environment, they suffer one of three fates which include:

- i. Biodegradation into carbon dioxide and water
- ii. Undergo some form of degradation to form metabolites
- iii. Persist in the environment unmodified. The amount of the compound that is broken down depends on several factors such as bioavailability and compound structure among others.

2.2. Biodegradation of xenobiotics

Biodegradation is the complete breakdown of the complex and toxic contaminants to non-toxic simple elements by the action of microbes. Hence, these contaminants act as the microbial food substrate. Biodegradation, in general can be considered as a series of steps of biological degradation or pathway that ultimately result in the oxidation of the compound which most often results in the generation of energy while xenobiotics in the human body are removed by a process called xenobiotic metabolism, in which these compounds are degraded by the liver enzymes such as Cytochrome P₄₅₀ which activate the xenobiotics by the process of oxidation, or hydration, reduction or hydration followed by conjugation with glucuronic acid, sulphuric acid or glutathione, and the compound conjugates are excreted by urination, exhalation, sweating and defecation. The xenobiotics in the environment are degraded by the microbes. Microbes have the capacity to degrade all naturally occurring compounds by the principle of microbial infallibility proposed by Alexander in 1985. Microbes can degrade many of the xenobiotic compounds, but not all. The compounds that resist biodegradation and persist in the environment are called "recalcitrant or Environmental persistent pharmaceutical pollutants (EPPP).

For complete biodegradation, oxidation of parent compound occurs to form carbon dioxide and water. Each step in the degradation pathway is catalyzed by a specific enzyme produced by the degrading cell. Degradation of some xenobiotics depends on the presence of a specific compound, which includes the required enzymes. These enzymes are metabolized to provide both energy and reducing equivalent for the degradation of xenobiotics compounds. Example of such enzymes are the oxygenases; a group of enzymes that catalyses the reactions that transforms the hydrophobic nature of the organic compound to water soluble forms which can be broken down by a larger number of other micro organisms. The oxygenases are of two main classes; mono-oxygenases and dioxygenases. These enzymes participate in the oxidative metabolism of a wide variety of chemicals of pharmaceutical, agricultural and environmental significance. Some of the most widely recognized substrate for this class of enzymes are the aliphatic and aromatic hydrocarbons of both endobiotic and xenobiotic sources. Monooxygenases are a class of enzymes that insert one atom of the oxygen molecule into the substrate; the other atom becomes reduced to water. They are also more complex and can catalyze several different types of oxygen atom insertion reactions. Since they can oxidize more than one substrate, they are called mixed functions oxidizers or oxidases. Also, since one of the main substrates gets hydrolysed they are also called hydroxylases. The general stoichiometry of the is reaction is as follows:



Dioxygenases incorporate both atoms of the oxygen molecule into substrate and are crucial in initiating the decomposition of a variety of chlorinated and nitro-aromatic compounds as well as non-substituted polycyclic aromatic hydrocarbons. Many of these compounds are first degraded to catechol protocatechuate by oxygenases (dioxygenases and monooxygenases). The intermediates are metabolized by ring cleavage type of dioxygenases to either beta-keto adipate or 2keto-4-hydroxy valerate. These intermediates then enter the TCA cycle. When the organism evolves to start tolerating the xenobiotic compound, it can lead to the phenomenon

called 'resistance'. An example is the resistance of the bacteria to certain antibiotics. In addition, some of these substances are resistant to degradation, for example plastics and certain pesticides. However it is understood that microbes have the ability to degrade many of these recalcitrant compounds.

2.2.1. Environmental Pharmaceutical Persistent Pollutants (EPPP)

The term Environmental Pharmaceutical Persistent Pollutants (EPPP) was suggested in the nomination 2010 of Pharmaceutical and Environment as an emerging issue to Strategic Approach to International Chemical Management (SAICM) by the International Society of Doctors for the Environment (ISDE). Pharmaceuticals are synthetic chemicals belonging to a wide group of different chemical families and may also react differently in the environment. There are documented evidences that some pharmaceuticals enter and persist in the environment. (Ruhoy and Daughton 2008, Segura et al 2005) Some are endocrine disruptors, particularly the synthetic hormones (Fick 2009, Snyder et al 2003) some are designed to kill bacteria and viruses and may affect microorganisms and wild life in severe and unexpected ways (Segura et al 2005). Little is known about the possible negative effects and impacts of EPPP in humans and the environment by diffuse and systematic exposure for long periods of time especially during the vulnerable periods of development. As there are thousands of different synthesized chemicals present at the same time in the environment, different interactions may occur and the result of these multiple exposure in human and nature are not sufficiently studied or understood. EPP's are already found in water all over the world. The diffuse exposure might contribute to:

- i. Extinction of species and imbalance of sensible ecosystems ; since many EPPS affect the reproductive systems of for example frogs, fish and mussels.
- ii. Genetic, developmental, immune and hormonal health effects to humans and other species in the same way as oestrogen- like chemicals.
- iii. Development of microbes resistant to antibiotics, as is found in India.

Some pharmaceuticals are degraded to various extents in sewage treatment plants but others leave the plant in active forms. Active residues have been detected in surface water, and they may persist in the environment for long periods of time. Large amounts of antibiotics and other pharmaceuticals have also been found downstream from sewage plants for pharmaceutical industries. EPPs from sewage sludge used as fertilizers can be absorbed by soya, and antibiotics have been found in the leaves. Many EPP's have been detected in drinking water and these include Atenolol (beta blocker) citalopram (anti depressive drugs Diclofenc (analgesic) Ibuprofen, (analgesic) metoprolol (beta blocker) Naproxen (anti inflammatory) and Trimetoprin (antibiotic) have been found in drinking water of Stockholm, Sweden Fish caught downstream from the sewage plants of Stockholm contained citalopram (antidepressant drug) and propoxyphene (narcotic/anaesthetic) (Daughton 2008, Hernando et al 2006). Several broad – spectrum antibiotics in very high concentrations as well as bacteria resistant to all known antibiotics were found downstream from a sewage plant in India. Also in Indian drinking water, cetirizine (antihistaminic) aprothoxacin (antibiotic), enoxacin (antibiotic) terbinafin

(antimycotic) and cataboprain (anti depressant drug) were found. Furthermore, up to 14 different pharmaceuticals have been found in the drinking water of big cities around the world. Some of these environmental pharmaceutical chemicals are well known to have serious genotoxic effects in humans. Half life in nature varies depending on the environment (air, water, soil, sludge) but is more than one year for several compounds (Neeman et al 2004, Conroy et al 1999, Sevage et al 2005). Clofibric acid, a metabolite of the lipid lowering agent clofibrate can still be found in surface as well as well-water despite the fact that clofibrate has been withdrawn from use long ago. Concentrations of EPPs can vary from 1ng to 1mg per litre. Serious effects of EPPPS on water living organisms especially on reproductive systems and microbial communities have been observed (Elloriaga et al 2013, Suartz and Perez-coll 2013) Halling- Sorensen 2007, Daughton and Hernes 1999).

3. Effect of xenobiotics on the environment and the ecosystem

More than 13 million deaths every year have been associated with environmental pollutants and as much as 24% of diseases are estimated to be caused by environmental exposures which can be averted. Today, detectable levels of pharmaceutical preparations either as parent drug or metabolite are found in food stuffs, water, i.e both rivers and seas. Although the levels might not be toxic in a single exposure, low dose chronic toxicity should be anticipated. (Jorgenson and Halling-Sorensen 2000).The prescribing and usage of medications for both humans and domestic animals have ramifications extending far beyond the traditional objectives of conventional medical care. The healthcare industry has an environmental footprint that includes the active pharmaceutical ingredients (API) from medications, residues of which can establish themselves as environmental pollutants. Many parallels exist between healthcare and the protection and remediation of the environment, spanning the stages from symptomatology and diagnosis and treatment (Daughton and Ruhoy 2008).

3.1. Drug – environment interaction (DXE)

Drugs interact with the environment in diverse ways and these include the aquatic system, ground water and surface water, sewage systems, flora and fauna of the ecosystems, causing various modifications including bacterial drug resistance. (Bound and Voulvoulis 2004)

3.1.1. Aquatic system

A growing number of pharmaceuticals are found in surface waters worldwide, raising concerns about their effects on aquatic organisms and it is major challenge to develop a rational strategy for prioritizing drugs on which to focus the most expensive environmental research efforts. Among aquatic organisms, fish most often share drug targets with humans. Very little is known about the long-term effect of drugs in aquatic organisms. A study suggested that anti- depressants like fluoxetine could trigger spawning in some shell fish (Pailla va Balga 2004), thereby disturbing the ecosystem (Jones at al 2001)

An Indian study of effluents from industrial site in which a large number of pharmaceutical companies send their waste water revealed the presence of pharmaceuticals in the treated effluents. In the said study of aquatic rainbow trout (*Oncorhynchus mykiss*) exposed to 0.2% of the effluent for 5 days, induction of hepatic cytochrome P450IA (CYPIA) genes expression as well as enzyme activity were observed. In addition, clinical blood chemistry analysis revealed an increase in plasma phosphate levels which by interpolation in humans, indicates impaired kidney function. In addition, several oxidative stress related genes were found to be induced in the livers, however, no significant changes were observed in antioxidant enzyme activities or in the hepatic glutathione levels. Furthermore, estrogen-regulated genes were slightly up-regulated following the exposure and moderate levels of estriol were detected in the effluent. The pattern of regulated gene may contribute to the identification of mechanism of sub-lethal toxicity as well as illuminate possible causative agent (Gunnarsson et al 2009).

Ketoprofan and diclofenac are non steroidal anti-inflammatory drugs (NSAIDS) often used for similar indications and both are frequently found in surface waters. Diclofenac affects organ histology and gene expression in fish when exposed to a concentration of 1µg/l. of this drug (Cuklev et al 2012). In another Indian study, five common non steroidal anti-inflammatory drugs (NSAIDS) namely diclofenac, ketoprofen, naproxen, ibuprofen and acetylsalicylic acid were detected in various concentrations in surface water from 27 locations of the Kaveri vellar and Tami rapani Rivers in southern India. The samples were extracted by solid-phase extraction and analyzed by gas liquid chromatography mass spectrometry (GC-MS). The concentrations of four of the five drugs in this reconnaissance were relatively smaller to those reported elsewhere being of a value of 200ng/l, however, acetylsalicylic acid, the most readily degradable of the drugs, investigated was found at all sites and at considerably higher concentrations of up to 660ng/l, compared to levels reported in European surface waters. The finding of elevated concentrations of acetylsalicylic acid could be as a result of direct discharges of untreated sewage. Therefore, readily degradable pharmaceutical may present larger concern in those regions without consistent sewage treatment. This situation poses risks of direct toxicity to aquatic wildlife and humans consuming the water (Shanmugan et al 2013).

In another case, effluent from a treatment plant; named Patencheru Environment Technology (PETL) which is located in an industrial area just outside Hyderabad in India, was observed to be capable of causing deleterious effects on aquatic vertebrates. In the study, an embryo toxicity test carried out, observed that as little as 0.2% of the effluent, reduced the growth of tadpoles by 40%, however the growth of zebra fish (*Danio rerio*) was not impeded. The median lethal concentration (LC₅₀) of effluent for zebra fish (*Danio rerio*) at 144hr after fertilization varied between 2.7 and 8.1% in different experiments. Although the study focused on fish, it also increased knowledge about how aquatic vertebrates are possibly affected by effluent exposures, which substances in the effluent are causing the toxic effects and at what dilutions of effluent are the fish likely to be affected (Shanmugan et al 2013).

3.1.2. Biofilm

Streams and rivers have been known to be exposed to combinations of different drugs. These include commonly prescribed medications like anti- diabetics, anti- histamine diphenylhydri-

amine of which Benachryl is a brand were (Karatan and Watrick 2009) observed to cause significant disruption to the biofilm community which is important to the ecosystem. Biofilms form the slippery coating in stream rocks and are quite vital to stream health where they contribute to water quality by recycling nutrients and organic matter. They are also a major food source for invertebrates that in turn feed larger animals like fish. The effects of diphenylamines on biofilm could therefore have repercussion for animals in stream food web such as insects and fish (Rosi- Marshall 2013). Other effects of drug contamination of the environmental were observed in activities of anti-depressant which could trigger spawning in some shellfish thereby disturbing the ecological balance. Furthermore, propranolol and fluoxetine were observed to have deleterious effects on zooplakton and benthic organisms ((Hoffman et al 2005, Rosi-Marshall 2013, NIH 2002).

One of the most important considerations for environmental pharmacology is the impact of drug contaminants on biofilms. Biofilms are aggregates of microorganisms in which cells that are frequently embedded within a self produced matrix of extracellular polymeric substances (EPS) adhere to each other and or to a surface. Biofilm EPS: also referred to as “slime” is a polymeric conglomeration generally composed of extra cellular DNA proteins and Polysaccharides. Biofilms may form on living or non living surfaces and can be prevalent in natural, industrial or hospital setting (Hall –Stoodley et al 2002, IUPAC 2012, Lear and Lewis 2012). Quite characteristic of biofilms is their ability to grow in the most extreme environments. For example, they can survive in the most extremely hot, briny water of hot springs, acidic and very alkaline waters and frozen glaciers. They can be found on rocks and pebbles at the bottom of most streams or rivers and often form on the surface of stagnant pools of which they are an important components of food chains in rivers and streams which are grazed by the aquatic invertebrates upon which may fish feed. Biofilms are ubiquitous, nearly every species of micro organism, including bacteria and archea have mechanism by which they can adhere to surfaces and to each other. They will form on virtually every non shedding surface in a non sterile aqueous or very human environment. With reference to the immediate human environment, they can be found growing in shower easily because it provides a moist and warm environment for them to thrive. They also form inside water sewage pipes causing clogging and contributing to about 20% of their corrosion.

Biofilms can also affect marine engineering systems such as pipelines of the offshore oil and gas industry where they may be found causing a substantial corrosion problem. Although corrosion in the circumstance is mainly due to abiotic reasons. Furthermore, bacterial adhesion is the foundation for bio fouling of sea bound vessels. Once a film of bacteria forms, it is easy for other marine organisms such as barnacles to attach. Such fouling can reduce the maximum speed of the vessels by up to 20% therefore prolonging the voyages with resultant extra consumption of fuel. They have also been found to be involved in a wide variety of microbial infections in the body. By one estimate 80% of all infections including intestinal tract infections, dental plaques gingivitis, catheter infections, and middle ear infections have been associated with them (NIH 2002). Most recently, it has been observed that bacterial biofilms may impair cutaneous wound healing and reduce topical anti bacterial efficiency in healing or treating

infected skin wounds (Davis et al 2008). In many animals including man, they build up in teeth forming dental plaques and causing gum diseases (Rogers 2008, Mihai et al 2010)

The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organisms which by contrast are single cells that may float or swim in a liquid medium. Microbes form a biofilm in response to many factors which may include cellular recognition of specific or non specific attachment sites on a surface, nutritional cues, or in some cases by exposure of planktonic cells to sub-inhibitory concentration of antibiotics (Hoffman et al 2005; Karatan and Watnick 2009). It is suffice to note that biofilms exhibit both negative and positive properties. For instance, many sewage treatment plants include a treatment stage in which waste water passes over biofilms growing on filters which extract and digest organic compounds. In such biofilms, bacteria are mainly responsible for the organic matter, i.e biochemical oxygen demand. (BOD) While protozoa and rotifiers are mainly responsible for the removal of suspended solids including pathogens and micro organisms. Slow sand filters rely on biofilm development in the same way to filter surface water from lake, spring or river sources for drinking purposes. Thus what is regarded as clean water is effectively a waste material to these micro cellular organisms. In addition, biofilms can help eliminate petroleum oil from contaminated oceans or marine systems. The oil eliminated by the hydrocarbon degrading activities of microbial communities, in particular by a remarkable recently discovered group of specialists called "Hydro-carbon-clastic bacteria" (HCB) (Martins dos Santos et al 2008).

Stromatolites are layered accretionary structures formed in shallow water by the trapping, binding and cementation of sedimentary grains by microbial films especially of *Cyanobacteria*. Stromatolites include some of the most ancient aggregated structures on earth and are still forming today. Further, biofilms are also useful in microbial fuel cells (MFC) to generate electricity from a variety of starting materials including complex organic waste and renewable biomass (Lear and Lewis 2012). The social structure i.e cooperation and competition within a biofilm highly depends on the species present (Nadel et al 2009). Bacteria living in a biofilm usually have significantly different properties of free floating bacteria of the same species as the dense and protected environment of the film allows them to cooperate and interact in various ways. One benefit of this environment is increased resistance to detergent and antibiotic as the dense extra cellular matrix and the Outer layer cells protect the interior of the community. In some cases, antibiotics resistance can be increased a thousand fold (Stewart and Costerton 2001). Furthermore, lateral gene transfer is greatly facilitated in biofilms and this leads to a more stable biofilms structure (Motin and Tolker-Nielsen 2003) Other example of acquisition of resistance by organisms in biofilm could be found in the *Legionella* bacteria and *Staphylococcus aureus*. *Legionella* bacteria are known to grow under certain conditions in biofilms in which they are protected against disinfectants. Workers in cooling towers, people working in air conditioned rooms and those taking showers are exposed to legionella by inhalation when the systems are not well designed, constructed or maintained (Murga et al 2001). Sub-therapeutic levels of B-lactam antibiotics induce biofilms formation in *Staphylococcus aureus*. This sub-therapeutic level of antibiotics may result from the use of antibiotics as growth promoters in agriculture, improper disposal of unused drugs or products of excretion

during the normal course of antibiotic therapy. The biofilms formation induced by low level methicilin was inhibited by DNase suggesting that the sub-therapeutic levels of the antibiotic also induce extracellular DNA release (Kaplan et al 2012)

However, biofilms are not always less susceptible to antibiotics. For instance, the biofilms form of *Pseudomonas aeruginosa* has no greater resistance to antimicrobials than do stationary phase planktonic cells, although when the biofilm is compared to logarithmic phase planktonic cells, the biofilm does have greater resistance to antimicrobials. This resistance to antibiotics in both stationary phase cells and biofilms may be due to the presence of persister cells (Spoering and Lewis 2001).

3.1.3. *Diethylstilbestrol (DES)*

Diethylstilbesterol (DES) is a synthetic estrogen that is used to prevent miscarriages in women between 1940s and 1960s. A moderate increase in breast cancer risk has been shown both in daughter of women who were treated with DES during pregnancy as well as in their granddaughters. The expression of 82 mi RNAs (91% of the 898 mRNAs) evaluated were observed in breast epithelial cell exposed to DES. In particular, the suppression of MIR-9-3 expression was accompanied by promoter hypermethylation of the MIR-9-5 coding gene in DES treated epithelial cells.

3.1.4. *Contamination with herbal products: Aristolochic acid*

Aristolochic Acid (AA) is a natural compound found in many plants of the Aristolochia genus. Aristolochia plants are commonly used in traditional herbal preparation as health supplements and remedies for various health problems including weight loss, menstrual symptoms and rheumatism (Vancher Weghem et al 1993, Kwak et al 2012,). In the 1990s, epidemiological studies revealed AA exposure was associated with a high risk of nephrotoxicity and upper urinary tract urothelial cell carcinoma (UTUC) (De Broe et al 1999, Grollman et al 2007, Debelle et al 2008, Notier et al 2000) caused by the ability of AA to bind DNA, forming DNA adduct. (Schmeiser et al 1998). These findings, consequently led to ban on the use of Aristolochia containing herbal preparations in Europe and North America since 2001 and in Asia since 2003 (Debelle et al 2008). Currently, AA is classified in the International Agency for Research on Cancer (IARC) monograph as a group 1 human carcinogen (IARC 2012). In Taiwan, AA associated DNA adducts was detected in the renal cortex of more than 50% of UTUC patients (Moriya et al 2011) and incidence of UTUC of 30% is strikingly higher than in the West with 3% (Yang et al 2002). This figure is consistent with AA playing a role in Asian UTUC. There is a possibility that AA may contaminate surface water, grain and vegetables during the processing of Aristolochia containing herbs and the disposal of its waste. Aristolochia species are commonly used in Chinese herbal medicine (Barceleux 2008 et al, Henrich 2009, Gluhovschi et al 2011). Aristolochic acid I is the most abundant of the Aristolochic acids found in almost all Aristolochia species and are often accompanied by aristolactam which has been implicated as the causative agent of Balkan endemic nephropathy (BEN), Chinese herb nephropathy (CHN) and urethelial cancer (UC). When hay meant for feeding horses was

contaminated with *Aristolochia* plants, the horses were observed to develop chronic renal failure. (Grollman 2013).

TP 53 mutation signature in urothelial tumours and the presence of aristolaclam DNA adducts in the renal cortex defined in the course of research proved to be a robust bio marker of exposure to this potent nephrotoxin and human carcinogen (Moriya et al 2011). With the growing influence of herbal drugs worldwide, botanical plants with pharmacological properties should be cautiously handled in order not to contaminate crops, vegetables and surface water. Another common herb which may contaminate the ecosystem is marijuana with the potential ability to interfere with the biological system of aquatic flora and fauna. In addition, herbs like St. John's wort has been observed to cause modulation of Cytochrome p₄₅₀ and may interfere with prescribed therapeutic agents (Guegenrich 1997).

3.2. Toxin-environment interaction

It is a well known fact that toxins interact frequently with the environment and some of these interactions may have deleterious effects on plants animals and man. Some of these effects are discussed further in this chapter.

3.2.1. Autoimmune diseases

There is a current view that environmental exposure play a role in the development and or the exacerbation of autoimmune diseases. (Ritz 2010, Bashir et al 2011). Auto immune diseases result from an immune response directed against the body's own tissues. There are so many different auto immune diseases and though many of the individual immune diseases are rare, autoimmune diseases collectively afflict approximately 24.5million Americans with women disproportionately affected. The causes of auto immune disorders remain largely unknown. During reproductive ages (18-40), there is a distinct female preponderance of autoimmune diseases including and sex hormones and/or sex chromosomes may be responsible for this enhanced susceptibility (Whitcare et al 1999, Voskuhi 2011). Genetic risk factors have been and continue to be studied and account for a portion of the risk for autoimmune disorders with concordance studies in identical; twins generally in the 25 - 40% range. It is becoming clear from human studies as well as animal model and in vitro research that the etiology of autoimmune diseases is multi-factoral involving both genetic and environmental influences.

Existing data and epidemiological evidence supports a role for the contribution of a number of environmental exposures to the development of specific auto immune outcomes. Included in this current line of thought were crystalline silica exposure and the development of several autoimmune diseases including rheumatoid arthritis (RA) systemic sclerosis (SSc) systematic lupus erythematosus (SLE) and anti-neutrophil cytoplasmic antibody (ANCA) related vasculitis; solvent exposure and the development of SSc, smoking and the development of seropositive RA; and an inverse relationship between ultraviolet radiation exposure and the risk of development of multiple sclerosis (MS). Furthermore, animal models of exposure provide additional support for the role of xenobiotics in the development of autoimmune diseases. Examples of demonstrated links include: forms of inorganic mercury and the induction of a

transient systematic autoimmune diseases in rats and mice and the mineral oil component 2, 6, 10, 14 tetramethylphenylenediamine (TMPD or pristane) and an induction of chronic lupus like disease and inflammatory arthritis in several strains of mice. Animal model studies have shown additional exposures with likely link to the induction and or exacerbation of auto immune diseases, including gold, silica, trichloroethylene (TCE), 2, 3, 7, 8, - Tetra dichloro-dibenzo-p-dioxin (TCDD), organochlorine pesticides and ultraviolet (UV) radiation. The mechanisms by which environmental factors alter basic biological processes to induce auto immune diseases continue to be examined, but remain largely unknown. A growing body of literature points to a number of mechanisms likely involved in environmental exposure-based auto immunity and include a role for xenobiotics in the activation of Toll-like receptors (TLR); B Cell activation; impairment of T. helper 17 (Th 17) and T-regulatory (T-reg) cell immune function; modifications of self antigens; and alteration of DNA methylation profiles. The cumulative body of research findings is increasing the confidence that specific exposures and mechanisms are involved in the development of auto immune diseases. Despite growing advances in the field, an understanding of the interactive roles of the environment and genetics in the auto immune process is still lacking and additional progress is needed on many fronts (NIEHS).

3.2.2. *Endocrine disruptors*

An important aspect of environment toxin interaction (TXE) is the effects of endocrine disruptors on aquatic system (Le Parge et al 2011). Environmental xenobiotics having oestrogenic effects include plants, pesticides, surfactants, plastics and animal genistein, methoxychlor and bisphenol. They cause lowering of cholesterol on oestrogen dependent animal models and have been associated as a cause of osteoporosis. Endocrine disruptors (EDC) are chemicals that may interfere with the production or activity of hormones in living organisms. Perhaps are the synthetic hormonal drugs such as birth control pills. Others are dioxins polychlorinated biophenyls (PCB) pesticides, bisphenol A, phthalates, lead, mercury cadmium, arsenic herbicides; atrazine, plastic residues, and cleaning products. These chemicals can enter the aquatic system through improper medical waste disposal, runoff from land fill and sewage discharge from storms (Daughton and Ruhoy 2008, Fent 2006, Genius et al 2012).

3.2.2.1. *Wildlife*

Because a large proportion of potential endocrine disruptors end up in surface waters, aquatic species are particularly vulnerable to their potential adverse effects. Recent studies identified a number of brain targets for EDC commonly present in environmentally relevant concentrations in surface waters. Among those normal systems disrupted by EDC are the gonadotrophin releasing hormone (GnRH) neurons, the dopaminergic circuits and more recently the Kiss/GPR54 system, which regulates gonadotrophin release. However, one of the most striking effects of EDC, notably estrogen mimics, is their impact on the Cyp19a1b. gene that encodes the brain aromatase isoform in fish. This is an example in which the molecular basis of endocrine disruption is fully understood. (Le Page et al 2011). More commonly observed effects of EDC is impaired reproduction and development in aquatic

animals (Kidd et al 2007). Furthermore, masculinization (imposex) had been observed in female marine snails exposed to tributyltin (TBT), a biocide used in anti-fouling paints. The dog whelk “(*Nucella lapillus*) a species of predatory sea snail’ after is particularly sensitive and imposex has resulted in decline or extinction of local populations worldwide, including coastal areas all over Europe and in the open North Sea. DDE – induced egg-shell thinning in birds is probably the best example of reproductive impairment causing several population declines in a number of raptor in species Europe and North America. Developmental exposure to the DDT complex has been formerly linked to the induction of ovotestis in male western gulls. EDGs have adversely affected a variety of fish species in the vicinity of certain sources, for example effluents of water treatment and in the most contaminated areas, this exposure is causally linked with effects on reproductive organs which could have implication for fish populations. Turtles can also be affected in the in the same way (Cleuvers 2003, Le Parge et al 2011).

In mammals, the best evidence came from the field studies on Baltic grey and ringed seals and from the semi-field studies on Wadden Sea harbour seals, where both reproduction and immune function have been impaired by PCBs in the food chain other mammals affected include the polar bear, rabbit and guinea pig. Distorted sex-organ development and function in alligators has been linked with a major pesticide spill into a lake in Florida in USA. Furthermore, the oestrogenic and androgenic effects observed in this reptile have been causally linked in experimental studies with alligator eggs to the DDT complex. For terrestrial (land living) wildlife, including aquatic mammals, exposure is primarily expected to be of dietary origin. The situation is however different for aquatic wild life where direct uptake of dissolved chemicals from the water is a significant route to exposure.

3.2.2.2. *Effect on humans*

The possible pathway of exposure to endocrine disruptors in humans, include, direct exposure at work place, and via consumer products such as food, certain plastic, paints, detergents and cosmetics as well as indirect exposure via the environment, viz air, water and soil.

In general, the vulnerability of a given species will depend on the intrinsic properties of the chemical, magnitude, duration, frequency means of exposure and the way in which a given specie can absorb, distribute, transform and eliminate substances. It will also depend on the sensitivity of specific organ at different stages of development. The endocrine- disruptor hypothesis was originally formulated for xenoestrogens i.e chemical which affect the estrogen signaling pathway. The greatest attention to endocrine disruption has focused on estrogenic effects but a clear cause-effect relationship has not yet been established. (Meeker 2012, EC2013,)

However, it is now becoming generally accepted that compounds of various types can interact with different component in several cell regulatory systems including the steroid and thyroid hormone receptor families. Apart from the drug DES (synthetic oestrogens), environmental oestrogens have never been proven to cause human health problems. At best there can only be speculation on possible human health effects by interpolation of documents obtained from animal studies (Hollander 1997) EDCs which act via receptors of the steroid receptor super family can have effect on many organs of the body. Steroid receptors for oestrogens, androgens

and adrenocorticoid and thyroid hormones are found in practically all cells of the body. The functions of the brain, the cardiovascular, the skeletal and the urogenital system are regulated by these hormones and can therefore be affected by EDCS. In addition, an EDC with a defined action in one organ; for example estrogenic activity can extent similar or non-oestrogenic effect or even antagonistic effects in other organs.

3.2.2.3. Potential effect in males

EDCS has the potential to cause poor semen quality including low sperm counts, low ejaculate volume, high number of abnormal spermatozoa motility. Other effects may include testicular cancer, malformed reproductive tissue. viz undescended testes, small penis size, prostate disease and other unrecognized abnormalities of male reproductive tissues. In infertility studies, the effects of environmental pollution including occupational exposures are currently being given due consideration.

3.2.2.4. Potential effect in females

There are currently putative links between EDC and some female diseases including breast and reproductive organ tissue cancers, fibrocystic disease of the breasts, polycystic ovarian syndrome, endometriosis, uterine fibroid and pelvic inflammatory diseases.

3.2.2.5. Potential effect in children

EDCS have been linked with impaired behavior, mental, immune and thyroid functions in developing children. Other include precocious puberty, osteoporosis, foetal growth, child development, and obesity (Meeker 2012)

3.3. Environmental pharmacology and the pediatrics population

Although non therapeutic xenobiotics represent the vast majority of environmental exposures during childhood, studies of these compounds in children has lagged behind drug studies. The paediatric group is unique with respect to environmental contaminants in that there is a lot of hand to mouth activities ranging from food stuff to drug and plastic toys. They are also quite vulnerable to poisoning from unprescribed medications. However, an increased impetus for paediatric pharmacology studies resulted from evidence of short comings in algorithmic approaches to dosing and the recognition of differing efficacy. While in some drugs developmental differences resulted in increase toxicity or failed efficacy. In others decreased toxicity was observed and toxicity in children compared to adults. Thus the paediatric patients may not be classified arbitrarily as a susceptible population, but however, certainly a different group compared with the adults. Better designed pediatric pharmacology studies use well documented, non linear changes in body composition across childhood, as well as knowledge about the impact of physical growth, mediated by complex normal changes. Developmental differences in all component of drug disposition, including absorption, distribution, metabolism and excretion have been characterized. Of these, the ontogeny of metabolism, particularly tissue specific metabolism, is the most complex. Many knowledge gaps still persist within

developmental pharmacology (McCarver 2004). The most common concern for pediatric environmental pharmacology are the EDCs. While human epidemiology studies of exposure to EDCs and children's health remain extremely limited, a growing body of evidence show that exposure to a number of chemicals commonly found in consumer goods, personal care products, food drinking water, and other sources may adversely affect child development through altered endocrine function. Some of which include persistent organic pollutants (POP) phthalates, bisphenol A and contemporary use pesticides had earlier been discussed in this chapter.

Each year, nearly 1,500 children under 6 years old are treated in U.S. emergency department as a result of accidental ingestion of buprenorphine. A new study examines 2,3800 of these cases for October 2009 to March 2012. Buprenorphine brand name (subutex) is used alone or in combination with another drug called naloxone (brand name suboxone) to ease the symptoms of withdrawal in people trying to beat addictions to heroin, certain prescription pain killers and other opioid drugs. One dose of this medication can be fatal to a small child. Other commonly used medications such as high blood pressure medicals, some diabetic drugs, strong pain killers, some medications used for arthritis, and some drugs for attention deficit/hyperactivity disorder can potentially be fatal to a small child with just a single dose. (Lavonas 2013).

3.4. Miscellaneous toxicity

Bisphenol A, a component used in many plastic products binds to the local anaesthetic receptor site to block the human cardiac sodium channel (O'Really et al 2012). Nickel, chromium arsenic and lead are well known environmental toxicant. Many household products like insecticides, paints, cosmetics, cleaning fluids, nano-materials based items are known to contain some of these toxicants. While they may not be directly toxic, their interaction with cellular organelles may result in cancer. Lead is known to be hepatotoxic while cadmium is a well known nephrotoxic agent. Phthalates which is commonly used in cosmetics like nail polish are thought to affect the endocrine system and are being investigated for a link with infertility in women

3.4.1. Household products

Many household products are potentially dangerous substances, and these include oven and drain cleaners, laundry powder, floor polish, paint and pesticides. Even arts and craft supplies and yard care products can be hazardous. Many household products can harm children pets and the environment if not correctly stored, used and disposed of. Toxic substances in these products can cause harm if inhaled, swallowed or absorbed through the skin.

Based on genetic composition, people respond to toxic substance might cause birth defects or other sources problems including brain damage or death. The concept of 3Rs viz reduction, reuse and recycling is bound to minimize amount of drugs in the environment.

Household waste (HHW): reduction, reuse, recycling and finally disposal

- Reduction and recycling of HHW conserves resources and energy that would be expended in the production of more products

- Re use of hazardous household products can save money and reduce the need for generating hazardous substances
- Proper disposal prevents pollution that could endanger human health and the environment

3.4.2. Pesticides

Pesticides can be fungicides, herbicides and rodenticides. Pests live where they are not wanted or cause harm to crops, people or animals and pesticides have been useful agents in getting rid of them..Pesticides can be very helpful in the sense that they protect man's health by killing germs, animals or plants that can hurt us. However, most pesticides can be harmful to people and pets. Disposing of pesticides properly is also important as it can protect the environment. Biologically based pesticides are becoming more popular as they often safer than traditional pesticides. They come in the form of pheromones and microbial pesticides.

Pesticides are classified as semi volatile organic compounds and include a variety of chemicals in various forms. They are chemicals that are used to kill or control pests which include bacteria, fungi and other organisms. In addition, to insects and rodents Pesticides are inherently toxic. Health effects of pesticides include irritation to eye, nose, throat, damage to central nervous system and kidney and increased risk of cancer. Symptoms of pesticide toxicity include headache, dizziness, muscular weakness and nausea. Chronic exposure to some pesticides can result in damage to the liver, kidneys, endocrine and nervous systems both the active and inert ingredients in pesticides can be organic compounds; therefore, both could add to the levels of airborne organics inside homes. Furthermore, both types of ingredients can cause the type of effects obtained in either house hold chemical products like those seen in volatile organic compounds (VOCs). However, as with other household products, there is an insufficient understanding at present about what pesticide concentrations are necessary to produce these effects. Exposure to high levels of cyclodiene pesticides commonly associated with misapplication had caused various symptoms, including headaches, dizziness, muscle twitching, weakness, tingling sensation, and nausea. It is also thought that cyclodienes might cause long-term damage to the liver and the central nervous system as well as an increased risk of cancer. Subsequently, in the U.S, no further sales or commercial use were permitted for the following cyclodiene or related pesticides: chlordene, aldrin, dieldrin and heptachlor. The only exception however is the use of heptachlor by utility companies to control fire ants in underground cable boxes.

Safe pesticides

Ninety percent of pesticides currently in use are synthetic, however, in the last two decades there had been conscious attempts to develop safe and environmentally friendly pesticides. Organic or natural pesticides have received the most acclaim and certain have the endorsement of environmentalists. It has also become increasingly likely that some synthetic pesticides such as (DDT), were not poor choices, but misused and overused thus leading to many reputable environmental groups urging that the use of DDT be reconsidered because its effectiveness is unrivaled and causes minimal collateral damage when properly applied. At the same time, organic pesticides are becoming increasingly effective and affordable. They now command

over 10% of the pesticide market in the United States. The paradox of organic or biopesticides is that the product and genetically engineered organochlorine is natural, being a fungus, virus or bacteria making it an interesting products to the environmentalists. Synthetic pesticides such as organophosphate and organochlorine insecticides have been associated with everything from cancer to neurological disorders and lung irritations in humans. However, these symptoms are unlikely, if not impossible to get from a healthy dose of fruits or vegetables. A variety of pesticides such as mineral oil, malathion, sulphur dimethylamine and many others re used to control fungi and insects on wheat and cereals. It is therefore naïve to think that humans can totally avoid ingestion of pesticides. Chlorinated hydrocarbons present in synthetic pesticides such as methoxychlor, endosulfan and captain accumulate in fatty tissue because it is not completely filtered from the system. Healthy humans can detoxify the body over time and the levels are rarely high enough to do any real harm (Ecoworld 2004).

Biological pesticides or biopesticides

Biological pesticides are pesticides based on microorganisms or natural products (Coombs 2013). They are typically created by growing and concentrating naturally occurring organisms and or their metabolites including bacteria and other microbes, fungi, nematodes, proteins etc. They are often considered to be important components of integrated pest management (IPM) programmes and have received much practical attention as substitutes to synthetic chemical plant protection productions (PPPs). (Coping 2009). Biopesticides are divided into three major classes and these include:

- i. Microbial pesticides which consist of bacteria, entomopathogenic fungi or viruses and sometimes includes the metabolites that bacteria of fungi produce. Entomopathogenic hematodes are also often classified as microbial pesticides, even though they are multi-cellular (Coombs 2013).
- ii. Biochemical pesticides: these are naturally occurring substances that control or monitor in the case of pheromones, pests.
- iii. Plant: incorporated protectants (PIPs): which have genetic material from other species incorporated into their genetic material i.e. genetically modified crops (GMCs). There have been a lot of controversies on (GMSs). In many European countries it is pertinent to note that apart from the biological activity of biopesticides against insect pests, nematodes, fungi and other organism, they usually have no known function in photosynthesis, growth and other basic aspects of plant physiology.

Applications of biopesticides is similar to chemical pesticides and are becoming widely used due to their environmental friendliness and biodegradability.

Examples of biopesticides include

- i. *Bacillus thuringiensis* toxin, (BE Toxin) genetically incorporated into plants as insecticide. There is however controversies associated with use of BT toxin which has been observed to have a negative impact on the liver and kidneys of mammals with contaminated Bt toxin in their diet (Kilie and Akay 2008).

- ii. Entomopathogenic fungi (e.g. *Beauveria bassiana*, *Laccanicillium spp*, *Metarizium spp*).
- iii. Disease controlling agents such as *Trichoderma spp* and *Ampelomyces quisqualis*
- iv. Beneficial nematodes attacking insects e.g. *Sleinernema fetiae* or slug pest such as *Phasmarhabditis hermaphrodita*.
- v. Entomopathogenic viruses e.g. *Cydia pomonella granulo virus*.
- vi. Insect pheromones and other semio chemicals.
- vii. Fermentation products such as spinosad (a macro-cyclic lactone)
- viii. Chitosan: a plant to which this product is applied will naturally induce systemic resistance to allow the plant to defend itself against disease, pathogen and pests (Benhamou et al 1994).
- ix. Natural plant derived products such as alkaloides terpenoids, phenolics and secondary chemicals. Certain vegetable oils are known to have pesticidal properties. In addition, products based on extracts of plants such as garlic have now been registered in the European Union and other places.
- x. Naturally occurring minerals such as baking soda are also thought to have pesticide properties.

Biopesticides have advantages and disadvantages over the conventional chemical pesticides and these advantages include non detection of harmful residues, cost effective, biodegradable and in some cases more effective Their disadvantages include high specificity, slow pace of action, variable efficacy and the potential of ability of target organism to acquire tolerance as living organisms evolving.

3.4.3. Triclosan

Triclosan (TCS) is a broad spectrum antimicrobial compound that is incorporated into numerous consumer products. Triclosan which is contained in about half of liquid soaps functions by slowing or stopping the growth of bacteria, fungi and mildew. Triclosan frequently gets into streams and rivers through domestic waste water, leaking sewerage and sewage overflows. The bacterial resistance caused by triclosan was found to disrupt aquatic life by changing natural bacterial communities leading to emergence of resistance bacteria that could diminish the usefulness of important antibiotics (Drury et al 2013).

3.4.4. Criminal Environmental xenobiotic pollution

There had been recent cases of pollution of the environment with criminal intents. One of the most notable is the use of Aldicarb (Temik) a carbamate insecticide which is extremely toxic to mammals and has been widely used by wildlife poachers in South Africa for many years to poison rhinos and other wild life species. Aldicarb is also used by burglars to poison sentry dogs, and the general public to destroy rats and stray dogs. Although banned in South Africa, it is readily available in Zimbabwe and Mozambique and large quantities are smuggled into South Africa for illegal sale there.

Temik is widely used normally as a pesticide on crops such as cotton, potatoes and peanuts and it is registered under the terms of The Fertilizers, Farm Seeds, Agricultural and Stock Remedies Act of 1947. As a member of the carbamate pesticides, classification is divided into super high and medium toxicity. By implication, it falls within the super toxic class which makes it highly toxic. It takes ½ - 1½ hour for symptoms to show and intoxication lasts up to six to eight hours. Fatality is due to asphyxiation as the lungs are flooded with secretion from the stomach. A teaspoon is enough to kill a grown rhinoceros while 1 µg can kill a rodent, making it more poisonous than arsenic. Rhinos are targets in Africa and Asia for their horns which fetch high prices in Yemen where they are prized for making dagger handles and in East Asia where they are used in traditional medicine. Application of Temik and other xenobiotics like cyanide for criminal intent of poaching is by lacing watering holes of the animals with the said substances. (Promed 2013)

Successful treatment of animals poisoned with aldicarb is the timely treatment with anti-muscarinic drugs such as atropine with additional supportive treatment options including fluid therapy, diphenylhydrazine, benzodiazepams and prevention of further absorption using activated charcoal (Amot et al 2011). Early treatment can be very successful. In areas like South Africa where such practice is endemic, pet owners are advised to keep their dogs inside or in a backyard at night and pets should be fed at night to prevent them from eating poisoned baits. Furthermore obedience training of dog to prevent food acceptance from strangers is also advised. At one of the veterinary clinics in Gauteng Province in South Africa, in 2003, 97 cases of aldicarb poisoning were diagnosed (Amot et al 2011). However, the intentional, malicious poisoning of dogs and other species is not restricted to South Africa alone, there are reports of other large scale practices in USA and Spain (Wassem et al 2012). It has also been reported that aldicarb is illegally used as a household rodenticide in Brazil and the Caribbean Island and sometimes human beings are victims. (Ragoucy-Sengler 2000).

3.4.5. *Natural health products (NHPs)*

Recent evidence have shown that natural health products (NHPs) therapies are increasingly recommended by various health providers, including conventional physicians leading to increased consumption of vitamins and many herbal agents worldwide. According to WHO estimates, the present demand for medicinal plants is about US \$14 billion a year and it is estimated to likely increase to about \$5 trillion by the year 2050. The high prices and sometimes established side effects of synthetic drugs have caused many people to find alternatives in herbal medicine when faced with options. There are however concerns about safety and efficacy of herbal medicine due to lack of regulation and some reported adverse effects. Although the WHO has developed guidelines for the quality control of herbal drugs which provide a detailed description of the techniques and measures required for the appropriate cultivation and collection of medicinal plants, there is still a lacuna between this available knowledge and implementation because the cultivators of herbs for medicinal uses are usually unaware of the regulations and these products may be contaminated with banned pesticides, microbial agents like fungi, heavy metals and chemical toxins which may cause adverse outcomes such as sensori-neural defects, congenital paralysis, liver and kidney damage. These

contaminants may be related to the source of the herbal drugs. Chemical toxins may come from unfavorable post harvest techniques, wrong storage conditions or through chemical treatment during storage period. Some of these environmental factors may be controlled by implementing good source; good agricultural practices and standard operating procedures (SOP) for producing good quality herbal products.

In a recent study conducted in Boston, USA on Indian ayurvedic medicines, it was observed that ayurvedic medicine obtained from 30 South Asian store in the Boston area had potentially harmful levels of lead, mercury and arsenic. These metals were found in the products like “bal guti”, mahayograj guggulu”, mahalaxmi vilas ras” safi, shilajit and etc in some of the leading stores within the ayurvedic communities. Therefore, users of the medicines may be at risk of heavy metal toxicity similarly; Koh and Woo (2000) reported excessive toxic heavy metals in Chinese proprietary medicine in Singapore during the year 1990-1997.

In addition Wong et al (1993) also reported concentration of nine heavy metals; viz cadmium, cobalt, copper, iron, manganese, nickel lead, zinc and mercury in 42 Chinese herbal drugs. The concentration range of the stores of metals were comparable to that reported in many of the East Asian vegetables and fruits. Few samples contained a higher concentration of toxic metals such as calcium, lead and mercury. This report suggested that the presence of heavy metals was probably caused by contamination during air drying and preservation (Rai and Mehrotra 2005). Whether an element is toxic or not is determined by many factors including route of exposure, dose, site of accumulation, nutritional status, detoxification biochemistry and the particular form of species in which the elements exists within the body. Different species of elements have the potential to display distinct toxicity patterns for example, hexavalent chromium (chromium VI) is highly toxic and carcinogenic while trivalent chromium (chromium III) is an essential metal involved in lipid and carbohydrate metabolism. Similarly, inorganic and organic arsenic are both naturally occurring compounds that display different toxicities. While certain inorganic arsenic species are classified as human carcinogens, some form of organic arsenic such as arsenobetaine (which accumulates in some aquatic organisms such as shrimp) are relatively non – toxic specific forms of some elements also have the potential to be converted within the body to different forms; which changes their properties and potential toxicity.

Most natural health products tested showed detectable contamination with one or more toxic elements, the degree of contamination appears to be linked to the country of manufacture with higher contamination from mercury, arsenic and aluminum primarily found in products imported from China. Marine –sourced NHPs usually have the highest level of lead contamination while non-marine sourced NHPs manufactured in North America generally demonstrated the least contamination among samples tested. Although marine sourced and ayurvedic NHPs were almost often contaminated, the levels rarely exceeded established toxicity guidelines (Genius et al 2012).

3.4.6. Chlorination of water

The most common disinfection method involves some form of chlorine or its compounds such as chloramines or chlorine dioxides. Chlorine is a strong oxidant that rapidly kills many

harmful micro organisms. Because chlorine is a toxic gas, there is a danger of a release associated with its use. This problem can be avoided by the use of sodium hypochlorite, which is a relatively inexpensive solution that releases free chlorine when dissolved in water. The generation of liquid sodium hypochlorite is both inexpensive and safer than the use of gas or solid chlorine. One drain back is that chlorine from any source reacts with natural organic compounds in the water to form potentially harmful chemical by products. These by-products include triethylin, trihalomethanes (THMs) and haloacetic acids (HAAs) are carcinogenic in large quantities and are regulated in the United States of America by the Environmental Protection Agency. For example, rats chronically intoxicated with triethylin drinking water, demonstrated cerebral oedema as well as an increase in phosphatidyl ethanolamine-n-methyl transferase activity. The increased methylation might be a compensatory mechanisms for counteracting the membranes damages induced by triethylin. Furthermore, chloroform, dichloroacetic acid (DCA) and trichloroacetic acid (TCA) which are known liver and kidney carcinogens are by product of chlorine disinfection found in drinking water. In mice treated with these three chlorine by products, hypermethylation and increased expression of c-myc a proto-oncogene involved in liver and kidney tumours were observed. Trihalomethanes viz chloroform, bromo-dichloromethane, chloro-bromomethane and bromoform are regulated organic contaminants in drinking water. Experimental evidences in the female B6C3F1 mouse liver demonstrated carcinogenic activities of the tri-halomethanes. Chloroform and bromo-dichloromethane were observed to decrease the concentration of 5-methyl-cystosine in hepatic DNA. Methylation in the promoter region of the c-myc gene was reduced by the tri-halome-thanes, a process consistent with carcinogenic activities. The formation of THMs and haloacetic acids may be minimized by effective removal of as many organics from the water as possible prior to chlorine addition. Although chlorine is effective in killing bacteria, it has limited effectiveness against protozoa that form cysts in water (*Girdia lamblia* and *Cryptosporidium*, both of which are pathogenic).

Alternatives to elemental chlorine disinfection include chlorine dioxide disinfection, chloramines disinfection, ozone disinfection and ultraviolet disinfection.

- i. Chloride dioxide disinfection: Chlorine dioxide is a faster acting disinfection than elemental chlorine. It is relatively rarely used because in some circumstances it may create excessive amount of chlorite, which is a by-product regulated to low allowable levels in the United States. Chlorine dioxide is supplied as an aqueun solution and added to water to avoid gas handling problems, chlorine dioxide gas accumulations may spontaneously detonate.
- ii. Chloramines disinfection: the use of chloramines is becoming more common as a disinfectant. Although chloramines is not as strong as oxidant, it does provide a longer-lasting residual than free chlorine and it won't form THM's or haloacetic acids. It is also possible to convent chlorine to chlorine by colding ammonia to the water after addition of chlorine. The chlorine and ammonia reacts to form chloramines.

3.5. Gene environment interaction (GXE)

Every human start life with a particular set of genes of about 20,000 to 25,000. Chemicals may not necessarily cause mutation of genes but may send subtle signals that silence them or switch them on at the wrong times. Chemicals in our environment and food can alter the genes, leaving the exposed, vulnerable to a variety of diseases and disorders including diabetes, asthma, cancer, and obesity. It may therefore be expedient to start testing chemicals for these effects.

3.5.1. Toxicogenomics: environmental epigenetics

Toxicogenomics is a field that emerged from conventional toxicology with functional genomics. In recent years, this field contributed immensely in defining adverse biological effects resulting from environmental stressors; toxins, drugs and chemicals. Through micro array technology, large scale detection and quantification of mRNA transcripts and microRNA related to alterations in mRNA stability or gene regulation becomes feasible. Other omics technologies, notably proteomics and metabolomics soon joined in providing further fine turning in the gathering and interpretation of toxicological data. A field that will inevitably modify the landscape of toxicogenomics is epigenetics, a term referring to heritable changes in gene expression without the accompanying alterations in the DNA sequence. These epigenetic changes may result from mechanism such as DNA methylation, histone modification and non coding RNAs in the regulation of gene expression. Epigenetic mechanisms are essential in normal development and differentiation but these can be misdirected leading to diseases notably cancer. There is now a mounting body of evidence that environmental exposure particularly in early development can induce epigenetic changes which may be transmitted in subsequent generations or serve as basis for diseases developed in later life. Either way, epigenetic mechanisms will help interpret toxicological data or toxicogenomic approaches to identify epigenetic effects of environmental exposures. Thus a full understanding of environmental interactions with the genome requires keeping abreast of epigenetic mechanisms as well as conducting routine analyses of epigenetic modifications as part of the mechanisms of actions of environmental exposure. For example epigenetic modification was observed in the tumour suppressor gene *Tsrc1* (*1-gsf4a*) obtained from transgenic mouse models.

The genome is dynamic and responsive to environmental signals not only during development but also throughout life and it is becoming increasingly apparent that chemicals cause the changes in gene expression that persist long after the exposure has ceased. Commonly used pharmaceutical drugs can cause persistent epigenetic changes. By altering epigenetic homeostasis by direct or indirect mechanisms. Direct effects may be caused by drugs which affect chromatin architecture or DNA methylation. For example the anti-hypertensive hydrazine inhibits DNA methylation while isotretinoin has transcription factor activity. A two tier mechanism is postulated for indirect effect in which acute exposure to a drug influences signaling pathways that may lead to an alternative of transcription factor activity of gene promoters. This stimulation results in the altered expression of receptors signaling molecules and other proteins necessary to alter genetic regulatory circuits. With more chronic exposure,

cells adapt by an unknown process that results in more permanent modification to DNA methylation and chromatin structure leading to enduring alteration of a given genetic network. Therefore, any genetic side-effects caused by a drug may persist after the drug is discontinued.

Some iatrogenic diseases such as 'tardive dyskinesia' and drug induced systemic lupus erythematosus (SLE) may be epigenetic in nature. Furthermore, epigenetic side effects of pharmaceuticals may be involved in the aetiology of heart disease, cancer neurological and cognitive disorders, obesity, diabetes, infertility and sexual dysfunction. It is suggested that a systems biology approach employing micro array analyses of gene expression and methylation patterns can lead to a better understanding of long term side effects of drugs and in future epigenetic assays should be incorporated into the safety assessment of all pharmaceuticals drugs. Some environmental chemicals enable methyl group to attack normal genes turning them off or muting them at a time when they should be turned on. When such genes are turned off, they can't direct the manufacture of proteins that are essential for proper cell function. Chemicals can also uncoil parts of the chromosomes causing genes to be expressed or turned on at inappropriate times. In a recent study in New York City, it was observed that children exposed in the womb to high levels of polycyclic aromatic hydrocarbons (PAH), a common air pollutant from traffic emissions were more likely to have asthma than those not exposed. Using cord blood for the analysis, it was observed that a particular gene (AC SL3) was methylated in the exposed children but not methylated in the unexposed ones. The result was suggestive that the abnormal methylation pattern probably caused the asthma. (Weksberg et al 2010).

Epigenetic changes have also been observed with children conceived with assisted reproductive technologies. One of the disorders that occur at a higher rate in these children is 'Beckwith-Wiedemann syndrome', a disease characterized by abnormal wall defects and a higher risk of certain childhood cancer (Weksberg et al 2010). Beckwith-Wiedemann syndrome is a condition that affects many parts of the body. It is classified as an overgrowth syndrome, which means that affected infants are considerably larger than normal (macrosomia) and continue to grow and gain weight at an unusual rate during childhood. Growth begins to slow by about age 8, and adults with this condition are not unusually tall. In some children with Beckwith-Wiedemann syndrome, specific parts of the body may grow abnormally large, leading to an asymmetric or uneven appearance. This unusual growth pattern is known as hemihyperplasia. The signs and symptoms of Beckwith-Wiedemann syndrome vary among affected individuals. Many people with this condition are born with an opening in the wall of the abdomen (an omphalocele) that allows the abdominal organs to protrude through the navel. Other abdominal wall defects, such as a soft out-pouching around the belly-button (an umbilical hernia), are also common. Most infants with Beckwith-Wiedemann syndrome have an abnormally large tongue (macroglossia), which may interfere with breathing, swallowing, and speaking. Other major features of this condition include abnormally large abdominal organs (visceromegaly), creases or pits in the skin near the ears, low blood sugar (hypoglycemia) in infancy, and kidney abnormalities. Children with Beckwith-Wiedemann syndrome are at an increased risk of developing several types of cancerous and noncancerous tumors, particularly a rare form of kidney cancer called Wilm's tumor, a cancer of muscle tissue called rhabdomyosarcoma, and

a form of liver cancer called hepatoblastoma. Tumors develop in about 10 percent of people with this condition and almost always appear in childhood. About one in five infants with Beckwith-Wiedemann syndrome dies early in life from complications related to the disorder. Older children and adults are much less likely to have serious medical problems associated with the condition. With respect to *in vitro* fertilization, it was postulated that the culture medium where fertilized eggs were grown for several days before implantation probably caused the syndrome. The possibility that the different media used for the eggs might contain a chemical contaminant that stimulate the addition of methyl group to the cells.

Some toxic metals have also been implicated to having epigenetic effects. For example nickel, chromium and arsenic are well known not because they are toxic to cells, directly but due to the fact that they cause increased DNA methylation which may result in gene silencing, cell transformation and subsequently cancer (EHN 2011)

3.5.2. *Histone modifications*

In humans, protection and packaging of the genetic materials are largely performed by histone proteins which also offer a mechanism for regulation of DNA, transcription, replication and repair. Histone are nuclear globular proteins that can be covalently modified by acetylation (AC) methylation, phosphorylation, glycosylation, sumoylation ubiquitination and adenosine diphosphate (ADP) ribosylation thus influencing chromatin structure and gene expression. The most common histone modification that have been observed in environmental chemical exposure are acetylation and methylation of lysine residues in the amino terminal of histone 3 (H3) and H4. Histone acetylation with only a single acetyl group added to each amino acid residue usually increases gene transcription activity. Whereas, histone methylation found as mono (me) dimethyl (me₂) and trimethyl (me₃) groups states can inhibit the increased gene expression depending on the amino acid position that is modified.

3.5.3. *Environmental antibiotic resistome*

Antimicrobial drug resistance is caused by microbial gene products that attenuate the activity of an antibiotic in an otherwise- drug sensitive organism. Bacteria easily acquire resistance gene even in the absence of selection. Hospitals are well known hot spots for the acquisition amplification and dissemination of resistance genes because of the steady supply of strong selective pressure through the prevalent use of antibiotic therapy (Gaze et al 2013)

The failure of antibiotics that were previously effective in controlling infectious diseases is a serious phenomenon that gravely affects the human health. The gene that confers resistance to pathogen was thought to originate from non pathogenic environmental microbes. This environmental resistome, its mobilization and the conditions that facilitate its entry into human pathogens are at the heart of the current public health crisis in antibiotic resistance. Therefore understanding the origins, evolution and mechanism of transfer of resistance elements is vital to the ability to adequately address this public health issue. Recent advances in microbial ecology have revealed the extensive presence of antibiotic resistance genes in environmental bacteria from human polluted; agricultural and pristine soils. The bioactive

products and the mechanism for resistance are diverse bearing in mind the varieties of microbes on the earth. The environmental resistome offers a vast reservoir of genes that have the potential to be mobilized into the antibiotic drug-sensitive cadre of bacterial human pathogens.

One of the distinctive characteristics of microbial genomics is the movement of genes vertically through populations by cell division and horizontally across species and genera. This movement is enabled by the "mobilome", the genetic element that enable and contribute to the horizontal gene transfer (HGT) (Si et al 2009). The mobilome is key to the spread of genes encoding resistance to antimicrobial drugs and heavy metals and for pathogenic traits among bacteria. Because these functions are often co-located on the same mobile elements, selection for 1 phenotype inadvertently selects for its unintended (and often recognized) companion. For example, selection for heavy metal or biocide resistance is often accompanied by antimicrobial drug resistance elements selection for resistance to 1 drug can co-select for 1 of many other (45 genes in 1 notable example) The scale of genetic transfer ranges from short gene segments to mega-bases of DNA depending on the transfer mechanism involved. Thus, even physically distant genes can be co-selected. These facts form a reality that offers cautionary tales for the substitution of 1 drug for another in response to resistance in clinical or agricultural setting or for the use of metals (or exposure to them) that can co-select for antimicrobial drug resistance. Furthermore, plasmids can encode toxin/antitoxin systems that result in plasmid addiction even in the absence of selection. The net result is an exploded mobile meta-genome of shared genetic traits that is fluid and readily promulgated through microbial populations. The rapid movement of water, plants, animals, soil, and humans across the planet virtually ensures that such traits and associated organism, once easily ecological segregated, can move seamlessly through habitats across the globe. The result is that no regions are safe or can escape the introduction and movement of antimicrobial drug-resistant organisms and their genes.

In addition, the antimicrobial drugs themselves, toxins and other compounds can favour genetic exchange and increase genetic diversity. Three principal mechanisms are involved in HGT and these include; conjugation (direct cell to cell transfer), transduction (phage-assisted transfer) and natural transformation (DNA to cell transfer). These mechanisms mobilize genetic elements such as plasmids, genetic islands and phages that can contain resistance elements (Colomer-Llurch et al 2011). The environmental hot spots for horizontal gene transfer (HGT) have been identified and they include the soil particle pores, air-water interfaces in the aquatic environments and biofilms formed on multiple surfaces. Other hot spots include sewage treatment plants where a wide range of chemicals meet human and environmental bacteria in high numbers and manure lagoons where bacterial densities and antimicrobial drug concentrations can be very high and exposure periods lengthy; aquaculture ponds that are routinely treated with antimicrobial drugs, biofilters used in degrading pesticides and environments contaminated by discharge from waste water treatment plants from antimicrobial drug manufacturing. It is therefore instructive that all efforts should be made to minimize the release of antibiotics into the environment.

3.5.4. Insecticide resistance gene in insects: the case of bed bugs (*Cumox lecturlaries*)

Perhaps one of the fall out of environmental pollution with insecticides is the development of resistance gene in insects. Although no putative link has been proven, recent discovery of resistance genes in bed bugs makes it plausible. Recent advances in genomic and post genomic technologies have facilitated a genome wide analysis of the insecticides resistance - associated genes in insects. A good example is the observation of resistant genes in bed bugs. Bed bugs (*Cumox lecturlaries*) are parasites that suck human blood. They come out at night and take five to eight minutes to feed and then return to cracks and crevices where they aggregate. In a recent study, a survey of the entire genome of 21 different bed bug population from cities around the Midwestern region of the United States of America (USA), identified 14 genes (molecular markers) that are associated with pyrethroid resistance. Pyrethroids are the chemicals that have been used as the first-line agent against bed bug infestation. Most of the resistance associated genes are functioning in diverse mechanisms and are expressed in the epidermal layer of the integument, which could prevent or slow down the toxin from reaching the target sites on the nerve cells where an additional layer of resistance (kdr) is possible. This strategy that has evolved in bed bugs is based on their unique morphological, physiological and behavioural characteristics and has not been reported in any other insect species. RNA interference – aided knock down of resistance associated genes showed the relative contribution of each mechanism towards overall development. Blocking these special genetic defenses in the laboratory is a relatively straight forward process. The bugs were simply injected with strands of RNA that interfere with gene expression. When these genes are blocked, the bed bugs once again became susceptible to pyrethroids. The challenge now is how to transmit strands of interfering RNA to wild bed bugs. Furthermore, understanding the complexity of adaptive strategies employed by bed bugs will help in designing the most effective and sustainable bed bug control methods (Zhu et al 2013)

4. Pharmaceutical disposal and environment standard

4.1. Good manufacturing practice (GMP)

Good manufacturing practice or (GMP) are practices and the systems required to be adapted in pharmaceutical manufacturing, quality control, quality system covering the manufacture and testing of pharmaceutical or drugs including active pharmaceutical ingredients, diagnostics, foods, pharmaceutical industry in over one hundred countries worldwide, primarily in the developing world. So far, emissions into the environment are not included.

4.2. Disposal of unused medicines

Although medicine play an important role in the treatment of many conditions and diseases, when they are no longer needed, it is important to dispose of them properly to avoid harm to others and the environment. Some of the well established methods of disposal include.

- i. Medicine take back programs: this is a good way of removing expired, unwanted or unused medicine. In most advanced countries, these are well established but such programs are absent in developing economies.
- ii. Disposal in household trash: this should be placed in a sealed plastic bag to prevent them from getting to the environment before they reach the treatment sites.
- iii. Flushing of certain medicines. There is a small number of medicines that may be especially harmful and in some cases fatal with just one dose if they are used by someone other than the person for whom the medication was prescribed. To prevent accidental ingestion by children, pets or any other persons, such medicines are flushed down the toilets or sinks as soon as they are no longer needed. However, there should be caution in the application of this disposal method due to current environmental concern of xenobiotics which is still emerging. Usually, medicines recommended for flushing are indicated in the medication guide of such drugs.

4.3. European Union (EU) regulations

The new directive for human pharmaceuticals explicitly requires that all member states should establish collection systems for unused or expired medicines such systems were already in use in several member countries at the time new legislation went into action in 2004. However the directive does not regulate how the collected pharmaceuticals should be handled. Disposal into the sewage system is still the legally accepted route of elimination. However, incineration at high temperature (1200°C) is a preferred alternative to avoid environmental pollution.

4.4. United States Environmental Protection Agency (EPA) recommendation on disposal of household pharmaceuticals

Many state and local enforcement agencies, communities and organizations have established take back events, mail – back and other collection programs to collect old, expired or simply unwanted prescription and over the counter pharmaceuticals from households. The progress the safe disposal of household pharmaceuticals has become more prevalent throughout communities in order to reduce the misuse and abuse of drugs and to prevent the practice of flushing consumer pharmaceuticals which may result in their entry into the environment. There are laws put in place enforced by the Drug Enforcement Administration's (DEA) to protect public health and safety. Many of such drugs are household pharmaceuticals which are collected through the take-back events. In October 2010, the Secure and Responsible Drug Disposal Act of 2010 was enacted. The Act and implementing regulations will provide the basic framework to allow the public who are the ultimate users to dispose of their unwanted or expired controlled substance pharmaceuticals in a secure and responsible manner. Currently, pharmaceuticals collected from ultimate users in a take back event are mostly destroyed by incineration EPA is currently recommending incineration as the preferred disposal method for household take back programme. Since it is believed that incineration will address both environmental and diversion concerns (EPA 2012). It is an accepted fact that excretion of drugs after human and veterinary therapeutic use dominates the global input of pharmaceuticals

into the environment. Other sources include effluent discharges from hospitals and manufacturing sites if emissions are not properly treated and controlled. If appropriate preventive methods are put in place, disposal of unused drugs can be effectively managed. A drug disposal programme is therefore inevitable and may include guidance for patients, take back schemes and distinct disposal procedures.

Unlike manufacturing related sources and unused drugs, pharmaceutical residues in the environment as a consequence of patients drug use are inevitable. The level of effective sewage treatment in a particular region may reduce the resulting concentrations; but there will still be some residues remaining. Therefore the challenge to scientists and environmentalists is to determine the acceptable level that will pose no significant health risk.

4.5. WHO guideline for safe disposal of unwanted pharmaceuticals

The WHO guidelines on safe disposal of unwanted pharmaceuticals stemmed from experiences of handling large quantities of drugs donated as part of humanitarian assistance during conflicts and natural disasters. Undoubtedly many of the pharmaceuticals save lives and alleviate suffering, but some donations given with good intention may not only be utterly useless but may result in waste and disposal challenges. Pharmaceuticals may arrive past or near their expiry date, may be inappropriate for the needs, be unrecognizable because they are labeled in a foreign language or may have been sent in unwanted quantities. Furthermore, donated pharmaceuticals with a long shelf – life may be mismanaged, particularly in the confusion during and after armed conflict or natural disasters. Staff and storage space may be lacking and the pharmaceutical management system in disarray. Smaller quantities of pharmaceutical waste may accumulate in the absence of emergency situations due to inadequacies in stock management and distribution and to lack of a routine system of disposal. Safe disposal of these unwanted or expired drugs often creates a major problem. To mitigate these challenges, the World Health Organization (WHO) has developed a guidelines for safe disposal of pharmaceutical wastes and unwanted drugs and these include.

a. Disposal methods

- i.** return to donor or manufacturers
- ii.** dispatch to landfill
- iii.** waste immobilization: encapsulation
- iv.** waste immobilization: inertization
- v.** dispatch to sewers
- vi.** Burning in open containers: this can only be used for small quantities to prevent air pollution due to release of smoke and residues.
- vii.** Medium temperature incineration
- viii.** Novel high temperature incineration
- ix.** Chemical decomposition.

b. Sorting

Drugs are better managed when sorted into categories e.g. anti-histamines, expired or unwanted drugs, hazardous or potentially hazardous non-pharmaceutical materials like aerosol cans and recyclable materials.

- c. Recommended disposal methods by sorting category sorting leads to application of appropriate disposal methods. For example solids, semi solids and powders, liquids, ampoules, anti-infective drugs, controlled substances, anti-neoplastics, disinfectants and aerosol canisters are disposed differently

4.6. Environmental ethics

The recent identification of single nucleotide polymorphism (SNPs) and their correlation with the modulation of enzyme activity in human cases has further crystallized the links between factors implicated in individual susceptibility and exposure to toxic substances in the environment. The frequently evident opposition between causal interpretations based either on genetic heritage or an external environmental facts serves to focus attention on the possibility that the triumph of “constitutionalist” views may reject the more complex and costly primary prevention measures to deal with pollution. In ethical terms the class between the genetic susceptibility of certain population groups and the vulnerability of the human “condition” bound up with our interdependence on the environment requires that we overcome the prevailing opinion that holds exposure to toxic substances to be normal. The supposed congenital deficient of some subjects or disorders linked to age, health condition or lifestyles fails to recognize that the risks to human health affect every single person without exception.

Environmental pharmacology or eco-pharmacology, though an evolving scientific concept possesses a great potential to promote “green healthcare”, reduce health costs, protect the environment and public health. From all indications environmental-drug contamination from pharmaceutical residues, drug manufacturing, cosmetics, veterinary drugs, and herbicides are of a global concern that calls for drugs environmental fate profiling. Gleaning a lesson from the principle of environmental protection which is prevention, it is important to emphasize prudent drug prescription by physicians. In addition, regulatory agencies should establish functional ecopharmacovigilance programme. This will not only protect the environment but will reduce the evolution of antibiotic stains of pathogens and reduce waste of resources on unused drugs.

4.7. Environmental Risk Assessment (ERA) and environmental classification of drugs

In most developed countries, there are regulatory requirements governing the environmental risk assessment of pharmaceuticals. In most cases, a new regulatory submission or line extension has to be accompanied by an ERA which requires environmental fate and effects tests to be taken (Holm et al 2012). Procedure for ERA involves the generation of a risk quotient; i.e. the ratio of the predicted environmental concentration (PEC) to the predicted no – effect concentration (PNEC) ratio (PEC: PNEC). The PEC provides an estimate of maximum concentration anticipated to occur in the environment resulting from patient use and subse-

quent excretion into the waste water systems. Currently, the concept of ecopharmacovigilance is evolving. This implies environmental protection from pharmaceutical contamination. Although slightly distinct from pharmacovigilance, which is patient oriented, there is nevertheless a measure of provision for ecopharmacovigilance within the European Pharmacovigilance Framework, which includes a reference to the pollution of waters and soils with pharmaceutical residues and states that “member states should consider measures to monitor and evaluate the risk of environmental effects *including those which may have an impact on public health*”. Furthermore, it should be observed that the general industrial practices include environmental impact assessment (EIA) therefore, the concept of ecopharmacovigilance which has drugs, cosmetics and household products as its focus is an additional provision for environment protection

In Sweden, the industry together with universities and health care sector had developed a method for environmental risk assessment and environmental classification of drugs. Environmental risk refers to the risk of toxicity to the aquatic environment. It is based on the ratio between predicted environmental concentration (PEC) and the highest concentration of the substance that does not have a harmful effect in the environment (PNEC). Environmental hazard expresses the inherent environmentally damaging characteristics of the substance in terms of persistence, bioaccumulation and toxicity. The toxicity tests used are usually acute toxicity of fish, acute toxicity of *Daphnia* specie and growth inhibition test for algae. Most medication on the Swedish market are now classified. This gives the health care practitioner the possibility to make better choices when prescribing medicine (Fent 2006, Daughton and Ruhoy 2008).

5. Ecopharmacovigilance (EPV)

The term “vigilance” according to the dictionary is the ability to maintain attention and alertness over a prolonged period of time. Traditionally, the focus of pharmacovigilance has been directed towards detecting, monitoring collecting, assessing and evaluating data regarding the human hazard posed by medicine, with the primary objective to reduce the occurrence of adverse drug reaction to the patients. While pharmacovigilance has a clinical focus, ecopharmacovigilance has an environmental focus. It will therefore be erroneous to attempt to group the two together. For example, the role of pharmacovigilance commences from post marketing surveillance while ecopharmacovigilance commences from the point of production to the point of disposal. However there is a little provision for environmental protection by those responsible for pharmaco vigilance from unintentional contact with the active ingredient in pharmaceuticals. Therefore as the practice of ecopharmacovigilance evolves, for practical purposes it will be expedient to concede the responsibility to the environmental health or public health department. In an attempt to rightly define the procedure for ecopharmacovigilance, it is important to examine the procedural framework for pharmacovigilance. This include (a) Post marketing surveillance and other methods of adverse drug reactive (ADR) monitoring such as voluntary reporting by doctors (b) dissemination of ADR data through “drug alerts” or “medical alerts” adversaries sent to doctors by pharma-

ceuticals and regulatory agencies such as FDA in the USA, committee on safety of medicines in UK and NAFDAC in Nigeria. (c) Changes in the labeling of medicine indicating restrictions in use or statutory warnings, precautions or even withdrawal of the drug. (Cuklev et al 2012, Fick 2010, Holn et al 2008)

From all indications while pharmacovigilance is patient oriented, ecopharmacovigilance is environment and public health oriented. However, it is rational to have a cooperation between the two. Thus, ecopharmacovigilance can be defined as the “science and activities relating to the safe discharge of effluents from drug manufacturing plants, safe handling and disposal of drugs and syringes in the hospital and sewage plants. While pharmacovigilance falls within the purview of pharmacists, doctors and nurses who deals with administration of drugs, ecopharmacovigilance entails the dedication of environmental health officers, public health engineers, veterinary physicians and agriculturists. However, those to be charged with environmental monitoring of drugs should be knowledgeable in same. In ecopharmacovigilance, there are some practical measures that can be taken to assess environmental risks across the product life-cycle; particularly after the launch of a new drug, to ensure that risk assessment and scientific understanding of pharmaceuticals in the environment remain scientifically and ecologically relevant (Kummerer and Velo 2006, Rahman et al 2007)

These measures include:

- a. Ensuring that the effluent from production source are treated and properly disposed of.
- b. Tracking environmental risks after launch of the product. If not locally manufactured, via literature monitoring for emerging data on exposure and effects.
- c. Using environmental risks management plans, (ERMPs) as a centralized resource to assess and manage the risks of a drug throughout its life cycle.
- d. Further research, testing or monitoring in the environment when a risk is identified.
- e. Keeping a global EPV perspective
- f. Increasing transparency and availability of environmental data for medicinal products.

These measures could help to ensure that any significant environmental issues associated with pharmaceuticals in the environment (PIE) are identified in a timely way and can be managed appropriately (Holn et al 2008). Thus pharmacovigilance should employ a wide spectrum of means to minimize the ecological footprint of medications as well as the possibility of their causing harm to humans and domestic animals, not just by way of intended use, but also by their unintended use as well as cessation of use. Many drugs have double lives. Once the active pharmaceutical ingredients (API) in administered medications have completed their intended purposes in therapy, disease prevention diagnosis or cosmetics, they can take on another lives in the environment. APIs from a large and diverse spectrum of pharmaceuticals can enter the environment as trace contaminants especially in waters, at individual concentrations generally less than a part per billion (mg/l) but sometimes more. The predominant route by which API's gain entry into the environment is via the discharge of treated and untreated sewage contaminate with PAI simply as a result of medications used for the purposes for which they were

designed. Residues of APIs from drugs that are administered parenterally (e.g. via injection and infusion) and enterally (e.g. via ingestion) are often excreted in faeces and urine and topically applied medications can be washed from skin during bathing. For most APIs, the fraction of unchanged, parent API transferred to the environment is altered as a result of metabolic activities in the body or transformation within a sewage treatment facility such as microbial degradation, for some APIs, only a small percentage of the total amount used is ever transported to the environment. For others, this percentage can approach 100%. The secondary route of transfer of API to the environment is from the purposeful, direct disposal of left over or unwanted medications to sewers as trash (Daughton and Ruhoy 2008).

In ecopharmacovigilance, pharmacovigilance and environmental protection are intimately tied just as are human health and ecological integrity (Daughton 2003a). The importance of ecopharmacovigilance cannot be over emphasized and if properly applied, there will be reduction in ecological exposure to drug contaminants and reduction of drug residues though at low levels making their way into our food and drinking water supplies, thereby further reducing human exposure. The ultimate goal of ecopharmacovigilance should be the design and implementation of changes in the aspects of the drug distribution/consumption chain in order to minimize or eliminate the generation of left over medications so that disposal is not needed to begin with. Ecopharmacovigilance has the potential to influence the re-designing of the healthcare system so that only the most efficacious medications are presented in minimal doses and dispersed in quantities and for durations to ensure their full consumption. The ideal outcome would be the absence of left over drugs which require disposal. EPV also promotes the concept of “greener” healthcare system which not only protects the environment but ensure more official utilization of healthcare resources, reduced healthcare costs, improved healthcare outcome and reduced incidence of purposeful drug abuse and accidental poisonings from diversion of stock piled drugs.

6. Discussion

Drugs and house hold products are part of man’s needs for various purposes. Drugs are prescribed for various medical conditions and cosmetics and house hold products are consumables of daily need. However, there is a growing concern among scientists and environmentalists on the impact of drug contaminants on the environment. This phenomenon is not limited to any region of the world but global. In many countries, low levels of medicines have been detected in sewage treatment plant (STP), effluents, surface water, ground water and drinking water. Effects of some drugs have been investigated in aquatic ecosystems and different species of the aquatic environment are known to be affected by disposed drugs when present.

The dramatic decline in vulture population in Asia which was linked to the use of diclofenac to treat cattle is a classical case of the importance of ecopharmacology. An alarming decline in the number of vultures poses the threat of outbreak of epidemics in parts of Asia due to potential increase in the number of decaying carcasses as a result of this. The three species of

Asia vultures viz Asian white-backed vultures, (*Gyps bengalensis*) Indian vultures (*Gyps indicus*) and slender-billed vultures (*Gyps tenuirostris*) are listed as critically endangered by the International Union of Conservation of Nature (IUCN) based in Switzerland. Furthermore, the decline in the vulture population has threatened the tradition of "Parsis"; a sect of the Zoroastrians who traditionally expose human corpses to vultures for disposal. As a sequelae in Mumbai, India, it had been reported that Parsis have stopped leaving human corpses in the "Tower of Silence" because the vultures that once quickly consumed the carcasses are vanishing (Houston 1990).

There have been specific cases of intersex fish in European rivers. The fact that traces of different drugs and personal care products including steroids and insect repellent have been detected in the American waters and the fact that traces of cocaine have been detected in River Thames in London underscores the global dimension of environmental drugs contamination. In India, different studies have shown that effluent from waste water treatment plant serving several pharmaceutical companies contain residues of many drugs, particularly antibiotics. However, it is generally accepted that the major sources of release of drugs to the ecosystem is from treated and untreated sewage end products due to human consumption and disposal of drugs. An important factor that promotes the release of pharmaceutical and house products into the environment is decaying infrastructure whereby aging sewage pipes are cause leaks of effluents into the environment.

Perhaps another area of neglect currently in ecopharmacology is the potential effect of herbal preparations. Herbal drugs have pharmacological properties which are likely to be ignored with respect to their environmental impact due to the assumption that plants are generally natural entities and therefore environmentally friendly. There is therefore the necessity for more studies of environmental impact of herbarium processes. In the same respect the effect of marijuana" probably the most commonly consumed narcotic worldwide on the ecosystem is likely to be dramatic. For example, it was observed that hay contaminated with the Chinese herb *Aristolochia* specie caused renal failure in horses (Grollman 2013). In addition, Parsley; another commonly used herbal preparation was said to have the negative attribute of absorbing toxic metals from the environment and therefore, its cultivation near waste water or using waste water for its irrigation is discouraged (Awe and Banjoko 2012). It is also important to note that preventing environmental drug contamination will protect the very important biofilm of the ecosystem and of the sewage system for proper functioning. The basic principle of environmental protection is prevention of environmental pollution, minimizing unavoidable emissions and remediating any existing damage. With reference to environmental pharmacology, it is therefore expedient to reduce as much as possible the use and circulation of drugs and establish a program or coordinated disposal of unused drugs. For example, there could be a take-back scheme whereby unused drugs are returned to the source of dispensing for proper disposal. There should be more prudent prescribing of drugs and patient use. Private stock piling of drugs and improper storage should be discouraged and the established drug disposal route should be monitored periodically. The very important impact of unsafe disposal of drugs is the potential of promoting the evolution of antimicrobial resistant

pathogens. This has a significant impact on clinical practice, public health, health economics and even ecopharmacology.

7. Conclusion and recommendations

The scope of human exposure to pharmaceuticals and personal care products from the environment is a complex function of many factors. These factors include the concentrations, types and distribution of pharmaceuticals in the environment, the pharmacokinetics of each drug, the structural transformation of the chemical compounds either through metabolism or natural degradation processes and the potential bioaccumulation of the drugs. Furthermore, the full effects of mixtures of low concentrations of different PPCPs is also unknown there is therefore concern about the potential they have for harm because they may act unpredictably when mixed with other chemicals from the environment or concentration in the food chain. In addition, some PPCPs are active at very low concentrations, and are often released continuously in large or widespread quantities. Proper destruction of pharmaceutical residues should yield rest products without any environmental formation of such new products. Incineration at a high temperature greater than 1000 degrees Celsius is considered to fulfill the requirements, but even following such incineration residual ashes from the incineration should be properly taken care of. Pharmaceuticals used in veterinary medicine or as additives to animal food, pose a different problem, since they are excreted into soil or possibly open surface waters. It is well known that such excretions may affect terrestrial organisms directly, leading to extinction of exposed species e.g. dung beetles. Furthermore, lipid – soluble pharma residues from veterinary use may bind strongly to soil particles, with little tendency to leak out to ground water or to local surface waters. In addition more water – soluble residues may be washed out with rain or melting snow and reach both ground water and surface water streams.

8. Recommendations

- Contamination of drinking water must be avoided and landfills must be sited and constructed in a way that minimizes the possibility of leachate entering an aquifer, surface water or drinking water system.
- Non- biodegradable drugs, antibiotics, anti-neoplastics and disinfectants should not be disposed into the sewage system as they may kill bacteria necessary for the treatment of sewage. Anti-neoplastics should not be flushed into water courses as they may damage aquatic life or contaminate drinking water. Similarly, large quantities of disinfectants should not be discharged into sewerage system or water course but can be introduced if well diluted.
- Burning pharmaceuticals at low temperature or in open containers results in release of toxic pollutants into the air. Ideally this should be avoided.

- Inefficient and insecure sorting and disposal may allow drugs beyond their expiry date to be diverted for resale to the general public. In some countries, scavenging in unprotected insecure landfills is a hazard.
- In the absence of suitable disposal sites and qualified personnel to supervise disposal, unwanted pharmaceuticals present no risk provided they are securely stored in dry conditions. If stored in their original packing, there is a risk of diversion and to avoid this, they are best stored in drums with the pharmaceuticals immobilized.
- Effective environmental detection methods have to be developed and global detection strategy applied to map the current global situation.
- There are currently no test methods to assess whether negative effects may occur after long term environmental diffuse exposure in humans, during the vulnerable periods of development, on aquatic micro organisms or how it may affect other animals. Therefore the precautionary principle must be guiding.
- Concentrations in surface water alone are not sufficient to assess the risk of negative environmental effects of these synthetic chemicals. Consideration must be taken to bio-accumulation in fish and other aquatic foods consumed by humans, as well as to additive and synergistic effects between pharmaceuticals and other chemicals in the contaminated water

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Safety and Efficacy of *Moringa oleifera* Lamarck (1785) – Therapeutic and Toxicological Properties

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Additional information is available at the end of the chapter

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1. Introduction

About 80% of the worldwide population use herbal products for their basic health care (primary care), such as extracts, teas and their active principles [1]. Despite the interest in molecular modeling, combinatorial chemistry and other chemical synthesis techniques by institutions and pharmaceutical industries, the natural products, particularly medicinal plants, persist as an important source of new therapeutic agents against infectious (fungal or bacterial) and cardiovascular diseases, insects, cancer, immunomodulation and on nervous system diseases [2-7].

According to the World Health Organization, medicinal plant is any plant that contains, in one or more of its organs, substances that can be employed for therapeutic purposes or used as precursors of substances utilized for such purposes [1]. The phytotherapeutic, in turn, is a drug obtained exclusively based on active vegetables raw material and is characterized by knowledge of its effectiveness and risks of their consumption as well as the

reproducibility and consistency of its quality [8]. Therefore, the production of vegetal drugs obeys specific laws in a way to maintain attributes and properties from manufacturing to importation and marketing, whatever use (oral and topical) or manner of preparation (infusions, decoctions and macerations) [9].

The most hazardous concept is that which declares that medicinal plants are nontoxic and without risks to human health since they are natural and have been tested worldwide through centuries. Adverse events, including 101 deaths associated with dietary supplements were reported to the FDA (Food and Drug Administration), but these adverse effects were not well reported whereas there is no an efficient monitoring system in the United States like that for allopathic medicines [10]. Researches conducted in the United Kingdom suggest an incidence around 7% attributed to plants and phytotherapics. Studies conducted in Taiwan and Hong Kong hospitals showed an admission rate caused by plants ranging from 0.2 to 0.5% [11,12]. In Brazil, there were 1037 reports of human poisoning with plants in 2009 (1.29% of total), with 61.9% of poisonings occurred in patients with 1-9 years old. About 0.31% deaths were directly linked to herbal poisoning [13]. The quality of the commercialized medicinal plants, the population inexperience, the origin of the plant, period and methods of collection, storage, drying, packaging, contamination by fungi and other microorganisms and the quantity ingested are factors that obscure the diagnosis and complicate the treatment in cases of poisoning by toxic plants [14,15].

The folk usage of the different parts of *Moringa oleifera* reproduces the general and indiscriminate use of plants in order to treat or (even) cure diseases without regard to their toxic potential. Thus, this chapter aims to review the pharmacological and toxicological potential of *M. oleifera* and their purposes for use and consumption.

2. Taxonomy, distribution and general use and consumption

Moringa oleifera Lamarck, 1785 (synonymy *Moringa pterygosperma* Gaertn.) is the most widespread species belonging to the Moringaceae family (Papaverales Order, Figure 1A), which possess additional 13 species of trees and shrubs originally spread in several Asian countries, such as India, Pakistan, Bangladesh, Afghanistan and Sri Lanka [16,17]. However, *M. oleifera* has been cultivated and introduced in several parts of tropical regions in the world such as Malaysia, Philippines, Singapore, Thailand, Mexico, Peru, the Caribbean Islands, Paraguay and Brazil [16,18].

With several popular names such as “morunga”, “árbol de rábano”, “árbol de los espárragos”, horseradish tree, drumstick tree, never die tree, “sajna”, Ben oil tree, “lírio-branco” and “quiabo de quina” [16-19]. *M. oleifera* is a deciduous and allogamous plant which grows even in poor soils (pH 5-9) and arid climates, being slightly affected by drought (250-300 mm/year). Its fruits present 12 seeds (in average); they are dry, simple and brown (when mature), possessing a dehiscent loculicide capsule with a triangular aspect (Figure 1B). Its embryo is oleaginous, has a pair of cotyledons and a cryptocotyledonary hypogeal germination that

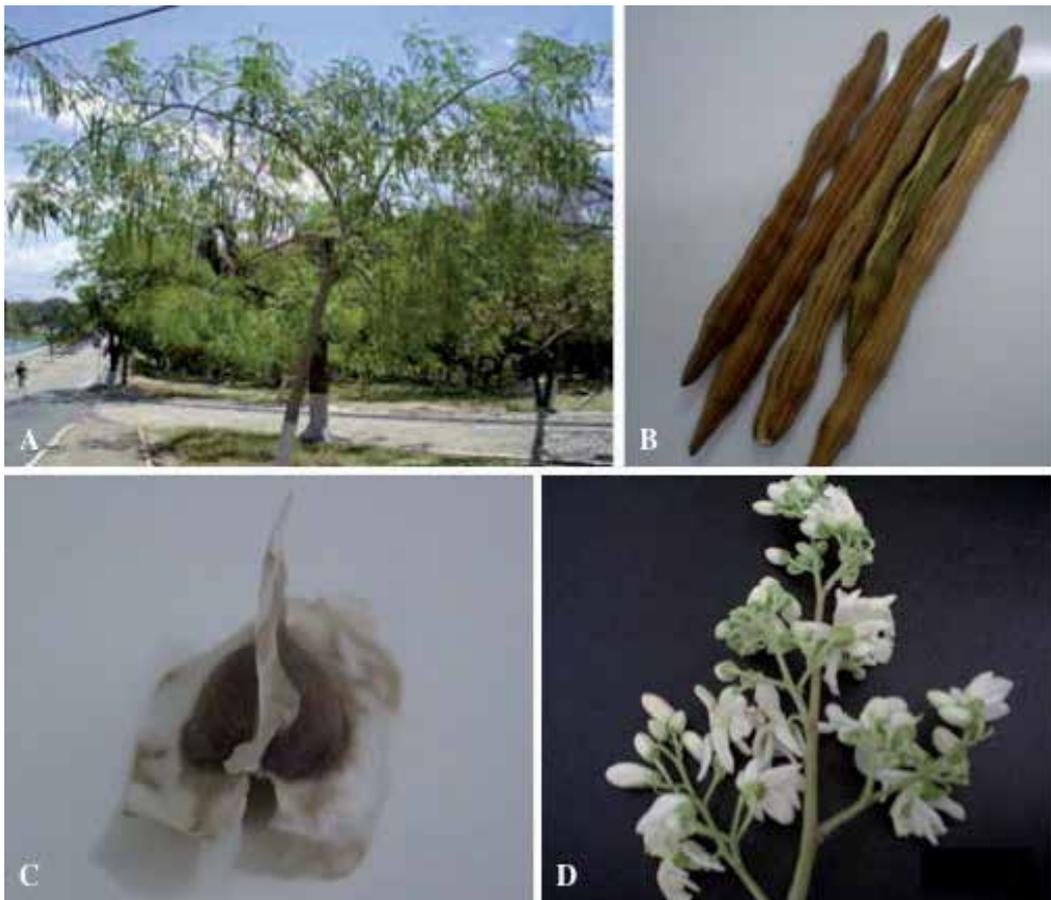


Figure 1. Parts of *Moringa oleifera* Lam. A-Plant; B-Pods; C-Seed; D-Inflorescence. Source: Personal archives.

begins between 5-8 days after seeding [20,21]. Root development presents positive geotropism; the central root is thick, long and with secondary ramifications [21].

Its seeds are anemochoric, bitegumetend, exalbuminous and winged (Figure 1C), making seed dispersal more effective [21]. They can be introduced directly in a definitive way or in seedbeds, though their dissemination can also be made by poles, without previous exceptional requirements, growing quickly up to 4m in the first year and 15 m in height in development later stages. Under favorable conditions, a plant might produce 50-70 kg of fruits/year [18,20,21].

Historically, ancient Romans, Greeks and Egyptians utilized all parts of the plant for human consumption as well as Asian communities have been made now [3, 16]. This primeval use in the East World has been attributed to its Asian origin and a massive popular use of the flora in the Asiatic continent [22]. The flowers (Figure 1D) are rich in Ca^{2+} and K^{+} and leaves are widely used as food complement, with appreciable amounts of vitamins [(A, 7-fold higher than in oranges), B and C], Fe^{2+} and proteins [23-25]. Leaves put in soups are used by Philippines'

women to improve the breast milk production and possess 4-fold the calcium in milk [16,26]. The roots, presenting alkaloids (0.2% of total), are scarcely consumed [16, 27]. However, when powdered, are appreciated as a spicy flavor similar to that showed by horseradish, explaining why the plant is commonly called "Horseradish Tree".

The seed oil is used in industry to manufacture cosmetics, lubricate machines and clocks, such as cooking oil, fuel for lamps and it is highly appreciated in perfume industries due to its ability in retaining fragrances [16] and high stability to oxidative rancidity [28]. Cooked fresh pods are very consumed in Haiti due to its taste comparable to asparagus or green beans; when dried and crushed, they show suitable characteristics to substitute traditional beds of laboratory animals (pine, for example), exhibiting a high absorptive capacity, low concentration of antinutritional compounds and endurance to autoclaving [29]. Stems are extensively used in paper factories and construction of furniture and fences [24].

The approximate composition of *M. oleifera* seeds shows levels of proteins (377.5 ± 1.9 g/kg dry matter) higher than those found in important legumes for human nutrition (149-220 g/kg) [30-32]. In fact, cytochemistry analysis performed in [33] detected a large amount of cotyledonary protein bodies. The oil content (363.2 ± 2.6 g / kg) is greater than that of soybean varieties [32]. The main saturated fatty acids found in this oil are behenic, palmitic, stearic and arachidic, also containing appreciable quantities of unsaturated fatty acids, especially oleic (65-80%) [28,31], which are desirable in terms of applications and nutritional stability for cooking and frying. Vegetable oils with a high percentage of oleic acid has received much attention since the association of diets rich in saturated and unsaturated *trans* fatty acids and increased risk of cardiovascular diseases due to high cholesterol levels have been documented [34].

M. oleifera (leaves, in particular) have also shown a great potential for animal feeding but this approach is underexplored. A complete drying process takes 72 h and yields 1kg of flour from 10 kg of fresh material. Dried powdered leaves have shown promising results to feed fishes [35], chickens [36] and sheep [37,38].

Additionally, studies demonstrate that the high content of proteins has ideal levels of essential amino acids and good availability for intestinal absorption and rumen degradability of nitrogen comparable to soybean meal [30,39,40], indicating a great potential of the leaves as a food supplement for ruminants, though little is known about changes that these proteins may cause in the final composition of the milk or how they may affect the animal growth. Recently, leaves and twigs' flour prepared by drying and grinding was given in substitution to the standard feedstuff of grass (*Pennisetum purpureum*) during six days to lactating cows. Presenting an apparent digestibility index similar to the standard diet, no changes were found in milk composition. On the other hand, cows fed with concentrated soybean meal produced more milk (13.2 kg/day), revealing better energy content in comparison with those that consume moringa meal (12.3 kg/day) [41]. In this event, the hypothesis that meal would influence organoleptic characteristics of milk are not corroborated, since the color, taste and smell remained unchanged, an encouraging finding for farmers who usually face problems with beef cattle undernutrition due to limitations in quality and/or quantity of the feed available.

In a study of 45 days exposure, sheep were fed with 4-6 g/day of MO delipidated seeds. It was found a significant increase in body weight gain with 4g/day of supplementation, corroborated by a higher nitrogen retention and efficiency in the microbial nitrogen production. These animals also showed elevated levels of plasmatic glucose [38], suggesting a relationship between sugar absorption rate and metabolizable energy intake. This result indicates that intake of 4g/day improved diet energy value due to, at least in part, the alterations in gastrointestinal tract microbial population which led to upper fermentative efficiency than those cows fed only with soybean meal. In another report, rats that consumed the aqueous extract of seeds during 30 days showed increased serum albumin and retention of body nitrogen (67.53 ± 2.49 g/100 g) compared to the control group that consumed only tap water (59.55 ± 3.02 g/100g) [32]. The albumin capacity in acting as a reservoir of amino acids may explain the improvement in body nitrogen, since those amino acids not incorporated into a high molecular weight protein are rapidly eliminated by the urinary system [42].

It is known that the seed meal has high levels of essential amino acids, except to lysine, threonine and valine amino acids which are present at low amounts and are important for children nutrition between 2-5 years-old. Elevated contents of methionine and cysteine residues are close to that realized in human and cow's milk and hen eggs. This abundance in essential amino acids stimulates its use as an excellent food supplement for vegetables that are normally poor in sulfur amino acids. Concerning mice requirements in growth phase, the lysine is the first limiting amino acid in the seeds, followed by isoleucine and leucine [30,43]. When added as a supplement to a child's diet, just 25 g of the leaf powder supplies all the calcium and vitamin A daily needs, about half the protein and potassium, and about three-quarters of the iron daily needs [44]. With advances in molecular techniques to manipulate genes, the seeds serve as ideal model for improving the protein quality of foods.

3. Coagulating properties

There are troubles of water distribution for human consumption in many parts over the world. To treat this water before distribution, inorganic and synthetic compounds have been used for sedimentation, filtration and disinfection. Aluminum [aluminum sulphate, $Al_2(SO_4)_3$] and iron [ferric sulfate, $Fe_2(SO_4)_3$] salts, positively charged, lead to the flocculation of negative particles in water via neutralization [45]. Notwithstanding this extensive usage, these salts and synthetic polymers have high costs and low distribution, making their use in developing countries and impecunious sites an interfering economic factor that affects the quality of drinking water [45,46]. Although alum and iron salts are the most widely used chemical coagulants for community drinking water treatment, other coagulants have been and are being used to coagulate household water at point of use, including alum potash, crushed almonds or beans and seeds of *Moringa oleifera* [47]. Some reports describe organic coagulants consisting in polysaccharides, proteins and especially starches, among which are highlighted the cassava flour, arrowroot and potato starch [48], which emphasize the natural coagulants' value as safer and ecologically more acceptable.

Moringa oleifera seeds have been employed as an alternative source to clean water, replacing synthetic coagulants [17], which are often expensive and associated with diseases, such as cancer and Alzheimer [49,50]. Moringa seeds are also used to clean, by flocculation, vegetable oils and irrigation, tap and waste waters, removing algae, volatile organic compounds and heavy metals from the liquid under treatment [46,51-53]. In Brazilian Northeast, they are crushed and put in containers (such as pots, 30-200 mg of seeds/liter of water) to storage water temporarily [19].

Advantages in exploiting the seeds include coagulation efficiency comparable to aluminum salts, complete degradation, pH maintenance, water conductivity, concentration of anions and cations [46,54], and its ability to dramatically decrease bacteria content in 99.9% [55,56]. Stored seeds up to 18 months kept the turbidity reduction in similar percentages. On the other hand, seeds with 24 months displayed a significant reduction in flocculation efficiency. Flocculating effects are greater at pH 6.5 while low temperature (< 15 °C) drops the efficiency of this process [57]. Cationic peptides of low molecular mass (6-16 kDa) are considered the main responsible for sedimentation of the suspended material in water, juices and drinks [56,58,59]. On the other hand, a non-protein active component with 3kDa isolated from seeds was able to flocculate a kaolin suspension [60].

In reference [61] have shown that a seed recombinant protein (isoelectric point of 12.6) expressed in *Escherichia coli* was capable to flocculate rhizobacteria and clay, suggesting that microorganisms undergo sedimentation similar to the colloids. Recently, in [62] also showed the clarifier competence of tablets produced with moringa seeds, which were able to remove oil from water utilized in petroleum extraction with efficiency percentages ranging from 76% (coagulant extracted in aqueous medium) to 96% (coagulant extracted in saline). The principal inconvenience of seeds in water purification is the augmentation in organic matter during treatment [46]. Thus, the water treated with seeds should not be stored for a period longer than 24 h, since the richness in nutrients promotes quick growth of microorganisms.

4. Pharmacological properties

Many medicinal properties of *M. oleifera* have been constantly supported by scientific works and reflect the folk knowledge of its therapeutic qualities (Table 1).

4.1. Antioxidant, antiulcer, hypocholesterolemic and hypotensive

Medicinal plants are good sources of cytoprotective compounds [2]. A single dose (150 mg/kg body weight) of methanolic extract of *M. oleifera* leaves protected bone marrow against chromosomal alterations (aberrations, metaphasic chromosome breaks and micronucleus formation) in mice exposed to gamma irradiation, allowing regeneration of hematopoietic stem cells and increasing survival of the animals [63]. This anticlastogenic effect was also seen in animals treated for 14 consecutive days with a diet enriched with increasing percentage of pods (cooked and pre-frozen), decreasing the number of micronucleated peripheral erythrocytes induced by mitomycin C exposure [64].

Pharmacological Activity	Part of plant	Reference
Abortifacient	Leaves, roots	[113], [114]
Against <i>Plasmodium falciparum</i> and <i>Schistosoma mansoni</i> cercariae	Seeds	[105], [106]
Analgesic	Roots	[27], [84]
Antiatherosclerotic	Leaves	[69]
Anticlastogenic	Leaves, pods	[63], [64]
Anti-constipant	Flowers	[109]
Anticonvulsant	Leaves, roots	[27], [103], [104]
Antiespasmotic	Leaves, seeds	[80]
Anthelmintic	Seeds	[53]
Anti-inflammatory	Seeds, leaves, roots	[80], [81], [82], [83]
Antioxidant	Leaves, seeds	[24], [25], [26], [63], [67], [68], [70]
Antipyretic	Leaves	[18]
Antitumor	Seeds, stem, leaves	[68], [72], [87], [88]
Antiulcerogenic	Leaves, seeds	[65], [66]
Bactericidal	Leaves, stem, pods	[17], [93], [97], [94], [95], [96]
Bradycardic/Hipotensive	Seeds	[77], [78], [79]
Diuretic	Seeds	[80]
Fungicide	Leaves, seeds	[91], [92], [93]
Hepatoprotective	Seeds, leaves	[109], [121]
Hypocholesterolemic	Leaves, seeds, stem	[73], [74], [75], [76]
Immunomodulatory	Seeds	[32], [74], [98], [107]
Larvicidal	Seeds	[32], [100]
Pupicidal	Seeds	[98]
Purgative	Leaves	[18]
Repellent	Seeds	[98]

Table 1. Pharmacological properties of *Moringa oleifera* Lamarck, 1785 (Moringaceae).

The leaf methanolic extract (100 and 150mg/kg) inhibits significantly the formation of gastric lesions caused by acetylsalicylic acid (55 and 78.3%), serotonin (86.5 and 92.4%), indomethacin (86 and 88.8%) and acetic acid (66.2 and 73.4%), respectively, and improves the healing rate of gastric ulcers induced by acetic acid [65]. In a similar way, water extract of *M. oleifera* leaves caused an enhancement of enterochromaffin cell (EC) density with increased 5-hydroxytryptamine (5-HT) content as well as mucosa thickness, showing maximum stomach protection at a dose of 300 mg/kg against lesions induced by aspirin as evidenced by increased mean ulcer index. Treatment with this extract after 14 consecutive days also reduced the severity of ulcer formation [66]. 5-HT is a key regulator neurotransmitter of smooth muscles of cardiovascular as well as gastrointestinal tract, being found in high concentration in EC cells [42]. So, the healing of gastric damage proposed is likely related to the 5-HT releasing from EC cells, which augments mucus secretion via cyclooxygenase pathway, inducing prostaglandin (PG) synthesis, especially PGE₂ and PGI₂, and leading to cytoprotection.

Antioxidant compounds from *M. oleifera* have also been frequently pointed as responsible by the antiatherosclerotic, antigenotoxic, anti-ulcerogenic, hypocholesterolemic and anti-inflammatory properties in the plant. Indeed, leaves, stem bark, flowers and/or seeds have significant quantities of antioxidant molecules such as α -, β - and γ -tocopherols, stigmasterol, campesterol [28,67], quercetin, kaempferol, vitamin A and C and polyphenols [25,68-70]. In India and Philippines, fresh leaves are used to preserve foods, suggesting that they are suitable source of antioxidants [26]. β -sitosterol, a vegetal sterol similar to cholesterol existing in hybrid varieties of *M. oleifera*, seems to be a compound capable of lowering plasma LDL-C (low density lipoprotein cholesterol) [71,72].

The authors of the reference [73] demonstrated that treated rabbits with ground cooked seeds (200 mg/kg/day) showed reduction in plasma levels of total cholesterol (TC), phospholipids, triglycerides (TG), LDL-C and VLDL-C (very low density lipoprotein cholesterol) as well as decreasing in lipid content in kidney, liver, heart and aorta. In this way, [74] and [75] working with similar doses (400 and 300-600 mg/kg, respectively) showed substantial increase in HDL-C (high density lipoprotein cholesterol), the latter also demonstrating dropping in blood levels of TC, TG, LDL-C and VLDL-C. This HDL-C increasing is a desirable event in an ideal hypocholesterolemic agent, since it indicates a possible role in reducing the atherosclerosis incidence. Related findings were seen in [69] and [76], who also divulged the great therapeutic potential and prevention of cardiovascular diseases showed by the water extract of leaves, reducing serum TC and TG and declining formation of atheroma plaques with efficacy equivalent to simvastatin.

The research [69] suggest a direct relationship between phenolic compounds present in leaves and hypolipidemic action, demonstrating that the water extract inhibited oxidative modifications in LDL-C molecule and probably suppressed initiation and propagation of lipid peroxidation and cellular damage induced by free radicals at levels similar to vitamin E [42, 68]. Since vitamin C might scavenge free radicals and regenerate, indirectly, vitamin E [42], this synergism between vitamins A and C have attracted interest as agents to delay and/or blockade atherosclerosis by LDL-C oxidation reducing as a way to keep the intracellular redox state and avoid damage to endothelial cells. Then, it is possible that the atherogenic index decreasing

could represent an anti-inflammatory action of antioxidants present in *M. oleifera* leaves, seeds and stem bark, since atherosclerosis is a chronic inflammatory and degenerative process that affects blood vessels.

Correlated with cardioprotective effects, extracts from leaves, stem bark and pods and nitrile compounds, mustard glycosides and thiocarbamates isolated [4-(α -L-rhamnosyloxy)-benzyl isothiocyanate (Figure 2A), niazirin, niazinins (A and B) and niaziminin] has negative inotropic and chronotropic effects on heart musculature causing bradycardia and hypotension (1-20mg/kg), suggesting that amide or-N=C-moieties and/or sulfur atoms could be critical for the cardiodepressant action [77-79]. Smooth muscle relaxation studied in isolated ileum and uterus certainly corroborates the popular use of the plant in gastrointestinal disorders and explains its antispasmodic activity [80]. Since pre-treatment with atropine did not abolish the hypotensive effects of *M. oleifera* compounds, it is probably that these effects are not mediated by stimulation of M₂ muscarinic receptors and they could trigger independent non-acetylcholine pathway(s).

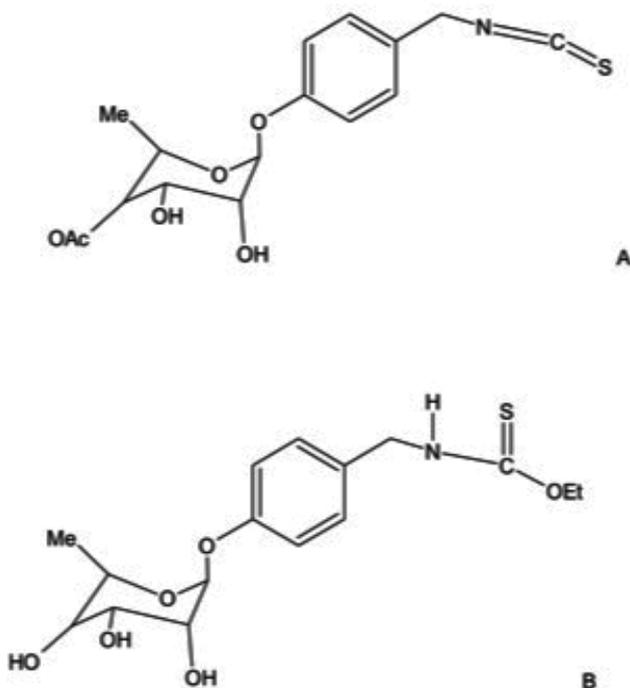


Figure 2. Structures of the compounds 4-(α -L-rhamnosyloxy)-benzyl isothiocyanate (A) and Niazimicin (B).

4.2. Anti-inflammatory and antitumor

The aqueous (1000 mg/kg), ethanolic, hexane and butanolic (3000 mg/kg) extracts of *M. oleifera* seeds reduced edema development in percentages ranging from 34 to 85% [80,81]. The

root methanolic extract, with oral IC_{50} value of 660 mg/kg body weight, also showed anti-inflammatory activity in classical models (paw edema induced by carrageenin and air bag), reducing fluid exudation in a dose dependent way, acute and chronic inflammation and accumulation of cells [82,83]. Compounds (aurantiamide acetate and 1,3-dibenzyl urea) isolated from alcoholic extract of roots decreased serum levels of tumor necrosis factor alpha (TNF- α) and interleukin-2 (IL-2), while 1,3-dibenzyl urea showed analgesic activity [84]. It is known that increased expression of pro-inflammatory cytokines are involved in a variety of autoimmune diseases such as psoriasis, arthritis, systemic lupus erythematosus and Graves' disease [85,86]. Then, compounds as aurantiamide acetate and 1,3-dibenzyl urea that reduce and/or inhibit cytokine production emerge as promising molecules to treat rheumatic diseases, preventing hyaline cartilage destruction and deformity of joints and avoiding the formation and establishment of a debilitating inflammatory process [85].

Inflammation, polycyclic aromatic hydrocarbons such as benzo[a]pyrene and 7,12-dimethylbenzanthracene (DMBA), alcohol, bacteria (*Helicobacter pylori* and *E. coli*) and viruses are involved in promoting carcinogenesis (Weinberg 2008). The text [72] showed that the ethanolic extract of seeds and the isolated molecules niazimicin (IC_{50} of 35.3 mg/mL, Figure 2B), 4-(α -L-rhamnosyloxy)-benzyl isothiocyanate (32.7 mg/mL), 3-O-(6-O-oleoyl- β -D-glucopyranosyl)- β -sitosterol (70.4 mg/mL) and β -sitosterol-3-O- β -D-glucopyranoside (27.9 mg/mL) inhibited *in vitro* leukemia induction by Epstein-Barr virus (EBV) and reduced the viability of Raji malignant cells. Other studies also exhibit cytotoxic activity of leaves on lymphocytic and myelocytic leukemia lines [87,88].

In carcinogenesis studies, niazimicin-treated animals showed delay in skin carcinoma formation induced by DMBA (initiator) and TPA (12-O-tetradecanoylphorbol-13-acetate, promoter) and they also revealed reducing in the number of papillomas, displaying greater activity than β -carotene and glycyrrhetic acid against cancer promoters [72]. The antimutagenic activity evidenced by micronucleus formation attenuation [63,64] may be a factor involved in deferring carcinoma progression. Hence, antioxidants like β -carotene and glycyrrhetic acid might be very effective in combating cancer. Moreover, since the methanolic extract of leaves caused emerging of apoptotic bodies, chromatin condensation, cell shrinking, DNA fragmentation and induce the generation of reactive oxygen species (ROS) in epidermoid carcinoma KB cells, it is believed that *M. oleifera* antiproliferative activity is related to apoptosis intrinsic pathway(s), probably because of the cytochrome *c* release from mitochondria following ROS production [89,90].

4.3. Antimicrobial

Seeds and leaves (and extracts) show activity against different species of fungi (*Trichophyton rubrum*, *Trichophyton metagrophytes*, *Microscoporum canis*, *Epidermophyton floccosum*, *Aspergillus flavus*, *Rhizopus stolonifer*, *Fusarium solani*, *Rhizopus solani* and *Mucor* sp.) [91-93], some of which being strictly anthropophilic dermatophytes. Correspondingly, these extracts have bactericidal and/or bacteriostatic action against *Staphylococcus aureus*, *Vibrio cholerae*, *V. parahaemolyticus*, *Enterococcus faecalis*, *Salmonella enteritidis*, *Aeromonas caviae*, *Pasturella multo-*

cida, *Bacillus subtilis*, *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter cloace*, *Proteus vulgaris* e *Micrococcus kristinae* [93-96].

Initially, it was difficult to accurately identify the responsible component(s) for the antimicrobial properties, since majority of studies was performed with seed and leaf crude extracts. Tannins and polyphenols found in *Moringa* species have shown antibacterial activity. However, some authors attributed this effect to the compounds 4-(α -L-rhamnosyloxy)-benzyl isothiocyanate, moriginin and 4-(α -L-rhamnosyloxy)-phenylacetoneitrile synthesized by the plant [17,97]. Molecules isolated from root barks [deoxy-niazimicin (N-benzyl, S-ethyl tioformate) and pterigospermin] also showed bactericide and fungicide action [24].

Outcomes have demonstrated that these extracts are more effective in low and moderate temperatures (4-37°C), whereas temperatures greater than 70°C lead to loss of antibacterial and antifungal activities, suggesting that specific bioactive compounds would be proteins capable of binding to negatively charged surfaces [32,93]. This finding partially explains the seed water purification efficiency to drop bacteria suspension after 1-2 h of treatment, whose ability has been accredited to basic flocculent proteins [55,61]. Besides, positive monovalent and divalent ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+}) diminished the antifungal and bactericidal activities of plant proteins due to plasma membrane structure stabilization [93].

4.4. Larvicidal

The search for novel products that improve the epidemiological control of vector-borne diseases is relevant, whereas the selective pressure of conventional and synthetic insecticides has amplified mosquitoes resistance for different classes of insecticides (e.g. DDT and other chlorinated hydrocarbons) and present undesirable effects on non-targets organisms, requesting innovative substances that are specific, biodegradable and safer environmentally as mosquito control agents [32,98]. In this event, products derived from plants have promising outcomes since they have been traditionally used by communities against insects [6].

Aqueous extract of moringa seeds exhibited larvicidal action against *Aedes aegypti* on different stages of larval cycle (LC_{50} of 1.260 $\mu\text{g}/\text{mL}$ for larvae in III instar). After 24 h exposure (5.2 mg/mL), this extract caused remarkable mortality (99.2%), though this activity had gone after heating the extract at 80°C/10 min [32]. Leaf extracts [hexane (52 and 61% mortality), ethyl acetate (78 and 68%) and methanol (100 and 100%), respectively] were quite active on *Culex gelidus* and *C. quinquefasciatus* in IV instar [99]. Similarly and more recently, [98] showed that methanolic extracts from seeds are also effective on different phases of the *Anopheles stephensi* malarial vector, presenting larvicidal [LC_{50} values ranging from 57.79 (I instar larvae) to 78.93 ppm (IV instar), pupicidal (67.77 ppm) and repellent activities.

At work [100] has associated this larvicidal potential with flocculating proteins such as lectins found in the seeds, which delay and/or impede the larvae development of the *A. aegypti* mosquito and other insects, especially extending larval early stages (L1 and L2). Lectin treated-larvae in IV instar presented morphological changes as enlarged intestinal lumen and hypertrophy or loss of the luminal epithelium. The peritrophic matrix dividing the gut lumen contents from intestinal epithelial layer contains glycosaminoglycans enclosed in a chitinous

matrix susceptible to enzymatic action. Thus, it is feasible that chitin-lectin complexes interfere with the peritrophic matrix integrity, leading to the death of larvae [101]. It is likely that bioactive organic chemicals as phenols, terpenoids, glycosides and alkaloids found in *M. oleifera* may jointly or independently contribute to cause oviposition deterrent and skin repellent [70,98,102].

Thus, low toxicity of “morunga” extracts, competence of dispersion and plant maintenance, resistance to inhospitable environments, low cost and simple technology are some factors that convert *M. oleifera* an alternative to unpolluted drinking water and add it in programs to control disease-transmitting mosquitoes, especially in rural areas and developing countries, where access to drinking water is problematical and its accumulation in artificial containers commonly found in and around human residences create an ideal site to lay eggs and breed larvae.

4.5. Action on Central Nervous System (CNS)

Aqueous (100-450 mg/k, oral) and methanolic (350-700 mg/kg, intraperitoneal) root extracts reduced locomotor activity of rats and the number of seizures induced by penicillin and strychnine [27, 103]. Aqueous extract also amplified rates of 5-HT and reduced levels of dopamine in the brain cortex, cerebellum and caudate nucleus and noradrenaline measure in the cerebral cortex [103]. Methanol extract produced CNS depression, decreases the mortality of strychnine-and leptazol-treated animals, increased the sleeping time, caused analgesia and potentiated morphine analgesic effects [27]. This sleepiness extension and anticonvulsant and analgesic activities can be justified by the 5-HT brain rising.

More recently, discoveries also showed that ethanol extract from *M. oleifera* leaves (250-2000 mg/kg) caused decreasing in rearing, grooming, head dips and locomotion of mice, enhanced learning and memory, increased angiogenic effect and reduced convulsions induced by pentylenetetrazol, though it has no effect on picrotoxin and strychnine induced convulsion. In this event, it is possible that these activities are mediated through the enhancement of central inhibitory mechanism involving release γ -amino butyric acid (GABA) [104]. These findings partially justified the traditional use of *M. oleifera* parts for the treatment of epilepsy.

Other pharmacological activities of *M. oleifera* include the seed biological action upon *Plasmodium falciparum* [105], *Schistosoma mansoni* cercariae [106] and helminth eggs [53], diuretic activity [80] and spleen and thymus enlargement [32,74]; the leaves are purgative, antipyretic [18], immunomodulatory [107] and inhibit conversion of thyroxine (T_4) in triiodothyronine (T_3), with high likelihood to be employed in the treatment of hyperthyroidism [108]; the flowers are aphrodisiac [68], hepatoprotective [109] and antidiabetic [110]; the roots, carminative and anti-constipant [99] and stem barks possess antitumor activity and prevent splenomegaly [68]. This notable pharmacological potential suggests that the beneficial effect of the plant may be associated with individual or combined action of its constituents, such as phenols, aromatic isothiocyanates, flavonoids and sterols [39,102].

5. Toxicological aspects

Plants have a variety of indispensable macro and micronutrients to feed heterotrophic organisms, including ruminants and monogastric animals such as sheep, rats, mice and humans. However, side effects and aversions to vegetal substances as alkaloids, tannins, cyanogenic glycosides, terpenes, lectins and glucosinolates are habitual [111]. Thus, animals can identify tastes from sweet (carbohydrate, for example, an indication of calories) to the unpleasant taste of toxins. Among these, some present bitter flavor (alkaloids, saponins and cyanogenic glycosides), astringent (tannins) or offensive odors (terpenes). Dislikes can be wild (temporary) or strong (permanent) depending on the toxin dosages and how they affect the gut and central nervous system. These aversions hardly develop if toxins act gradually (days to weeks). Furthermore, toxins can activate the emetic center, causing nausea and vomiting [112]. Tropical seeds usually have high content of antinutritional factors, specially tannins and lectins [111].

5.1. Leaves, flowers and roots

M. oleifera leaves possess minor quantities of tannin (12 g/kg dry material), phytic acid (21 g/kg) and absence of trypsin, amylase inhibitors, lectins and glucosinolates, an aspect which encourages their consumption. Pods and stem have negligible amounts of tannin, but saponins and alkaloids are found in significant quantities in leaves and stem, respectively, though they should be considered non-toxic to ruminants [39].

Water extract of roots inhibit development of uterus and blastocyst implantation [113], indicating an abortifacient effect that interferes in estrogen and progesterone levels, modifying the normal physiology of the genital tract during the fertile period. Relatedly, Indian women frequently use leaf extracts as natural oral contraceptives [114].

Extracts from roots and flowers (200 mg/kg/day) were able to maintain transaminase (aspartate aminotransferase, AST; alanine aminotransferase, ALT) and bilirubin levels, protect against hepatotoxicity induced by acetaminophen toxic metabolites produced by P₄₅₀ monooxygenase enzymes and presented slight acute toxicity, since it was found LD₅₀ values of 1023 and 1078 mg/kg for root and 1047 and 1092 mg/kg for flowers extracts (ethanolic and aqueous extracts, respectively) [109] (Table 2).

However, root methanolic extracts (intraperitoneally and weekly doses greater than 46 mg/kg/day) produced hepatotoxicity and nephrotoxicity associated with hematological and plasma changes, particularly, AST, ALT, cholesterol, bilirubin, urea, proteins and causing leukocytosis and clotting time increasing [115]. Histological examinations in guinea pigs also propose toxicity of root methanolic extract (3.5, 4.6 and 7.0 mg/kg), whereas balloon degeneration and micro and macrovesicular steatosis (in liver) and interstitial inflammation, tubular damage and amorphous eosinophilic materials (in kidneys) were seen, demonstrating reversible signals of histo-architectural distortions [116].

Reference [117] reported that acute and sub-chronic exposure to higher doses of aqueous leaf extracts (400 to 6400 mg/kg) revealed to be relatively safe for human and rodents, since any

Part of plant	Extract	LD ₅₀ value (mg/kg body weight)	Route of administration	Reference
Seeds	Aqueous	446.5	intraperitoneal	[32]
Leaves	Aqueous	1585	oral	[119]
		> 2000		[117], [118], [119],
	Ethanollic	> 6400	oral	[104]
	Methanolic	7420	intraperitoneal	[63]
	Flowers	Aqueous	1092	intraperitoneal
	Ethanollic	1047	intraperitoneal	[108]
	Root	Aqueous	1078	intraperitoneal
	Ethanollic	1023	intraperitoneal	[108]
	Methanolic	223.6	intraperitoneal	[116]
Stem	Ethanollic	> 5000	oral	[75]

Table 2. Lethal dose 50% (LD₅₀) of *Moringa oleifera* extracts upon laboratory mammals.

mortality was detected when administered orally. These results are in according to [118], who documented that moringa leaf extracts are non-lethal at 2000 mg/kg and [104], whose publication demonstrated that ethanol extract from moringa leaves were not toxic to mice and revealed a LD₅₀ higher than 6.4 g/kg in oral acute toxicity studies. Nevertheless, i.p. injection presented 20% and 80% mortality in Wistar albino mice at doses of 1000 and 2000 mg/kg, with LD₅₀ of 1585 mg/kg and acute administration at 3000mg/kg reduces urea and albumin levels, indicating liver and renal dysfunction [119] probably initiated by toxicants such as isothiocyanates and glycosides during biotransformation and corroborating those outcomes described by [115] and [118], whose mice presented biochemical alterations suggestive of renal damage. An opposing discovery to all previous researches divulged, for the first time, showed that *M. oleifera* has genotoxic potential at higher doses (3000 mg/kg), increasing significantly the number of polychromatic micronucleated erythrocytes derived from bone marrow of rodents (20.2 ± 4.0 cells/1000 cells) when compared to control (0.9% saline) [119].

5.2. Seeds

The best advantage of using *M. oleifera* seeds for water clarification is its low toxicity. The aqueous extract of seeds (400 mg/kg/day) caused no biochemical, histological and hematological alterations, while it increased albumin and HDL-C serum and reduced AST and ALT levels

[74]. *Ad libitum* intake of aqueous extract as the unique source of water in doses of 1300-1670mg/kg/day for a month was also harmless and no change suggestive of toxicity was observed [32].

In [120] verified that seeds orally administered for 5 days at 500 mg/kg/day protected against toxic arsenic effects and recovery physiological measures to normal values (hemoglobin, erythrocytes and levels of δ -aminolevulinic acid dehydratase and glutathione S-transferase), probably due to the arsenic tissue removal. Previously, [121] showed that oral administration of hydroalcoholic extract of *M. oleifera* fresh pods increased hepatic levels of cytochrome b₅, cytochrome P₄₅₀, glutathione peroxidase, catalase, reductase and S-transferase enzymes involved in reactions of Phases I and II responsible by detoxification of exogenous substances such as carcinogens and plant poisonous. These findings were corroborated by [122], who showed that seed hydroethanolic extract (1g/kg) avoided the development of hepatic fibrosis induced by carbon tetrachloride and reduced histopathological and biochemical characters of inflammatory necrosis on hepatocytes (cellular infiltration, fatty degeneration and levels of AST, ALT, myeloperoxidase, collagens and biomarkers of oxidative stress). These findings highlighted the chemopreventive properties that have been attributed to antioxidant compounds in the seeds [68,72,121].

Despite investigations have indicated absence of toxicity following oral consumption of the seed aqueous extract, reproducing the intake of treated water with clarifying agent [32,74,123], nutritional assessments reported that those growing rats fed during 10 days with a diet whose total protein content (10%) was replaced by seed flour and whose doses were 24-fold higher than the highest dose tested by [123], suffered from severe growth disorders, loss of appetite and weight, hyperplasia of the small and large intestine, liver, pancreas, kidneys, heart, stomach and atrophy of key organs like spleen and thymus, though protein digestibility is similar to the foodstuff presenting egg white [30]. The antinutritional compounds prevailing in mature seeds, mainly glucosinolates (65.5 mmol/g), phytic acid (41 g/kg) and lectins [30,39, 124,125] should be responsible for these effects. Phytates, when found in percentages between 1-6% and ingested for extended periods, they may reduce the bioavailability of minerals (Ca²⁺ and Zn²⁺), starch and proteins in monogastric animals. Glucosinolates disturb growth and reproduction.

Lectins, in turn, are proteins or glycoproteins with reversible binding sites to carbohydrates [111]. They interact with the intestinal mucosa and interfere with digestion and absorption of nutrients, reduce activity of amylase, establish stable complexes with trypsin/chymotrypsin [126], cause pancreatic hypertrophy [127] and decrease growth rate [30]. In fact, studies have emphasized the *M. oleifera* haemagglutinating activity and associated it with lectins detected in the seeds [30,100,128].

Additionally, weight gain reduction in sheep supplemented with 6 g/dia of *M. oleifera* delipidated seeds compared with animals feed with 4 g/day may be explicated by the abundant presence of cationic proteins with antimicrobial activity [38] and/or because of bitter taste [39]. Therefore, antibacterial activity of seeds should inhibit the animal growth, altering its intestinal flora and rates of fermentation efficiency.

The bitter taste in the seeds, important to provide its typical aroma, is alleviated by treatment [39], suggesting that its taste would not be a limiting factor for using them, since even cow's milk whose dairy cattle was treated with moringa meal did not reveal changes in quality [41]. Furthermore, it is known that most adverse effects are eliminated by suitable methods as washing, storage, drying and/or heating. For example, lectin biological properties are lost after protein denaturation by temperature and pH. Nevertheless, these techniques are expensive and prolonged cooking of seeds result in nutritional value reduction and loss of micronutrients, specifically vitamins and minerals. The text [129] showed that roasted seeds promote formation of mutagenic compounds [4-(alpha-L-rhamnosyloxy)phenylacetone nitrile, 4-hydroxyphenylacetone nitrile and 4-hydroxyphenyl-acetamide]. On the other hand, it was observed that moringa seed flour has post-treated proteins with good digestibility and absorption [39,41].

Environmental assessments with seed water extract using the microcrustaceans *Artemia salina* and *Daphnia magna* showed LC₅₀ values of 177.8 and 188.7 µg/mL, respectively [32,74]. The research [130], working with the *Scenedesmus obliquus* green algae, another aquatic organism, found LC₅₀ of 207.5 and 287.5 mg/mL (methanolic and aqueous seed extracts, respectively). In addition, acute toxicity tests in mammals (*Mus musculus*) revealed a LD₅₀ of 446.5 mg/kg body weight [32]. Thus, both studies with marine organisms as well as those performed in mice indicate low toxicity of moringa seed extracts [131,132].

In summary, results obtained by [30,32,74,104,116,117,119,123,133,] confirm that toxicity of *M. oleifera* depends on concentration, part of the plant used, and manner of preparation and routes of administration. Then, though the consumption of different parts of specie for various purposes has been widely accepted, it is important to note that intake without any pre-treatment should be done carefully, since the specific adverse(s) factor(s) remains unclear whereas the presence of other unknown toxicants is uncertain. Additionally, little has been done to define, optimize and standardize conditions for their use and a few government programs encourage or disseminate such treatment for household water or determine its acceptability, sustainability, costs and effectiveness.

6. Conclusion

Relatively safe for human, *M. oleifera* is a worthy pharmacological and nutritional alternative, especially taking into account that technology requirements for leaves and seeds' flour production is cheap and simple, which benefits small farmers and the general population by providing an abundant food supply and bioactive substances.

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Anticalcineurinic: Role of Mitochondrial Transition Pore on Nephrotoxicity

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Additional information is available at the end of the chapter

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1. Introduction

Cyclosporin A (CyA) and tacrolimus (TAC) are calcineurin phosphatase inhibitors which are currently used in immunosuppression therapy for solid organ and hematopoietic stem cell transplantation to prevent and treat allograft rejection, and for autoimmune disease treatment [1]. Although drugs are not structurally related (Figure 1, Materials and Methods), they have identical cellular and molecular actions, including nephrotoxicity as a major side effect [2-4].

It is known that CyA and TAC may cause acute as well as chronic tubulointerstitial nephropathy characterized by tubular atrophy, loss of tubular cells and interstitial fibrosis [5], although the mechanisms of immunosuppression-associated renal damage are not fully understood [6, 7]. In any case, it is generally agreed that acute haemodynamic effects of CyA may be mediated by an imbalance of different vasoconstrictors and vasodilators, including renin-angiotensin system and nitric oxide among others [1,3]. Histologically, vacuolization, calcification and necrosis of tubular epithelial cells are characteristics, and endothelial cell swelling with variable degrees of hyalinosis. Abnormalities similar to those are found with TAC [4,8]. For chronic CyA nephrotoxicity, different studies have suggested a toxic effect of the drug on afferent arterioles and tubular epithelial cells, vasoconstriction and endothelial injury leading

to ischemia, as well as a direct toxic effect of CyA on tubular epithelium [5,9]. Moreover, apoptosis has been clearly evidenced in tubular and interstitial cells in patients, animals and cell culture models [9]. We have described that loss of mitochondrial potential usually precedes caspase activation and endonuclease mediated DNA laddering when proximal tubule cells are incubated in the presence of increasing doses of CyA [1,7]. Similar effects are found in TAC-induced nephrotoxicity [7, 10]. Mitochondrial depolarisation, caspase activation, cytosolic nucleosomal formation, and other morphological features of apoptosis induction are commonly found with both CyA and TAC in proximal tubule cells [1, 7]. Transforming growth factor- β (TGF- β), hypoxia and oxidative stress have also been considered to be important mediators of CyA and TAC-induced renal injury [6,7]. Significantly, although both drugs have similar effects, TAC is recognized to induce fewer haemodynamic and fibrogenic alterations at equivalent doses of CyA [11], and studies have shown that the incidence of acute rejection is reduced by TAC use over CyA [12].

Although mitochondrial mediation in the damage is generally accepted to be common to both immunosuppressors, the exact mechanism has not been fully investigated. Several authors have found a decreased mitochondrial respiration with CyA, mediated by a reduction in complex II activity, with stage 3 and 4 activity reductions [13-15]. Others describe a preferential inhibition of respiration by CyA when substrates of complex I are used [16]. TAC also inhibits the succinate supported state 3 respiration [13]. ATP depletion is a key factor in cell death associated with renal failure; therefore phosphorylation has also been studied as a potential target for CyA induced nephrotoxicity. CyA induced inhibition of ATP synthesis ranges from 12% [17] to 40% [18], and may be partially prevented by calcium antagonists [19]. Like CyA, TAC inhibits net ATP uptake [13]. However, the most intriguing finding regarding mitochondrial mediation in CyA and TAC induced apoptosis is the relationship of both immunosuppressors with the permeability transition pore.

The mitochondrial permeability transition (MPT) pore is an unstable structure that mitochondria reversibly assemble in their inner membrane under several physiological and non-physiological conditions, mainly cell calcium overload. When assembled, inner and outer membranes physically touch each other at specific points; when assembled and opened, it allows non selective permeation of ions and solutes up to 1.5 kDa [20, 21], dragged by a water movement from cytosol into mitochondria resulting in mitochondria swelling that may be measured [22]. MPT pore assembly includes cyclophilin-dependent conformational changes in adenine nucleotide translocase (ANT), located in the inner membrane and in a voltage dependent anion channel (VDAC). Cyclophilin D, benzodiazepine receptor, creatine kinase, hexokinase, cytochrome c and Bax protein are also involved in MPT pore formation and regulation [23-25]. The exact molecular structure of the MPT pore is still controversial, as it is the MPT pore relation to energy transfer and apoptosis, and for these reasons the physiological function of the MPT pore is not well established [23, 26].

It is generally agreed that MPT pore opening is triggered by a sudden rise in mitochondrial matrix free calcium concentration, representing the third of three sequential phases of mitochondrial accumulation: respiratory chain acceleration, extramitochondrial calcium buffering and finally activation of the MPT pore [27]. Transitory openings are necessary to prevent mitochondrial calcium overload, by allowing calcium excesses release [26, 28, 29]. MPT

pore only closes again if intramitochondrial free calcium is reduced. However, no clear explanations have been offered about how mitochondria may release calcium without causing the death of the cell. Although there should be a physiological role for MPT pore transitory opening, many authors agree that a role of MPT pore in healthy cells has not been established, and it only plays a role in pathophysiological conditions [26, 28, 30], such as necrotic and apoptotic cell death, ischemia/reperfusion injury, heart failure and other cardiovascular or neurodegenerative diseases [26,31,32]. In opinion of Martin Crompton, a possible physiological function of MPT pore may be to establish contact between mitochondria in the formation of mitochondrial networks [28]. It is established that mitochondria can form tight intermitochondrial junctions, allowing the thus-conjugated mitochondria to operate as a bioenergetics continuum permitting efficient energy transfer between different parts of the cells [33]. Other authors have also hypothesized about other possible physiological roles of MPT pore, as the regulation of protein import, matrix volume and pH, cristae remodeling, redox equilibrium and control of metabolism [31, 34, 35].

In any case, it is accepted that MPT pore formation is considered a prelude of mitochondria triggered apoptosis; prolonged times of MPT pore activity are accompanied by loss of transmembrane mitochondrial potential [36, 37] and release of proapoptotic mitochondrial proteins as cytochrome c [38], SMAC/Diablo [39, 40], HtrA2/Omi [41], apoptosis inducing factor (AIF) [42] or endonuclease G [43]. Although there is no agreement as to whether these proapoptotic mediators leave the mitochondria through the MPT pore or by other pathways [44-46], or whether the leak takes place after or before pore opening [27], protein implicated in apoptotic balance Bcl-2, Bcl-XL, Bax and Bid [47-48] are implicated on MPT pore activity regulation. Agents capable to keep MPT pore timely opened as nitric oxide [49], free oxygen radicals [50, 51] or Bax [52] are proapoptotic. Those who block or interfere with MPT pore activity are antiapoptotic.

It is currently known that CyA is to date, the most specific inhibitor of the MPT pore by inhibiting the peptidylprolyl cistrans-isomerase activity of cyclophilin D [53, 54], and is considered as antiapoptotic on most cellular systems [26, 28]. On the other hand, no TAC binding proteins able to modify MPT pore activity have been reported to date. Therefore, MPT pore mediation on the nephrotoxicity of calcineurin inhibitors has always been considered a non-directly related phenomenon.

In this chapter we provide some insights into the proximal tubule mitochondria modifications that take place during CyA and TAC induced nephrotoxicity. We explore the potential implication of MPT modulation on cell protection, acute renal failure and mitochondrial ammoniogenesis.

2. Materials and methods

2.1. Drugs

CyA (Sandimmun Neoral®) was obtained from Novartis Farmacéutica S.A. (Spain) and TAC (Prograf®) from Fujisawa S.A. (Spain). Their chemical structures are represented in Figure 1. The

concentration used for both drugs were similar to the pharmacologically active recommended plasma level, and they were selected from our previous studies and experience [1, 7]. Cell cultures were exposed to different increasing concentrations of CyA (1-1000 ng/ml) and TAC (50-500 ng/ml), although most of the experiments were performed with 1000 ng/ml (CyA) and 50 ng/ml (TAC) based on the proven effectiveness of the drug to induce nephrotoxicity [1].

2.2. Experimental animals

All experiments were performed on miniature swine genetically selected to be isogenic for three loci of the major histocompatibility locus [55]. They were 3 months old and weighed 31.3 ± 0.7 kg (mean \pm SEM). Isogenic minipigs were housed under controlled conditions and the study was approved by the Institutional Board for Animal Experiments. Animals were handled at all times according to the applicable legal regulations in RD 1201/2005, of 10 October, on the protection of animals used for experimentation and other scientific purposes.

2.3. Isolation and primary culture of renal proximal tubule epithelial cells

Proximal tubule suspensions were obtained as described [56]. The kidneys were aseptically removed from the animals and placed in a sterilized beaker containing Ham's F-12 culture medium, pH 7.4. Briefly, renal cortex was sliced with a Staddie-Riggs microtome, and incubated with 0.6 mg/ml of collagenase A (Boehringer Mannheim, Germany) in Ham's F-12 medium for 30 minutes at 37 °C. Digested tissue was then filtered through a 250 μ m metal mesh, washed three times in Ham's F-12 medium and centrifuged at 20,000 \times g for 30 minutes in a 45% (v/v) Percoll gradient. The last band contains pure proximal tubules.

Proximal tubules were recovered from the deepest fraction, washed, and resuspended in culture medium (1:1 Ham's F-12:DMEN supplemented with 25 mM hepes, 3.7 mg/mL sodium bicarbonate, 2.5 mM glutamine, 1% non-essential aminoacids, 100 U/mL penicillin, 100 μ g/ml streptomycin, 5.10^{-8} M hydrocortisone, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/mL sodium selenite and 2% fetal bovine serum), at a final concentration of 0.66 mg/mL, and plated on plastic culture dishes (60 mm) [57]. The culture was incubated at 37 °C in a 95% air/5% CO₂ atmosphere. The medium was renewed on the fourth day and every two days thereafter.

CyA or TAC were added to cell cultures as specified in every experiment.

2.4. Isolation of renal cortical mitochondria

The renal cortex was rapidly removed and placed into 100 mL of ice-cold isolation buffer (300 mM mannitol, 1 mM EGTA, 10 mM TRIS HCl, 1 mM PO₄H₂K, 1.74 mg/mL phenylmethylsulfonyl fluoride (PMSF), 0.2% BSA, pH 7.4 and gassed with N₂) and homogenized. The homogenate was centrifuged at 1075 \times g for 10 minutes at 4 °C. The supernatant was centrifuged at 8635 \times g for 10 minutes. The resulting pellet was washed twice at 8635 \times g for 10 minutes and resuspended in isolation buffer to 32.5 ± 1.97 mg of protein/mL.

All experiments with isolated mitochondria are carried out in assay medium (300 mM mannitol, 10 mM TRIS HCl, 1mM PO₄H₂K, pH 7.4).

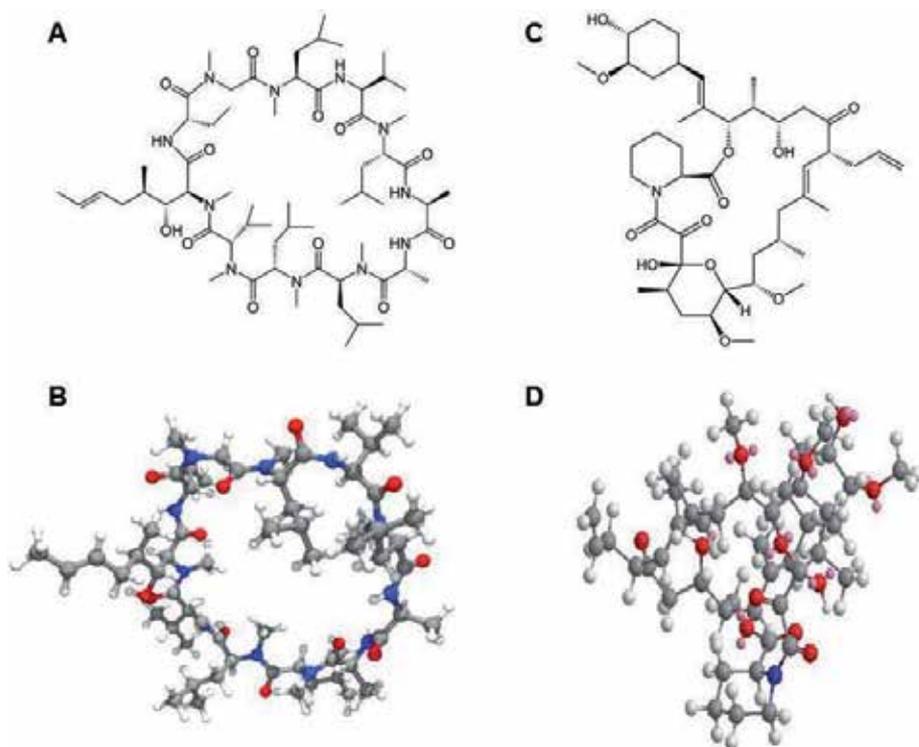


Figure 1. Formula of the nephrotoxic compounds. A and B represent the molecular structure of cyclosporin A; cyclic undecapeptide with chemical formula $C_{62}H_{111}N_{11}O_{12}$ and a molecular weight of 1202.16 g/mol. C and D represent the molecular structure of tacrolimus; macrolide whose empirical formula in its monohydrate form is $C_{44}H_{69}NO_{12}$, and with a molecular weight of 804.018 g/mol.

2.5. Mitochondrial oxygen consumption

State 3 and state 4 respiration was measured with a Clark-type electrode (YSI incorporated, Yellow Springs, Ohio, USA) in a metacrilate chamber at 37 °C equipped with magnetic stirring in the presence of 5 mM succinate before (state 4) and after (state 3) the addition of 2 mM ADP and 10 μ M atractilisyde. Mitochondria were suspended in continuously stirred assay medium, maintained at 37°C at a final mitochondrial protein concentration of 0.40 ± 0.02 mg/ml. In parallel experiments, CyA 1 μ g/ml and TAC 50 ng/ml were added 30 minutes prior to addition of effectors or simultaneously with then (no preincubation). The content of oxygen was calculated using the following equation:

$$O_2(\text{mol}) = 0.21 \times 0.024 \times V_{\text{cuvette}} \times (P_{\text{atm}} - P_{\text{H}_2\text{O}}) / 22.4 \quad (1)$$

where 0.21 is the $O_2\%$ in the air, 0.024 is the O_2 solubility coefficient, 22.4 is the molar volume of an ideal gas (L/mol). The cuvette volume is 807 μ l. Atmospheric pressure was 708.9 ± 1.96 mmHg and the vapour water pressure is 46.6 mmHg.

2.6. Calcium uptake

One mL of mitochondrial suspension was incubated with 5 μ M fura-2, AM (Molecular Probes) for 30 minutes at 4 °C, washed three times at 8635 x g for 10 minutes and resuspended in isolation buffer at 4°C in a 1:1 proportion.

Fura-2 fluorescence was monitored with a SLM Aminco 8000 fluorometer at 37°C in stirred cuvettes, by exciting it at 340 nm (λ_{ex1}) and 380 nm (λ_{ex2}) and rationing the fluorescence intensities detected at 510 nm (λ_{em}). Mitochondrial matrix $[Ca^{2+}]$ was determined using the following equation [58]:

$$[Ca^{2+}] = K_d Q \left(\frac{R - R_{min}}{R_{max} - R} \right) \quad (2)$$

where R represents the fluorescence intensity ratio F (340 nm)/ F (380 nm), R_{min} and R_{max} requires taking fluorescence measurements for the completely ion-free (EGTA 2 mM) and ion-saturated ($CaCl_2$ 5 mM) indicators. Q is the ratio of F_{min} to F_{max} at 380 nm. K_d is the dissociation constant of fura-2 for calcium (0.14 μ M).

2.7. Mitochondrial swelling: MPT pore

Ca^{2+} -induced mitochondrial swelling was determined spectrophotometrically as described by Beavis and Col. [59], adapted to our experimental conditions, by using authors equation:

$$\text{Mitochondrial volume } (\mu\text{l/mg}) = \left[\frac{1}{\text{Abs}_{520\text{ nm}}} - 0.1832 \right] \times [\text{Protein}] / 0.06 \quad (3)$$

where 0.1832 is the theoretician inverse of absorbance for an infinite protein concentration, and 0.06 is a proportionality constant established by the authors [59]. Mitochondria were suspended in 3 mL of continuously stirred assay medium, maintained at 37°C at a final mitochondrial protein concentration of 0.54 ± 0.1 mg/ml. Swelling was initiated by the addition of succinate to the sample cuvette, and the absorbance changes at 520 nm were monitored with an Uvikon 930 spectrophotometer.

A mitochondrial basal volume of 3.56 ± 0.06 μ l/mg protein was obtained.

2.8. Analysis of mitochondrial transmembrane potential by fluorescence microscopy

Mitochondrial transmembrane potential in intact renal proximal tubular epithelial cells (RPTECs) was followed by cellular distribution of Rhodamine. Cells were incubated at 37°C for 15 minutes in the presence of 10 mM Rhodamine 123 (Sigma) and washed twice in PBS. The cells were then observed with a fluorescence microscope Olympus IX70 (wide-band cube U-MWG; λ_{ex} : 510-550, λ_{em} : >590, λ_{dic} : 570).

2.9. Cellular glutamine metabolism

Glutamine uptake was determined on proximal tubules following spectrophotometrically the NADH production at 340 nm. Glutamine uptake was determined by difference with the

glutamate. Determinations are based on following reactions of glutaminase and glutamate dehydrogenase, respectively [60]:



2.10. Cytochrome c distribution

Release of cytochrome c from mitochondria into cytosol was measured using Western blot analysis. RPTECs treated for 48 h with 1 $\mu\text{g/ml}$ CyA or 50 ng/ml TAC were harvested, washed once with ice-cold PBS, and gently lysed for 10 minutes in ice with 90 μl of lysis buffer (250 mM sucrose, 80 mM KCl, 500 $\mu\text{g/ml}$ digitonin, 1 mM dithiothreitol, 0.1 mM PMSF, and protease inhibitors in PBS). Cell lysates were centrifuged at 12,000 \times g at 4°C for 5 minutes to obtain the supernatants (cytosolic extract free of mitochondria) and the pellets (fractions containing the mitochondria), which were resuspended in 90 μl of lysis buffer. Equal amounts of protein were loaded (30 μg in each lane) and electrophoresed on 15% polyacrylamide gels as previously described [56].

Rabbit polyclonal antibody anti-cytochrome c (Santa Cruz Biotechnology, Inc, CA, USA) was used at 1:500.

The membranes were also probed with goat polyclonal antibody against a peptide of voltage-dependent anion-selective channel 1 of human origin (VDAC-1, 1:500; Santa Cruz Biotechnology, Inc.) for mitochondrial fractions and monoclonal anti-tubulin Clone B-5-1-2 Mouse Ascites Fluid (mouse IgG1 isotype) antibody (1:10,000; Sigma-Aldrich) for cytosolic fractions as internal controls for the technique. Proteins were visualized with the enhanced chemiluminescence detection system (ECL, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.11. Apoptosis studies: Flow cytometry, microscopy and caspase activity assay

After CyA or TAC treatment, cell number was determined with a computer coupled video-microscopy system. Every count was assessed on 21 sectors images of 0.0775 mm^2 chosen at random from three different culture dishes.

Flow cytometry was performed on supernatant and monolayer harvested cells after dilution in 2×10^6 cells/mL in 2% paraformaldehyde/PBS (30 minutes, 4°C, pH 7.4), permeabilization with PBS-Tween 0.5% (15 minutes at room temperature) and incubation with 40 $\mu\text{g/mL}$ RNase and 250 $\mu\text{g/mL}$ propidium iodide (Sigma, St Louis, USA) at room temperature for 45 minutes. A minimum of 10,000 cells per sample were acquired in a FACScan equipped with a single argon-ion laser (Becton Dickinson, San Jose, CA), using standard Lysis II software. A gate was set on the basis of forward and scatter characteristics before fluorescence (FL2) was analysed.

Caspase 3 and caspase 9 activities were determined in RPTECs using the caspase 3 inhibitor, DEVD-FMK conjugated to FITC (FITC-DEVD-FMK) and the caspase 9 inhibitor LEHD-FMK conjugated to FITC (FITC-LEHD-FMK) as a fluorescent markers respectively, following the protocols of the CaspGLOW™ Fluorescein Active Caspase-3 and Caspase-9 Staining Kit from

BioVision, Inc., (Milpitas, CA, USA). Activated caspases detection was examined with the 20X PL-APO 0.7-numerical aperture objective of a Leica-SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany). Different intensity measurements were assessed with the Leica Confocal Software LCS-1537 (Leica Microsystems).

2.12. Statistics

Pooled data are presented as mean \pm SEM. Comparison between treatment groups were done by one or two ways factorial ANOVA. Least significant differences were computed for between-level comparisons. A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of CyA and TAC on primary growth of the renal proximal tubule cells

When primary cultures of pig RPTECs are exposed to increasing CyA or TAC concentrations, a dose dependent growth inhibition is produced, even with concentrations as low as CyA 1 $\mu\text{g}/\text{mL}$ or 50 ng/mL . Figure 2 shows the growth dynamics of cultures in the presence of CyA 1 $\mu\text{g}/\text{mL}$ and TAC 50 ng/mL , concentrations near to the peak plasma levels reached with oral administration of these two drugs. Both CyA and TAC inhibit growth from day 6, which impedes the culture confluence from day 8 (Figure 2).

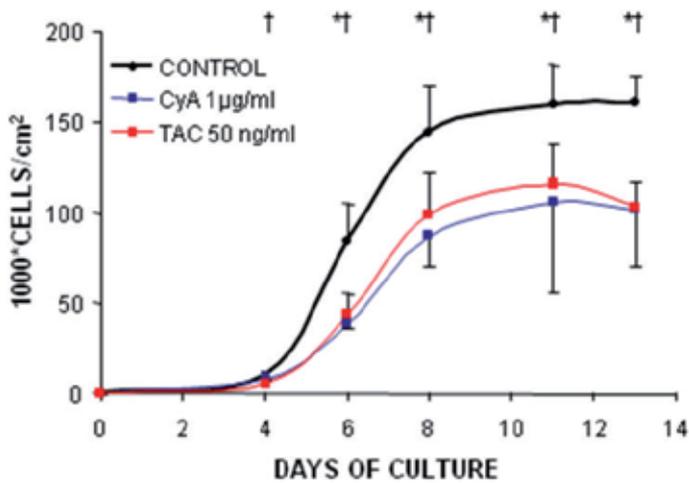


Figure 2. Cyclosporin A (CyA) and Tacrolimus (TAC)-induced cytotoxicity in pig kidney proximal tubule epithelial cells cultures. Culture cells were incubated with 1 $\mu\text{g}/\text{mL}$ CyA or 50 ng/mL TAC. Viable cell number was determined by cell counting in a phase-contrast inverted microscope. Values shown are the mean \pm SEM of cell counts per cm^2 . * $p<0.05$ CyA vs. control; † $p<0.05$ TAC vs. control.

Figure 3 gives a 3-D histogram of the cell flow cytometry in cultures treated with CyA or TAC for 8 days. A distinct hypodiploid population can be observed, indicative of apoptosis

induction with both treatments. Table 1 shows the dose dependence exhibited by the apoptosis induced by the two drugs.

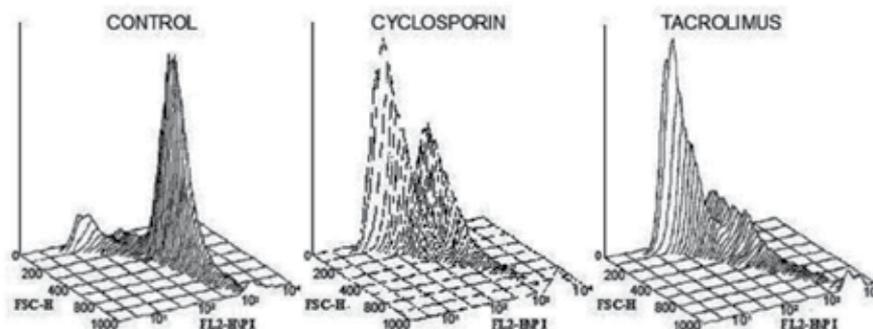


Figure 3. Effect of Cyclosporin A (CyA) and Tacrolimus (TAC) on apoptosis of pig kidney proximal tubule epithelial cells as assessed by flow cytometry analysis (FACScan). 3-D histograms of the cell flow cytometry in either control cultures or treated with 1 µg/mL CyA or 50 ng/mL TAC for 8 days. The hypodiploid population is greater in the CyA and TAC treated cells.

DOSE (ng/mL)	% hypodiploid cells (TAC)	% hypodiploid cells (CyA)
0	28.55 ± 2.9	28.55 ± 2.9
50	35.87 ± 5.8	
100		33.08 ± 3.8
500	44.42 ± 5.9*	
1000		42.32 ± 5.4*

Values are means ± SEM. *p<0.05 vs.dose 0 ng/ml. TAC, tacrolimus; CyA, cyclosporin A.

Table 1. Apoptosis assessed by flow cytometry analysis.

3.2. Effect of CyA and TAC on intramitochondrial free calcium

When suspensions of fura-2 AM loaded mitochondria were incubated in the presence of 21% oxygen and 5 mM succinate, but in the absence of ADP, an increase in intramitochondrial calcium concentration is observed (Figure 4A), paralleling an 8-fold increase in oxygen consumption (Table 2). Subsequent addition of 2 mM of ADP significantly increases the rate of oxygen consumption (Table 2) and drastically decreases intramitochondrial calcium concentration, although not reaching baseline levels (Figure 4A). This reduction in mitochondrial calcium when adding ADP is accompanied by a net calcium extrusion from mitochondria, as can be seen in Figure 4B where the same maneuver was repeated with non fura-2 loaded mitochondria suspended in the presence of the non-permanent acid form of fura-2. ADP

addition is followed by an increase in extramitochondrial free calcium, but there is a net difference between the initial free calcium mitochondria uptake when succinate was added, and free calcium release after ADP addition.

In contrast, if fura-2 loaded mitochondria are first incubated with ADP 2 mM, no changes in mitochondrial calcium occur (Figure 4A). Subsequent addition of 5 mM succinate caused a delayed and lower increase in mitochondrial calcium, reaching a final intramitochondrial concentration similar to the one observed in the previously described setting (Figure 4A). Atractilyside addition blocks mitochondrial calcium rise (data not shown) and reduce O₂ consumption in every condition.

In the presence of 1 µg/mL of CyA in the assay medium, mitochondrial calcium increases further when 5 mM of succinate is added, always in the absence of ADP (Figure 5). Simultaneously oxygen consumption increase is almost a 50% higher than observed in non-treated mitochondria (Table 2).

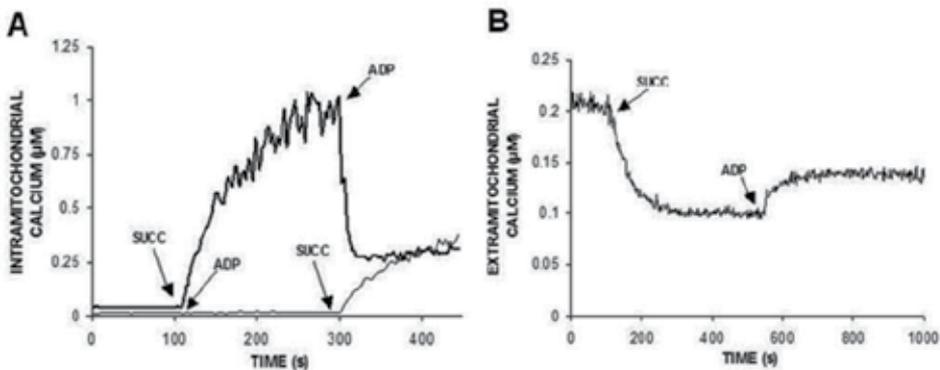


Figure 4. Succinate and ADP effects on mitochondrial calcium concentration. **A.)** The addition of 5 mM succinate (SUCC) to mitochondria in suspension induces an increase in the intramitochondrial calcium concentration. This calcium increase is inhibited by 2 mM ADP. **B.)** Decrease of the extramitochondrial calcium concentration induced by 5 mM SUCC. Mitochondrial suspension was incubated with 5 µM fura-2 and fluorescence was monitored with a fluorometer at 37°C.

The presence of TAC 50 mg/ml also causes a significantly greater increase in intramitochondrial calcium concentration and oxygen consumption when mitochondria begin succinate oxidation (Figure 5, Table 2).

In both cases, ADP addition significantly increases oxygen consumption (Table 2) and simultaneously the outflow of most of the calcium accumulated with or without CyA and TAC treatment (Figure 5). After ADP addition a stable intramitochondrial free calcium concentration is reached indicative of a new influx/efflux balance establishment. However, after 50-100 seconds a spontaneous increase in mitochondrial calcium concentration can be observed, although to a lesser extent. Again this new increase in mitochondrial calcium is higher and happens earlier with TAC than CyA.

Groups	Control	CyA 1 µg/mL (NP)	CyA 1µg/mL (30 min)	TAC 50 ng/mL (NP)	TAC 50ng/mL (30 min)
Basal	341 ± 44	309 ± 39	541 ± 234	378 ± 36	469 ± 161
SUCC	2587 ± 126 [†]	3290 ± 514 [†]	2929 ± 286 [†]	2918 ± 149 [†]	2810 ± 240 [†]
SUCC + ADP	3093 ± 187 [#]	3675 ± 386 [†]	3381 ± 163	3537 ± 335 [†]	3440 ± 63 [#]
SUCC+ ADP + Atractilosyde	2130 ± 151 ^{&}	2218 ± 322 ^{&}	2188 ± 177 ^{&}	2114 ± 167 ^{&}	2090 ± 135 ^{&}

Mitochondria were preincubated with both drugs 30 minutes prior the conducting experiment or simultaneously (NP: No preincubation). Values are means ± SEM, n=22 *p<0.05 vs. "control" column; †p<0.05 vs. "basal line"; #p<0.05 vs. "succinate line"; &p<0.05 vs. "succinate + ADP line". SUCC, succinate.

Table 2. Mitochondrial oxygen consumption (µmol/g*h) induced by cyclosporin A (CyA) and tacrolimus (TAC).

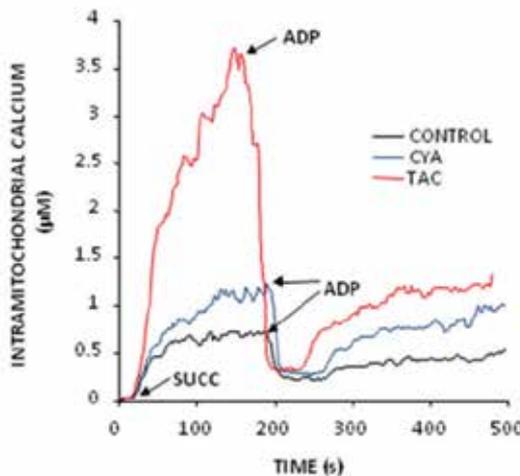


Figure 5. Cyclosporin A (CyA) and tacrolimus (TAC) increase intramitochondrial calcium concentration. In 5 mM succinate (SUCC) presence, 1 µg/mL CyA and 50 ng/mL TAC induce an increase in isolated mitochondria. 2 mM ADP causes the outflow of most of the calcium accumulated. Mitochondrial suspension was incubated with 5 µM fura-2 and fluorescence was monitored with a fluorometer at 37°C.

3.3. Effect of CyA and TAC on mitochondrial permeability transition pore

MPT pore opening was studied following mitochondrial electrochemical gradient restoration after respiratory chain activation (succinate addition), and subsequent mitochondrial calcium uptake. No extra calcium was added to the extramitochondrial medium, where free calcium was 0,2 µM (as determined in Figure 4B).

Figure 6 shows the temporary relationship between the changes in mitochondrial calcium influx, expressed as the first derivate of mitochondrial calcium concentration against time, and the change in mitochondrial volume expressed as the first derivate of the mitochondrial

volume versus time. Net calcium influx reaches a maximum of 7.3 ± 1.1 nM/s at 17.5 ± 0.9 seconds after addition of 5 mM succinate (Table 3).

Groups	Basal Calcium Influx (nM/s)	Maximal Calcium Influx (nM/s)	Time to Maximal Calcium Influx (seconds)
Control (n=11)	0.019 ± 0.011	7.3 ± 1.1	17.5 ± 0.9
CyA 1 $\mu\text{g}/\text{mL}$ (n=4)	$0.057 \pm 0.015^{\text{NS}}$	$17.5 \pm 2.7^*$	$16.5 \pm 0.9^{\text{NS}}$
TAC 50 ng/mL (n=4)	$0.006 \pm 0.008^{\text{NS}}$	$88.8 \pm 5.41^*$	$17.5 \pm 0.9^{\text{NS}}$

Values are means \pm SEM. * $p < 0.05$ vs. control. CyA, cyclosporin A; TAC, tacrolimus. NS, non-significant.

Table 3. Characteristics of calcium influx induction after oxidation of succinate 5 mM.

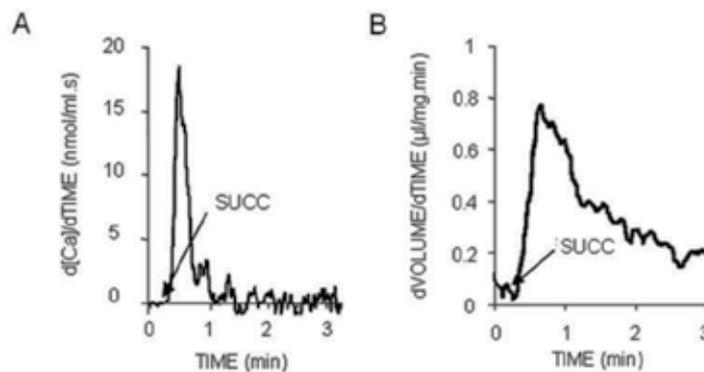


Figure 6. Temporary relationship between the changes in mitochondrial calcium influx and the change in mitochondrial volume. The addition of 5 mM succinate (SUCC) cause a net calcium influx (A) and at the same time a gradual change in mitochondrial volume occurs (B). Ca^{2+} -induced mitochondrial swelling was determined spectrophotometrically at 520 nm.

This influx subsequently decreases reaching a steady state when equilibrates with efflux (1st derivate = 0) (Figure 6A). A simultaneous gradual change in mitochondrial volume occurs, with a maximum value of 0.432 ± 0.055 $\mu\text{l}/\text{mg}$ mitochondrial protein*min at 16.0 ± 1.5 seconds (Table 4). The influx volume then decreases to baseline values (Figure 6B).

The characteristics of this volume influx correspond to those of the MPT pore. As can be seen in Figure 7A, if the oxidation of the succinate is blocked by potassium cyanide (CNK), or if calcium inflow is impeded by ruthenium red, or if the mitochondria are pre-incubated with 2 mM of ADP, change in mitochondrial volume is impeded.

ADP immediately blocks pore formation and could induce pore closing at any time during the process. This can be seen in Figure 7B where the addition of ADP at different time points to a suspension of mitochondria with the pore previously formed and open, closes the pore at any time during the process, causing a volume efflux (1st derivate < 0).

Groups	Mitochondrial Basal Inflow ($\mu\text{L}/\text{mg prot. min}$)	Maximal Inflow ($\mu\text{L}/\text{mg prot. min}$)	Time to Maximal Inflow (seconds)
Control (n=21)	0.057 ± 0.009	0.432 ± 0.055	16.0 ± 1.5
CyA $1 \mu\text{g}/\text{mL}$ (n=17)	$0.042 \pm 0.007^{\text{NS}}$	$0.235 \pm 0.018^*$	$21.1 \pm 1.9^*$
TAC $50 \text{ ng}/\text{mL}$ (n=20)	$0.062 \pm 0.013^{\text{NS}}$	$0.405 \pm 0.052^{\text{NS}}$	$16.1 \pm 1.5^{\text{NS}}$

Values are means \pm SEM. * $p < 0.05$ vs. control. NS: non-significant. CyA, cyclosporin A, TAC, tacrolimus.

Table 4. Characteristics of MPT pore induction after oxidation of succinate 5mM.

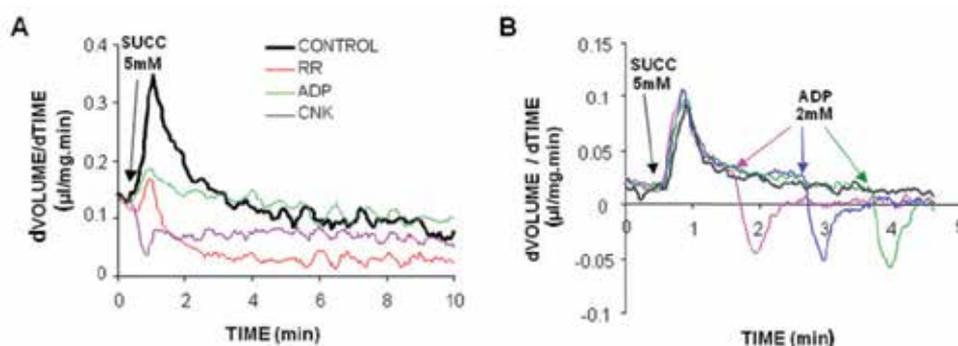


Figure 7. Inhibition of volume influx with well-known blocking of the mitochondrial permeability transition (MPT) pore. (A) Volume influx induced by 5 mM succinate (SUCC) is inhibited by 1 μM Ruthenium Red (RR), 1 mM potassium cyanide (CNK) and 2 mM ADP. (B) Close of the MPT pore and volume efflux induced by 2 mM ADP addition to a suspension of mitochondria, at different time point. Mitochondrial swelling was determined spectrophotometrically at 520 nm.

When CyA is present, 5 mM succinate addition to a mitochondria suspension causes a significantly reduced inflow through the MPT pore. The mitochondrial swelling observed in the presence of CyA is approximately half of that observed under control conditions (Table 4). It also takes significantly more time to reach its maximum (Table 4) and has an earlier closure (Figure 8A). Same results were obtained using the Met-Ile CyA, an analogue without anti-calcineurin activity (is devoid of immunosuppressive activity), but still capable of binding mitochondrial cyclophilin D (Figure 8A). However TAC did not modify overall kinetics of MPT pore opening (Table 4).

CyA-induced increase in mitochondrial calcium is not followed by a proportional opening of MPT pore, due to CyA dependent inhibition of MPT pore activation. TAC-induced increase in mitochondrial calcium is followed by the expected opening of MPT pore. To verify that volume inflow could be dragging calcium chelating anions into mitochondria, we studied again CyA and TAC effects on mitochondrial calcium in the presence of increasing doses of extramitochondrial phosphate, keeping constant the extramitochondrial calcium concentration. By increasing phosphate availability, the pore opening is followed by a much modest increase in intramitochondrial free calcium (Figure 9).

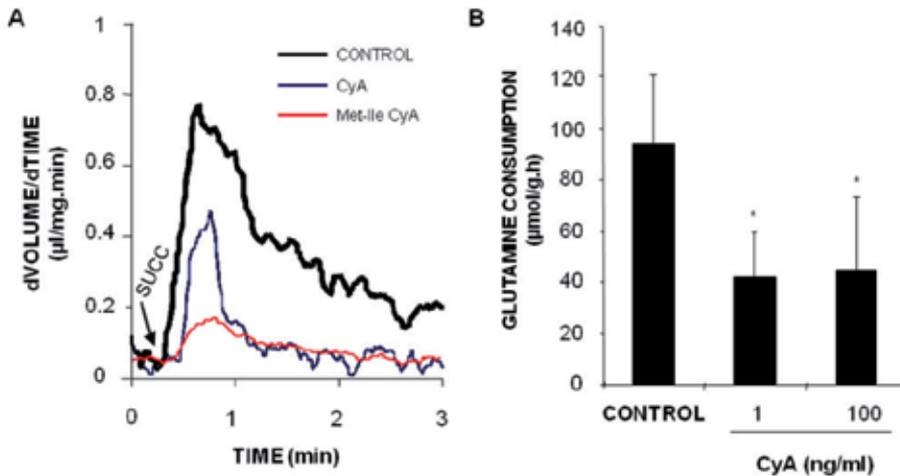


Figure 8. Effect of Cyclosporin A (CyA) on mitochondrial swelling and glutamine consumption. **(A)** In 1 µg/mL CyA presence as well as on Met-Ile CyA analogue, the mitochondrial swelling induced by 5 mM succinate (SUCC) is reduced. Mitochondrial swelling was determined spectrophotometrically at 520 nm. **(B)** CyA inhibited intramitochondrial phosphate-dependent glutaminase activity at 1 ng/mL and 100 ng/mL. Glutamine consumption was determined spectrophotometrically following the NADH production at 340 nm. * $p < 0.05$ vs. control.

MPT pore opening inhibition by CyA will reduce the acute influx of phosphate into the mitochondria, causing free calcium to increase. This finding was indirectly supported when mitochondrial phosphate-dependent glutaminase (a selective enzyme of the proximal tubule mitochondria) activity was studied in intact proximal cells. CyA but not TAC (data not shown) inhibited mitochondrial phosphate-dependent glutaminase activity (Figure 8B).

3.4. Early and delayed MPT pore opening: role in CyA and TAC-induced apoptosis

Following succinate oxidation, opening and closure of MPT pore may be observed during the first minutes. However, if incubation with CyA or TAC is prolonged for 30 minutes more, a second increase in mitochondrial volume is produced (Figure 10) that is independent of any change on oxygen consumption rate. This second spontaneous swelling has some of the MPT characteristic: it can be inhibited by ruthenium red and by ADP. However, in contrast to that observed with the first opening of the pore, this time the change does not appear to be reversible, it is maintained over time and the presence of CyA does not impede or reduce it. This second opening is not observed in control mitochondria.

3.5. Mitochondrial potential and cytochrome c: apoptosis induction

Despite the fact that CyA is capable of a premature closing of the pore activated by succinate oxidation in the absence of ADP, and that TAC does not modify the usual response of the pore to such mechanism, both CyA and TAC cause the spontaneous, delayed opening of the transition pore, loss of cytochrome c to the cytosol (Figure 11B), and the gradual collapse of the mitochondrial potential gradient. In Figure 11A the mitochondrial potential dye, Rhoda-

mine 123, which only stains the mitochondria when the mitochondrial potential is intact, diffuses out of the mitochondria and stains all the cytosol when the mitochondria is depolarised in the presence of CyA or TAC.

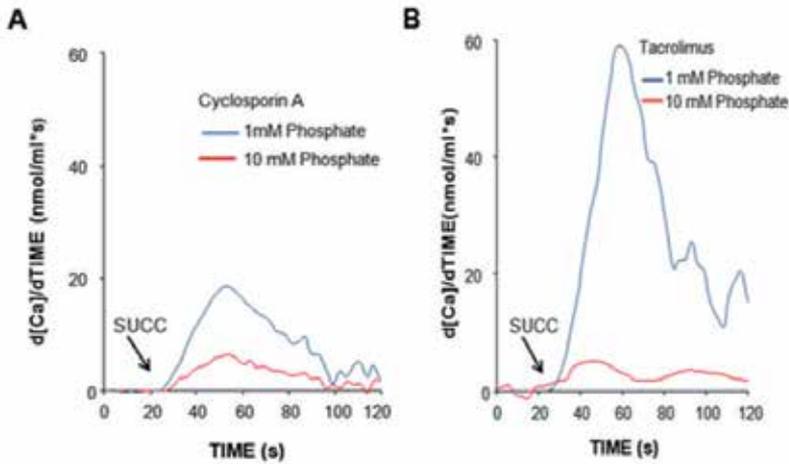


Figure 9. Effect of extramitochondrial phosphate on intramitochondrial calcium. When increasing extramitochondrial phosphate, the increase of intramitochondrial calcium induced by 5 mM succinate (SUCC) is much smaller so much in the presence of 1 $\mu\text{g}/\text{mL}$ cyclosporin A (A) like of 50 ng/mL tacrolimus (B). Mitochondrial suspension was incubated with 5 μM fura-2 and fluorescence was monitored with a fluorometer at 37°C.

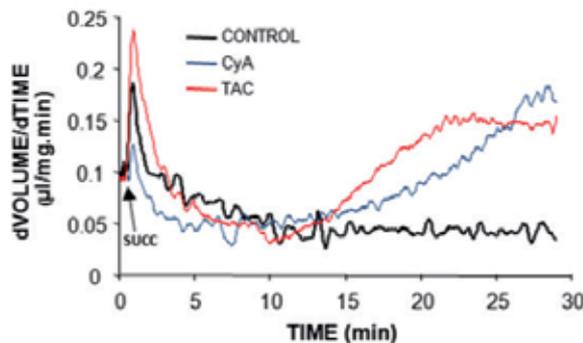


Figure 10. Early and delayed mitochondrial permeability transition pore opening by cyclosporin A (CyA) and tacrolimus (TAC). Incubation of a mitochondrial solution with 1 $\mu\text{g}/\text{mL}$ CyA or 50 ng/mL TAC during at least 30 minutes produced a second increase in mitochondrial volume. Succinate-induced mitochondrial swelling was determined spectrophotometrically at 520 nm.

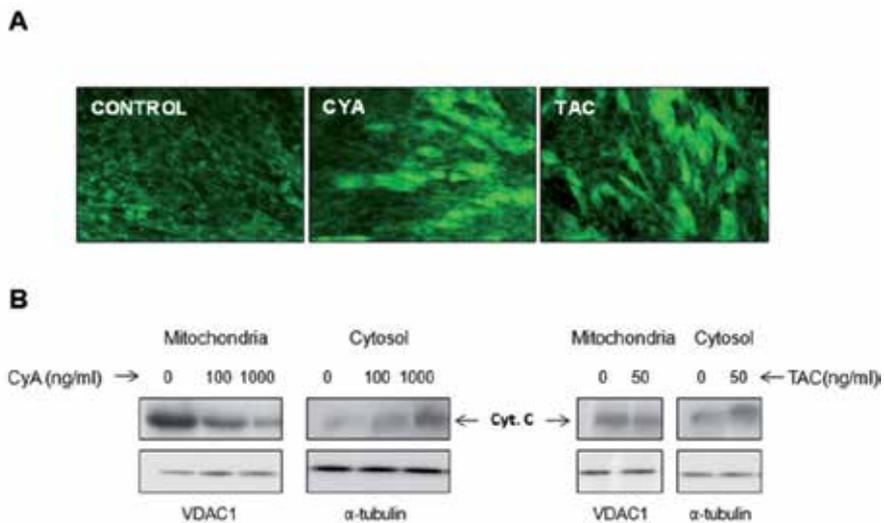


Figure 11. Mitochondrial depolarisation and cytochrome c release induced by Cyclosporin A (CyA) and Tacrolimus (TAC). **(A)** Renal proximal tubular epithelial cells (RPTECs) were treated during 48 hours with 1 $\mu\text{g}/\text{mL}$ CyA or 50 ng/mL TAC and incubated with 10 mM Rhodamine 123. Cells were then observed with a fluorescence microscope. Rhodamine 123 diffuses out of the mitochondria and stains all the cytosol when the mitochondria are depolarised. **(B)** Western blot analysis of cytochrome c of cytosolic and mitochondrial fractions of RPTECs treated with CyA and TAC for 48 hours. Voltage-dependent anion-selective channel (VDAC1) and α -tubulin antibodies were used as internal loading controls for mitochondrial and cytosolic fractions respectively.

4. Discussion and conclusion

Anticalcineurinic based immunosuppressors have dramatically changed the prognosis spectra of solid and bone marrow transplant. A decade ago, antiapoptotic properties of CyA and TAC on different tissues and animal models allowed to consider these drugs as the final solution in immunosuppression [61]. However, kidney injury showed to be an important drawback in their clinical applications, more dramatic when kidney function was normal prior to heart, liver or bone marrow transplantation. m-TOR inhibitors, the long-time awaited alternative to anticalcineurinics, have not fulfilled all the expectations because of the high incidence of tumors observed. A new look at the mechanisms and potential solutions to anticalcineurinic-induced nephrotoxicity deserves attention. We have discussed elsewhere the renal pathways involved in CyA or TAC apoptosis [1], but it may deserve to look at their effect on MPT pore activity to throw some light on largely described but not explained observations.

Both CyA and TAC cause increase in intramitochondrial calcium, even in the absence of rises in extramitochondrial calcium. But the mechanisms and consequences are different with both.

MPT pore will open as soon as the matrix free calcium reaches a given concentration able to drive the cyclophilin D dependent conformational change in ANT. Free calcium will be driven inside mitochondria when:

- a. The electrochemical gradient has been created,
- b. There is extramitochondrial calcium in significant concentrations.

Both conditions take place during kidney transplantation when organ is removed from the cold storage and grafted in a donor. In our *in vitro* model, we induced the electrochemical gradient by adding O₂ and succinate to our mitochondria suspensions. Most of the papers previously published on isolated mitochondria, use also extramitochondrial additions of calcium to study the MPT pore. However, this maneuver mimics too closely a near to death cell condition, and makes it difficult to extrapolate conclusions to MPT pore activity under physiological conditions.

We observe the same inhibition of CyA permeates mitochondria and binds cyclophilin D, sparing it from calcium activation, and MPT pore assembly is barely activated. Imported free calcium rises inside the matrix and reaches values well over control values. We observed the same reduction in MPT pore activity with CyA analogous able to bind cyclophilin but unable to block calcineurin. Other authors reported an increased capacity of calcium buffering of CyA-treated mitochondria [24, 27, 62].

CyA is considered as an antiapoptotic factor because of this inhibition of MPT pore activity [26, 27, 63-65]. However, CyA does not inhibit calcium entry into mitochondria. Crompton proposed MPT pore opening as the mechanism to prevent matrix free calcium accumulation and hypothesized that matrix calcium would be extruded through the pore, creating calcium currents until other cell calcium extrusion mechanisms would finally eject calcium out of the cell [24, 28]. Our results do not support such hypothesis. During pore opening, matrix calcium inflow stops, probably because VDAC is recruited to form the pore itself, but no calcium outflow was observed. Instead, based on our studies at different extramitochondrial phosphate concentrations, it is possible that water entry into mitochondria through the pore will have a "solvent drag" effect on phosphate or another anions able to reach the matrix, combine with free calcium, precipitate it as calcium phosphate granules, and release cyclophilin allowing the pore to close. If this mechanism is true, mitochondria will act as "calcium cleaner", trapping extramitochondrial calcium in order to keep cell calcium low, but with a limited capacity. As mitochondria gets older, more and more microcalcifications would be observed inside matrix and free calcium will progressively rise until opening again the pore, this time in an irreversible way as there is not a chance for free calcium to be precipitated any longer. Different indirect observations support this hypothesis from the early descriptions of calcium stores discovered in mitochondria of damaged tissues, to the mitochondria microcalcifications considered one of the hallmarks of CyA toxicity [66]. Interestingly, as CyA reduces influx into proximal tubule isolated mitochondria, we observe a dose-dependent inhibition of phosphate dependent glutaminase activity, an enzyme that is critical in proximal tubule ammoniogenesis extremely dependent on phosphate supply. It is possible that MPT pore inhibition by CyA is related to the reduced ammoniogenesis described with CyA in clinical and experimental settings.

When CyA or CyA analogues bind cyclophilin matrix free calcium rises faster than in control mitochondria. Although initially there is a transient reduced and delayed MPT pore opening, with time and calcium accumulation overwhelms CyA dependent cyclophilin inhibition, and

a second irreversible type of MPT pore opening takes place. Membrane potential begins to disappear and cytochrome c is released out of mitochondria.

We certainly show that MPT pore opening does not cause calcium entry into mitochondria as suggested by others [25].

Actually, there is another condition requested for MPT pore to open; there should be a short supply of ADP. Other way ANT will be recruited for ADP/ATP exchange and MPT pore will not be opened. We showed it clearly in Figure 7B. In other words, ADP delivery to mitochondria may prevent matrix calcium detoxification by disassembling the pore. Bruce Molitoris showed transient internalization and inactivation of tubule proximal Na⁺ pumps during ischemia-reperfusion [67,68]. Our group identified the same phenomenon in porcine models of toxicity with CyA [69]. ATPase inhibition use to be considered as a deleterious process leading to cell death. But at the same time, by inhibiting Na⁺K⁺ATPase activity ADP delivery to mitochondria is drastically reduced, giving the MPT pore a chance to detoxify mitochondria trapped calcium.

TAC shares with CyA some protective characteristics against apoptosis, as well as the ability to cause proximal tubule cell damage mediated by mitochondria [1, 7]. TAC does not bind cyclophilin, and is not implicated in changes in the normal function of MPT pore. Dynamics of aperture, maximal inflow, and mitochondria swelling is similar to those observed under control conditions.

TAC is able to bind several members of the FK-506 binding protein (FKBP) family; immunophilin homologs located in the outer membrane of mitochondria with chaperone activities on several enzymes. FK-506 binding protein 8 (FKBP-38) interacts with Bcl-2 [70], anchoring it to mitochondria outer membrane where exerts antiapoptotic properties [70]. FKBP-38 acts as a docking molecule to localize Bcl-2 at the mitochondrial membrane [70, 71]. Similar effects have been described for Bcl-XL [71].

However, TAC does not prevent MPT pore from opening. The most intriguing finding we got about TAC interaction with MPT pore was the higher increase in the calcium influx into mitochondria (more than 12 times control influx) when electrochemical gradient was built up by O₂ and succinate addition to mitochondria. Ruthenium Red inhibited such inflow, showing that calcium was imported through the VDAC channel (data not shown). Correspondingly, matrix calcium increase was followed by a 12% increase in O₂ consumption. We could not observe any direct effect on the oxidative activity, phosphorylation or MPT pore activity.

So, only mitochondrial calcium influx seems to be targeted by TAC. Recently several authors described the ability of FKBP-12, another TAC receptor in the outer membrane of mitochondria to modify VDAC ion channel function. This effect is related *in vivo* to the endoplasmic reticulum (ER)-mitochondria calcium signal transmission axis, and it is essential to explain coupling between these two organelles. A subfraction of the ER is localized in isolated mitochondria associated to VDAC (Mitochondria Associated Macromolecular Complex, MAM). Increase in VDAC calcium permeability observed in our experiments with TAC may be a reflection of what is taking place *in vivo* in the ER to mitochondria calcium transfer process.

Whatever mechanism lies under the relevant calcium influx into mitochondria under TAC treatment MPT pore will be opened, matrix calcium transiently reduced and MPT pore opened again as matrix calcium rises this time in an irreversible way. As with CyA, cytochrome c translocation and membrane potential lost were lately observed. Although both CyA and TAC actions seem to be able to converge into a final pathway of apoptosis and cell death, we have tested if time course activation on intermediate caspases is similar in CyA and TAC-treated proximal tubule cells. Not very surprisingly, time course was different (Figure 12). In CyA, caspases 3 and 9 activation followed a similar time course, but with TAC, caspase 9 activation was early and preceded caspase 3 activation.

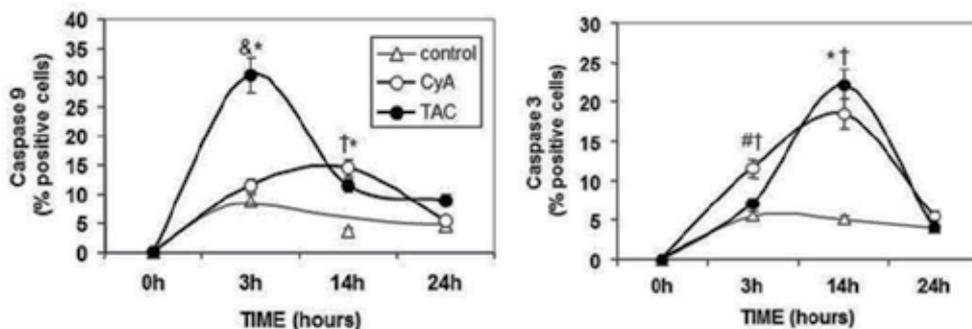


Figure 12. Effect of cyclosporin A (CyA) and tacrolimus (TAC) on time course activation of caspase 9 and caspase 3 in renal proximal tubular epithelial cells (RPTECs). RPTECs were treated along time with 1 $\mu\text{g}/\text{mL}$ CyA or 50 ng/mL TAC. The fluorescence label of caspases allows for direct detection and quantification of activated caspases by fluorescence microscopy. Data are represented as the mean \pm SEM of four experiments. * $p < 0.05$ TAC vs. CyA treatment; & $p < 0.05$ TAC vs. control; † $p < 0.05$ CyA vs. control; # $p < 0.05$ CyA vs. TAC treatment.

In summary, MPT pore has been classically considered as a mechanism aimed to cause cell death through mitochondria depolarisation. But MPT pore is only lethal when its activity becomes irreversible. Its physiological role is probably other, playing a role in intramitochondrial free Ca^{++} detoxification during periods of intense cytosolic Ca^{++} scavenging by mitochondria. Therefore, inhibition of the MPT pore opening may have unexpectedly bad results, as it may limit calcium detoxification. Phosphate availability to mitochondria may play a role in injury adaptation when mitochondrial calcium scavenging is necessary. So, these findings provide some insights about a potential physiological role of MPT pore activation in health and disease, a clear and distinct link between ADP production and delivery and mitochondria calcium detoxification capacity, the double-edged sword of CyA inhibition of MPT pore activity and the potential implication of TAC in the ER-mitochondria calcium transfer.

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Nanotechnology and Chemotherapy

Nanotherapeutics in Cancer Prevention, Diagnosis and Treatment

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Additional information is available at the end of the chapter

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1. Introduction

Definitions of nanotechnology vary widely. Some scientists restrict the definition to work with molecules and devices between 1 and 100nm, while others widen these parameters to 1-1000nm. Kostoff believes that nanotechnology is best defined by the capacity to artificially construct and manipulate structures at nanoscale, and nanoscale's novel properties [1].

Drug loaded nanoparticles are widely utilized in the treatment of a number of diseases, such as metabolic disorders, autoimmune diseases, inflammatory disorders, neurodegenerative diseases and cancer. For instance, nanomedicines have been extremely useful in improving the efficacy of small molecule drug delivery across the blood-brain barrier for the treatment of CNS diseases [2]. In addition, nanoparticles serve as artificial oxygen carriers that can act as a substitute for blood, saving the lives of those in dire need of transfusion [3]. Although liposome-encapsulated formulations of Doxorubicin were being widely administered as early as the 1990's, nanotherapeutics is still viewed as a new and emerging field. The current chapter will focus on the progress made using nanoparticles in cancer prevention, diagnosis and treatment. This is certainly an area of rapid progression, with current nanotherapeutics for cancer encompassing a vast array of nanomaterials and nanodevices [4, 5]. But some critics believe that nanotechnology has not fulfilled its early promise and have expressed concern that progress and investment in the laboratory has not been mirrored by comparable progress or significant clinical success in cancer treatment [5, 6], a concern echoed in the title of Vendito and Szoka Jr's 2013 review: 'Cancer nanomedicines: So many papers and so few drugs!' [7]

However, much investment, research and development into nanotechnology diagnostics, therapies, devices, biosensors, and microfluidics continues to provide advances in the prevention, diagnosis and treatment of cancer [4]. Many scientists believe that nanoparticles

are the future of diagnosis and drug delivery [8] with the potential to overcome many of the obstacles that cancer presents.

2. Obstacles in cancer diagnosis and treatment

2.1. Late stage diagnosis

Late detection and diagnosis of cancer remains one of the fundamental causes of low survival rates [9, 10], so developing a test that detects clinically apparent cancer before symptoms appear is obviously an important goal [9]. The traditional biomedical imaging tools of magnetic resonance imaging, ultrasound and positron emission tomography have several limitations in the diagnosis of cancer, including an inadequate imaging period, a risk of renal toxicity and an inability to detect tumor cells smaller than 1cm [6, 11]. Improvements in PET, CT and MRI, through the use of small molecule imaging agents, such as 2-deoxy-2-(¹⁸F) fluoro-D-glucose [FDG], iodinated small molecules and chelated gadolinium respectively, are routinely used in the diagnosis of cancer. However, poor stability, rapid clearance and low signal intensity have limited the use of these techniques and prompted more research into the use of nanoparticles as a diagnostic tool [12].

2.2. Challenges in targeting, transport and delivery of treatment

Chemotherapy's perennial problem has always been that, due to challenges presented by its targeting, transport and delivery, a pharmacologically active concentration in tumor cells is often only achieved at the expense of what Couvrer terms 'massive contamination of the rest of the body' [13]. This toxicity can result in the use of suboptimal and/or intermittent dosing, to allow the body to rest, or in some cases to forgo chemotherapy altogether [14].

Many traditional chemotherapeutics have poor stability and aqueous solubility. Due to this limitation, many drugs, despite significant biological activity, are disregarded at early stages of drug screening in the laboratory. In addition, distribution of some drugs is too general, with only a small fraction of drugs reaching the cancer site; injected agents are often cleared by the monocytes and macrophages of the reticuloendothelial system (RES) [15]. To be successful, a therapeutically sufficient quantity of the drug, still in a viable state, must survive clearing and be delivered to different regions of tumors via blood vessels, cross the vessel wall and then finally penetrate through the interstitial space to reach the target [16], where unpredictable blood flow and often abnormal vasculature in tumors, particularly in necrotic and semi-necrotic regions, can make accurate delivery even more difficult [17, 18].

Other than conventional chemotherapeutic drugs, biological molecules, such as antibodies and nucleic acids, are being widely explored for the treatment of different diseases, including cancer. Nucleic acid drugs, such as aptamers, anti-sense DNA/RNA, and small-interfering RNA, have shown great promise in the treatment of cancer. However, these drugs are greatly limited by serum nucleases, opsonization and clearance by macrophages and clearance by the renal system. Some of the nucleic acid therapeutics, such as stable nucleic-acid-lipid particle

(SNALP), have used nanocarriers to effectively overcome the above mentioned barriers and are being used in the treatment of cancer [19].

2.3. Chemoresistance

Chemoresistance, a major cause of cancer treatment failure, is linked to cancer stem cells (CSC). CSCs possess unique properties, such as quiescence, mesenchymal morphology, increased DNA repair ability, overexpression of antiapoptotic proteins, drug efflux transporters and detoxifying enzymes [20]. These properties, together with the favorable tumor microenvironment and hypoxic stability, mean that they often escape elimination by current radio and chemotherapies. Having survived through chemotherapy, they can give rise to metastases and recurrent tumors which then increase in malignancy and resistance [20].

Chemoresistance can be divided into two types: intrinsic and acquired. **Intrinsic chemoresistance** is displayed by tumor cells whose genetic and phenotypic characteristics make them ideally suited to withstand cytotoxic agents. **Acquired chemoresistance** can occur after prolonged exposure to chemotherapeutic agents, which disrupt only one of the many biochemical pathways involved in their pathogenesis. Unfortunately this approach often activates and strengthens the alternative pathways, resulting in chemo resistant mutations in the tumor cells and tumor relapse [18, 21, 22]. **Multidrug resistance, or MDR**, can also occur through a process of cross-resistance in which cancer cells mutate and acquire resistance to multiple structurally-related drugs and also to mechanistically different drugs, either via the overexpression of multidrug transporters or through altered apoptosis [21], resulting in decreased intracellular drug retention and altered tumor response [23].

2.4. The patient – Compliance and individuality

It may seem harsh to list the patient as an obstacle but, through no fault of their own, this is often the case. Genetic variation across individuals affects a drug's pharmacokinetics and pharmacodynamics [24], and a breakthrough with one patient cannot always be replicated in another. Also, each patient has different levels of tolerance to the discomfort and effects of chemotherapy, and in many cases a pre-existing condition or illness may complicate their cancer treatment or lead them to refuse it. Patients with comorbid illnesses and elderly patients are those most likely to forgo or discontinue chemotherapy [14].

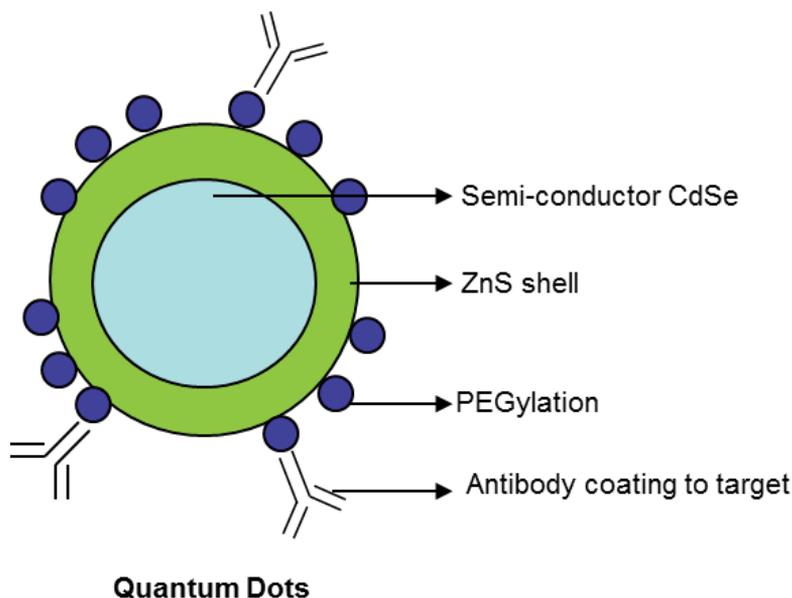
3. Current nanotherapeutics: Overcoming the obstacles

3.1. More accurate detection and diagnosis

Promising results have emerged from combining nanoparticle-based optical contrast agents with existing optical imaging technologies [9]. Their 'programmable' surface properties and potential for passive or active targeting make nanoparticles ideal for diagnostic imaging. The ability of nanoshells, constructed with a silica core and gold shell, to absorb specific wavelengths of light, has great potential for cancer imaging and therapeutic applications [4].

3.2. Quantum dots

Semiconductor quantum dots are luminescent nanocrystals with great potential in both biological and biomedical applications [25]. Their photostability, fluorescence intensity, small size (2–10 nm) and tunable surfaces make them ideal for optical imaging and detecting hundreds of cancer biomarkers in blood assays or tissue biopsies at pg/mL concentrations [25]. The most commonly used agents in the quantum dots are selenides or sulfides of cadmium and zinc [12]. The wavelength of light emitted by the quantum dots depends on their size. The light emitted is much more intense and stable than their other fluorescent counterparts and hence very useful in optical imaging [12]. Cadmium selenide (CdSe), cadmium telluride (CdTe), indium phosphide (InP), and indium arsenide (InAs) are the most common quantum dot formulations used in biological applications [25]. The inorganic core is covered by an inorganic shell, which imparts greater photostability and increases the fluorescence properties of the core [26]. The surface of the shell is coated with another layer that enhances solubility and stability of quantum dots in the blood [26]. Often times, the surface coating is PEG as it has low toxicity and is biodegradable. A major limitation of quantum dots in imaging is a process called “Blinking”. This is due to fluctuation of the quantum dots between the light emitting and non-emitting states. This limits the amount of signal obtained at a specific time [26].



3.3. Magnetic resonance imaging

Recently, the development of nanoparticle systems to improve MRI for cancer imaging and diagnosis has made significant progress [8]. Magnetic nanoparticles usually consist of an

inorganic nanoparticle core and a surface coating that provides stability in aqueous dispersions. This surface coating is manipulated to facilitate targeting, real-time monitoring or both [25]. Their success, particularly as contrast agents for MRI, is largely due to their enhanced proton relaxation and deep-tissue imaging capabilities, non-invasiveness and low toxicity [8, 25].

Supermagnetic iron oxide (SPIO) nanoparticles are now widely used as bowel contrast agents and have been used for some time in spleen/liver imaging. SPIO nanoparticles are readily taken up by macrophages present in the liver parenchyma (Kupffer cells) and, as liver tumors are usually devoid of macrophages, the macrophage-specific uptake of SPIOs increases the contrast between healthy and diseased tissue, allowing liver tumors or micro-metastases as small as 2–3 nm to be detected [25]. SPIO nanoparticles are biodegradable as the iron molecules released into the plasma upon degradation can bind hemoglobin. To avoid clearance of the SPIO nanoparticles, they are often coated with PEG, which enhances the circulation time during the imaging and treatment of prostate cancer. They are conjugated with an aptamer that binds with high specificity and affinity to a cell surface ligand on the prostate tumor cells. The aptamer binding to the cell surface antigen cause a localized increased accumulation of the SPIO that enables imaging. In addition, conjugation of doxorubicin to the SPIO nanoparticles allows the targeted delivery of the chemotherapeutic drug with minimal side effects [27].

3.4. Molecular diagnostics

AuNPs (gold nanoparticles) have brought great benefits in this area, with increased sensitivity and specificity, multiplexing capability and short turnaround times. Aptamer-conjugated NPs can also be used for the collection and detection of multiple cancer cells [8, 28]. Gold nanoparticles scatter light intensely. Based on the size and shape of the gold nanoparticles, the scattering properties of the gold nanoparticles are also changed [29]. The light scattered by the gold nanoparticles have greater photostability than the other imaging agents commonly used [29]. Gold nanorods exhibit a phenomenon called the Surface Enhanced Raman Scattering (SERS), which is also used in cancer diagnosis. In addition, gold nanoshells and gold nanorods have been used to induce photothermal therapy [29]. This is an example for a “theragnostic” agent, as the gold nanorods not only assist in diagnosis of the cancer, but also help in ablation.

3.5. Improving targeting, transport and delivery

Nanoparticles are increasingly utilized because of the multiple benefits that they offer [30]. Nanodelivery systems can make the use of chemotherapy drugs more safe and efficient by improving delivery, cell uptake and targeting, and have been shown to improve their pharmacokinetic profiles and enhance their targeting at the required site [21, 31]. This success relies on two main factors: 1] the EPR 2] The potential ability of nanodrug delivery systems to overcome the shortcomings of many anticancer drugs [20].

1. The EPR, or enhanced permeation retention effect, exists because of two properties of tumors. Firstly, tumor tissues have increased vasculature which allows the entry of macromolecules and colloidal particles of diameter up to 600nm. Secondly, the lymphatic

system is not effective in clearing the interstitial fluid from the tumor tissues [6]. Normal tissues other than the spleen, liver and kidney are impermeable to molecules that are larger than 2nm. Hence, nanoparticles can selectively target tumor tissues reducing toxic side effects [6]. Together, the enhanced permeation and retention properties of the tumor over the normal tissues cause the nanoparticle to have prolonged contact with the tumor cells. In addition, nanocarriers also release the drug slowly, ultimately resulting in reduced drug distribution and toxicity to normal tissues [6].

2. Once the nanoparticles reach the target tissue, cell surface receptors interact with ligand-coated nanoparticles leading to their uptake by endocytosis. Cellular uptake of uncoated nanoparticles is governed by their differences in size, shape and charge. It is suggested that positively charged nanoparticles are taken up more readily due to electrostatic attraction [32]. Interaction with specific serum proteins, results in the formation of a corona, promoting cell entry. Recent studies indicate that non-spherical molecules, such as rod-shaped structures, are internalized better than spherical structures [33]. Uptake of larger nanoparticles disrupts the membrane surface, thereby inducing cell death [34]. Non-specific uptake of the nanoparticles by the lung epithelial cells and red blood cells could cause toxic side effects.
3. Nanosized drug delivery systems can potentially overcome the shortcomings of many anticancer drugs, such as low aqueous solubility and stability and high nonspecific toxicity [20]. For example, paclitaxel nanoparticles stabilized with pluronic F68 are stable for years, while the same drug in dissolved form degrades completely in less than 48 hours [30, 35], and magnetic nanoparticles (MNPs) are increasingly used because their targeting ability reduces systemic distribution of cytotoxic compounds *in vivo* and enhances uptake at the target site, resulting in effective treatment at lower doses [8].

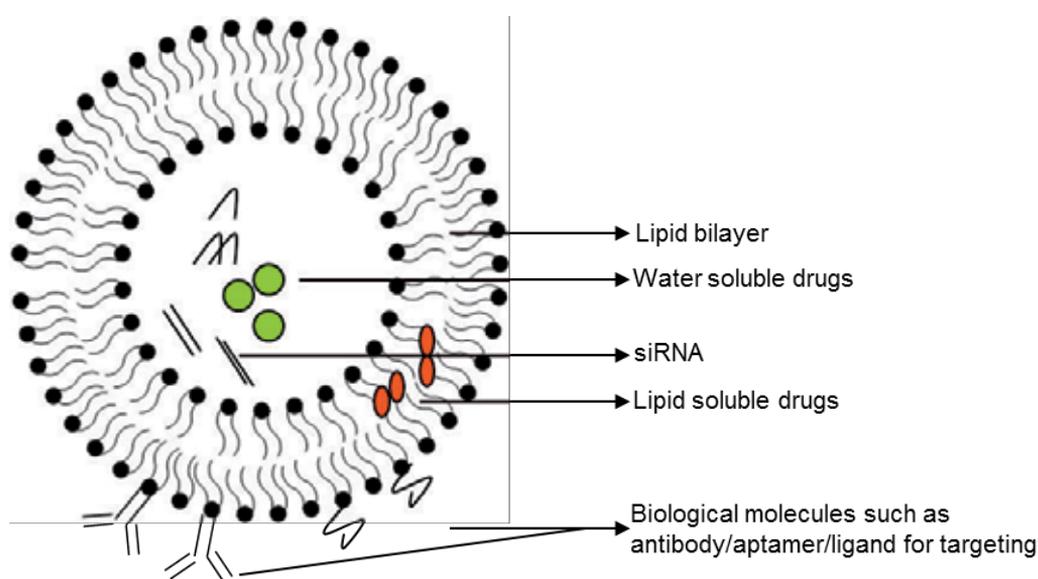
3.6. Nanodrug delivery systems

Nanodrug delivery can either use passive targeting mechanisms, such as the EPR effect, or active targeting mechanisms, using ligands directed against differentially overexpressed cell surface markers surface on tumor cells [20]. Drug encapsulation within nanoparticles can also enhance the bioavailability of drugs administered via routes other than intravenous; both insoluble and soluble drugs can be incorporated within nanoparticulate sols, extending their stability as they travel through the blood, which in turn improves their overall pharmacokinetic half-life [30].

By 'pre-programming' the degradation of nanoparticles in the body, prolonged drug release can be achieved, eliminating the need for repetitive dosages and enabling more sustained and consistent drug concentrations in the target area [30]. Brannon-Peppas and Blanchette have compared the uptake of nanoparticles with more hydrophobic surfaces with those of more hydrophilic surfaces. They concluded that a nanoparticle designed to be 100nm or less in diameter with a hydrophilic surface will have a longer circulation time and hence a greater ability to target the required site [17] due to reduced clearance by macrophages.

Nanodrug delivery systems can carry one or a combination of therapeutics, including cytotoxic agents, chemo sensitizers, small interference RNA (siRNA) and antiangiogenic agents [22]. The most commonly used platforms are described below.

Liposomes have been in use for the past several decades and are established as drug and imaging agent carriers with proven clinical efficacy [6]. They are artificial phospholipid vesicles 50 nm to $\geq 1 \mu\text{m}$ in size, either unilamellar or multilamellar (with one lipid layer or several, arranged in onion-like layers), with one or more aqueous compartments [6, 36]. The 'cargo' can be held in the aqueous compartment(s) or lipid layer [37]. Liposomal nanocarriers provide protection from degradation. Optimization of the pharmacokinetics of the encapsulated drug can improve drug accumulation in the tumor and reduce the adverse effects of bolus administration [37].



Liposome

Polymeric micelles consist of a hydrophobic core and a hydrophilic shell and are useful drug carriers, due to their tunable size and surface functions, high monodispersity and excellent stability. They have the ability to form hydrogels and are used for drug encapsulation or drug conjugation [38]. Under the right conditions, pluronics, the most well-known thermosensitive polymers, form a hydrogel at body temperature but are water soluble at 2-4°C. This allows them to be injected as a liquid but they form a hydrogel *in situ*, resulting in prolonged drug release of the encapsulated drug [36].

Dendrimers are globular macromolecular compounds consisting of an inner core, which can be manipulated to alter its shape and size, surrounded by a series of branches with surface

functional groups. They can carry a multiple payload of active targeting molecule, diagnostic agent and therapeutic drug, and those with a hydrophobic core and hydrophilic surface groups can form micelles, which can then be designed for site-specific release of their payload, via pH and enzyme dependent mechanisms [39, 40].

Inorganic nanoparticles, such as gold nanoparticles, can be used as a cargo for drug delivery. Gold has a number of appealing surface properties, such as light scattering, which makes them attractive inorganic biomaterials for drug delivery when combined with nanoparticles. Due to their ease of synthesis, biocompatibility, and affluent functionalization, many drugs can be conjugated to the surface of gold through hydrophobic interactions. Gold–thiol conjugates are the most common due to their accurate and predictive functionalization. Various antibiotics, anticancer agents and oligonucleotides are also conjugated with gold nanoparticles to yield more viable drug delivery agents. Other inorganic nanoparticles that are frequently used in drug delivery and diagnostics include silica and iron oxide, which forms the core constituent of many inorganic nanoparticles [41].

Porous silica based nanoparticles are highly suitable for carrying hydrophobic drugs. These nanoparticles have a high surface-to-volume ratio and consists of large pores. The drug can be loaded on the nanoparticles by physical adsorption and covalent linkage [42]. Nanostructured mesoporous silicon (PSi), fabricated by electrochemical etching, have nanometer range pores that facilitate high drug loading capabilities, irrespective of different surface chemistries.

3.7. Nanoparticles as therapeutic agents

Nanoparticles can be used as a therapeutic agent themselves. Their ability to alter the substrate molecule, through a process called “intercrossing” upon excitation by light, is used to treat cancer cells in the photodynamic therapy. While in the photothermal therapy, the property of small inorganic molecules to generate heat upon excitation is taken advantage of in the inducing apoptosis or necrosis of cells.

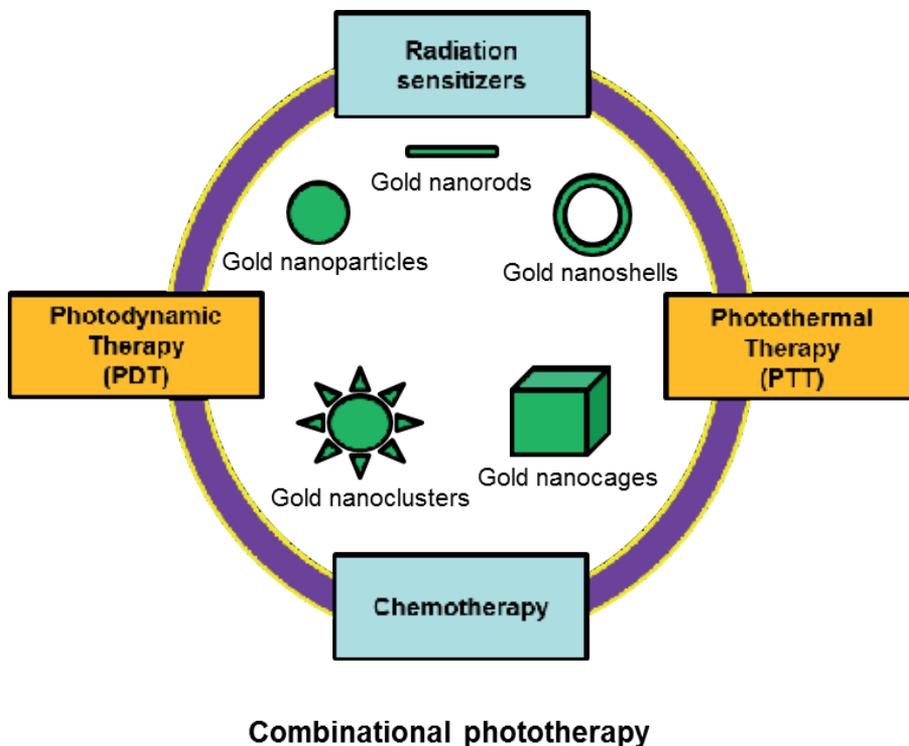
3.7.1. Photothermal therapy

Photothermal therapy (PTT) uses sensitizers that can absorb light in the near-infrared region and convert it to thermal energy, causing heat in the vicinity. The sensitizer used in PTT is usually inorganic molecules, such as gold or carbon nanoparticles. Thermal ablation therapy has been used in the treatment of cancer for many decades, but the damage to nearby tissues has limited the use of this technique in the treatment of cancer [43]. However, with the advent of photodynamic therapy (PDT), targeted destruction of the tumor cell has become possible. PEGylation and active targeting of Au nanotubes have been used in the treatment of many cancers [44].

3.7.2. Photodynamic therapy

Photodynamic therapy (PDT) uses photosensitizers in the treatment of cancer or other disorders. Photosensitizers are molecules that can be excited by light, which then alters molecules in the vicinity, causing the release of singlet oxygen species (reactive oxygen

species). ROS are capable of causing oxidative stress to the surrounding cells, causing apoptosis or necrosis [12]. Photosensitizers can be excited using lasers over a wide range of visible wavelengths. Because of the limited penetrability of visible light, photosensitizers can be used to treat only superficial tumors, such as skin, lung, esophagus, prostate, head and neck, colon and rectum to mention a few. Because the half-life of the reactive oxygen species is only a few milliseconds, this therapy can be used to cause targeted cell death in regions where the photosensitizer has accumulated. Photosensitizers can be coated with polyethylene glycol to prevent renal clearance and to enhance the circulation time in the blood. Further antibody conjugation to the surface can target the photosensitizer to the cancer cells that overexpress the antigen on the surface.



3.8. Overcoming resistance

Nanovehicles carrying therapeutic drug combinations that not only target the tumor cells selectively, but also overcome the mechanisms of drug resistance are the focus of intensive research. This method has been proved especially effective in circumventing multidrug resistance (MDR) in multiple cancer models [21, 36]. MDR was reversed in *in vitro* and *in*

vivo cancer models through the co-delivery of combinations of chemo sensitizing agents and chemotherapeutic agents [22].

3.9. Improving therapy

Nanoshells are nanoparticle beads with a thin gold outer shell and a central silica core. By manipulating the thickness of the shell and core, the beads can be tuned to absorb and scatter specific wavelengths of light across the visible and near-infrared (NIR) spectrum, which is very useful in enhancing imaging properties [4].

Arguably, however, this ability to absorb light is most usefully exploited in thermal ablation therapy. For maximum efficacy, nanoshells with a silica core diameter of ~120 nm and a 10-nm gold shell are used in this therapy as they strongly absorb NIR light (~800 nm) and can then create intense heat that is fatal to cells [4]. As tissue chromophores do not absorb much energy in the NIR range, NIR light can penetrate several centimeters of human tissue without causing harm [4].

3.10. Improving cancer prevention

The complete prevention of cancer occurrence, claims Siddiqui et al, as an unachievable goal; cancer prevention describes 'slowing the process of carcinogenesis' and inhibiting its reoccurrence [45]. Inefficient systemic delivery and bioavailability of chemopreventive agents has so far limited their applicability to human medicine. However, Siddiqui et al have experimented with encapsulating a chemopreventive agent, epigallocatechin-3-gallate (EGCG), in polylactic acid [46] and polyethylene glycol (PEG) nanoparticles [45]. Nano-EGCG had a significantly longer half-life and had more than a 10-fold dose advantage over nonencapsulated EGCG in cell growth inhibition, proapoptotic, and angiogenic inhibitory effects. Curcumin derived from turmeric, when conjugated with polymeric amphiphile, mPEG-PA or PEG, has been shown to have more significant antiproliferative effects than the free curcumin [47, 48]. Another nanoparticle-based formulation, called solid lipid nanoparticles (SLN), is also being used as newer therapeutic modality to address the area of chemoprevention. The advantage is that they act like colloidal carriers which remain as solids at room and body temperature, and so can be efficiently used as alternatives to liposomes and other polymeric nanoparticles [36]. A multitude of approaches utilizing nanoparticles to combat these existing deficits in the chemopreventive strategies will re-captivate the 'silver bullet' for chemoprevention in the near future.

3.11. Improving compliance

Nanotherapeutics can be less invasive than conventional diagnosis and treatment methods. This leads to shorter recovery times and a decreased risk of infection, and these advantages in turn should lead to a reduction in cost and improved life expectancy and quality [49].

4. The future: Potential risks, rewards and research

4.1. New risks: The voice of caution

With its obvious potential for breakthroughs in so many fields, it is easy to view nanotechnology as an exclusively positive concept. However, it is not without risk and nanomaterials may present greater risks than their larger counterparts, as their greater relative surface and unique quantum effects mean they have a tendency to be more active and reactive [50]. Their potential to cause harm is harder to predict, as it is determined using factors such as surface area, rather than molecular structure, which is used to risk assess most other chemical hazards, and there are no proven toxicity screening methods to evaluate them. The scarcity of information about how nanomaterials may impact safety, health and the environment, along with the growing number and diversity of nanotechnologies and their associated engineered properties, has raised serious concerns. If nanomaterials escape the laboratory or manufacturing site, their degradation and interaction with substances in the environment would be unpredictable and potentially hazardous [30, 51].

When assessing the risk to patients, it is important to bear in mind that preclinical trials of nanodrugs may be less indicative of human risks than trials of standard medicines, and that nanomaterials can utilize unique mechanisms and routes of exposure, potentially bypassing the blood-brain barrier [25]. If inhaled, they may aggregate in the alveoli, where their increased surface area places a burden on mucociliary and macrophage clearance [52].

Like any other emerging area of interest in human health, nanotechnology also has its own demerits. A word of caution is that this research is still in its infancy to determine the unforeseen side effects pertaining to nanoparticle related therapies [53]. Although our understanding on the concepts regarding nanotherapeutics has come a long way, the exact nature of nanoparticulate drug interactions has not been tested vigorously. Studies in animals suggest alarming facts affecting the brain function [54-56]. With the limited current literature in humans, it is almost impossible to judge their safety over efficacy. Hence, until a stringent risk assessment strategy is employed, nanotherapeutics should not be viewed exclusively as a positive concept. It is important that, if nanotechnology is to move forward safely and sustainably, a thorough assessment of the biocompatibility and toxicity of nanoparticles is undertaken, with potential toxicities identified and their underlying mechanisms understood [57]. Research into the avoidance of health risks associated with nanotechnology may potentially be used to guide therapy, and vice versa [58].

5. New rewards: A bright future

5.1. Multifunction nanovehicles

Advances in MRI contrasting agents promise a next generation of agents consisting of a core and coating conjugated to tumor-specific moieties for improved efficacy and tumor targeting [25]. Perche and Torchillin have suggested that a possible direction for research may be the

coupling of ligands of different natures (antibodies, proteins, peptides and chemokines, hormone analogs) to target at least two tumor cell populations, providing more sensitive malignant lesion detection and reducing relapses [24, 37]

Shapira looks forward to the development of ‘theragnostic’ nanovehicles that carry four major components: a selective targeting moiety, a diagnostic imaging aid for localization of the malignant tumor and its metastases, a cytotoxic small molecule drug(s) or innovative therapeutic biological matter, and a chemosensitizing agent to neutralize drug resistance – the advent of “quadrugnostic” nanomedicine [59].

5.2. New detection methods and diagnostic devices

Nanoparticle probes, nanocantilever, nanowire and nanotube arrays are the subject of intensive research and are expected to solve the problem of early detection in the future [9]. Accurate localization of tumors and their metastases, *via* nanoparticles loaded with a diagnostic aid, could in future facilitate the harnessing of other therapies, such as radiotherapy, photodynamic therapy and surgery [59].

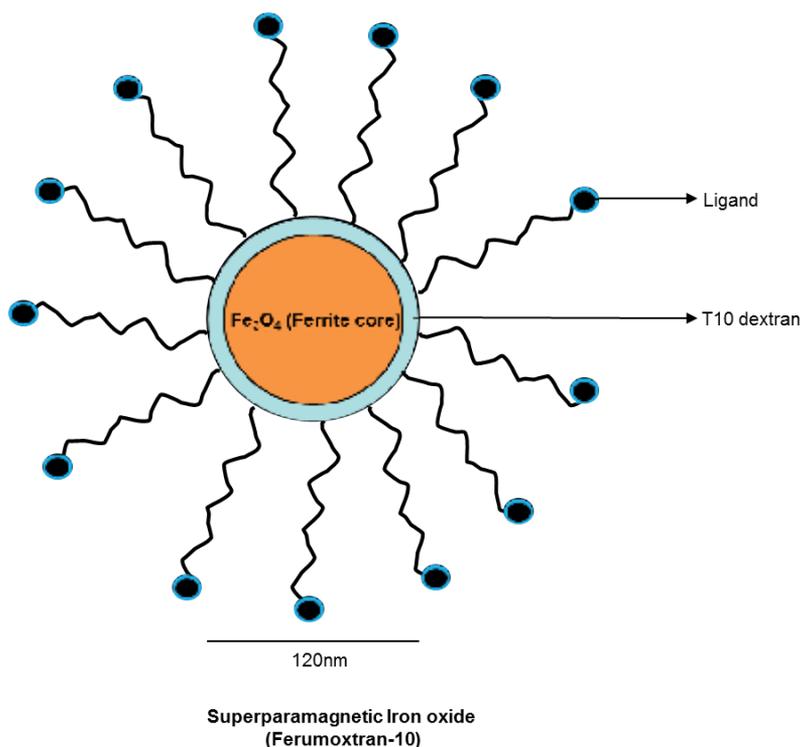
Heller group has described the goal of research as ‘the development of a cancer therapy monitoring/diagnostic platform device’. This would provide real-time monitoring of patient blood for cancer cells, cell derived nanoparticulates (such as high molecular weight DNA fragments), and carry out cancer-related genotyping, gene expression and immunochemical analysis [60].

5.3. New applications, new targets

Superparamagnetic iron oxide nanoparticles (crystalline magnetite structures coated with dextran and dextran derivatives) are promising candidates for a number of applications, including magnetic resonance imaging and drug delivery [6]. Bharali and Mousa believe that a major potential application of these nanoparticles is the diagnosis and treatment of central nervous system (CNS) tumors, particularly if USPIOs (ultrasmall supermagnetic iron oxide particles) are used, as they can be utilized as intravascular contrast agents, as well as for cellular imaging [25]. One USPIO already showing great promise is Combidex, which has been undergoing clinical trials for the detection of lymph node metastases [25].

Talekar et al predict that multifunctional superparamagnetic nanocarriers, with FR (folate receptor) targeting and pH mediated drug release, can be developed to achieve a decrease in tumor volume, as well as improved MRI sensitivity and decreased adverse effects [6]. Metal coordination complexes also offer a diversity of formulations and the prospect of mechanisms that differ from those of organic drugs, including ligand substitution and metal-and ligand-centered redox properties [31].

Therapeutic and imaging nanoparticles have normally used passive targeting to date, but active targeting needs to be used and further developed if drugs are to be delivered to specific classes of cells and specific intracellular sites in cancer cells [31].



5.4. Reducing the side effects

Nanoparticle encapsulation has already been shown to reduce unwanted accumulation of platinum in the kidneys from the platinum [59] prodrug mitaplatin, and reports show that metallodrugs loaded in nanoparticles cause less damage than the drugs on their own. So these formulations are predicted to be in line for further research and exploitation in the near future [31].

6. Bench to bedside: Translational perspectives

Nanotechnology has raised as many questions as it has answered, and spawned new and unpredicted fields [61]. With its vast array of potential applications in so many fields of science and industry, it is a prime candidate for multidisciplinary collaboration, and the urgent need to see laboratory breakthroughs translated to clinical successes is increasingly recognized. Biochemists are increasingly working with scientists from fields not usually associated with medicine, and the NIH's Nanomedicine Development Centers are staffed by multidisciplinary research teams, including biologists, physicians, mathematicians, engineers, and computer scientists, whose first task has been to research the chemical and physical properties of

nanoscale biological structures [58]. Baseline work of this sort is vital if clinicians are to have the knowledge to develop new therapies.

Murday et al claim that translational research has been 'a powerful process that drives the clinical research engine', but feel that a stronger research infrastructure is needed in future to 'strengthen and accelerate this critical part of the clinical research enterprise [58]. Kawasaki and Player agree that scientists from all fields must make a strenuous effort to integrate and coordinate the research in an approach that might now be described as 'systems biology'. They hold up the 2004 article 'Electronic structure and bonding of Au on a SiO₂ cluster: a nanobullet for tumors' [46], produced by physicists, as a prime example of how research in other fields can advance nanomedicine [46, 62].

7. Conclusions

Nanotherapeutics have already yielded significant breakthroughs in the detection, diagnosis and treatment of cancer, and appear to have the potential to yield many more, with extensive and focused routes of research planned for the future and the possibility of nanotechnology-based cancer prevention. But it is clear that nanotechnology must be thoroughly understood and its risks assessed if it is to be developed safely, and that the expertise of researchers in many fields needs to be brought together to move new discoveries out of the laboratory and into the clinical environment where patients can reap the benefits.

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Anticancer Drug – Friend or Foe

Tuğba Taşkın-Tok and Sivakumar Gowder

Additional information is available at the end of the chapter

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1. Introduction

Cancer is the third most lethal disease in the world after cardiovascular, parasitic and infectious diseases, based on reports from American Cancer Society (ACS) [1,2]. In 2011, nearly 13 million people are diagnosed with cancer and hence, cancer continues to be a great threat to people now [3]. Thus, the medical needs for cancer remain one of the most demanding areas in scientific research. Several studies have been carried out to prevent and treat cancers. Chemoprevention is defined as pharmacological intervention with synthetic or naturally occurring compounds that may inhibit or prevent carcinogenesis [4]. Cancer treatment involves surgery, radiation and drugs. Surgery-the first line of therapy, is used for early stage of cancer. Radiotherapy is most often applied in a localised setting and conjunction with surgical procedures. The last one, drugs are implemented with chemotherapy (CTX), which employs a wide group of drugs that have cytotoxic effects. The anticancer drugs inhibit cell division and proliferation and are less selectivity towards cancer cells. Thus, these drugs not only destroy cancer cells but also destroy normal cells.

In this chapter “Anti Cancer Drug: Friend or Foe” we have evaluated the beneficial and harmful effects of anticancer drugs.

2. Anticancer drugs – Beneficial effects

Anticancer or chemotherapy drugs are chemicals that can denature cancer cells by arresting their growth. Though anticancer drugs affect dividing cancer cells, normal cells are also affected in the course of the event. The most affected cells are:

- *bone marrow,*
- *gonads (sex organs),*

- *gastrointestinal tract*, and
- *skin (hair follicle cells)*.

In addition to the above organs, liver and kidneys (slow proliferating cells) are affected since they are the organs of metabolism or target organs of toxicity.

Today, more than 100 different drugs have been used for chemotherapy, either alone or in combination with other treatments. For several years, the most effective drugs used in chemotherapy were considered to be DNA damaging agents [5]. These drugs can be divided into different categories based on their mechanism of action. They are summarized in Figure 1.

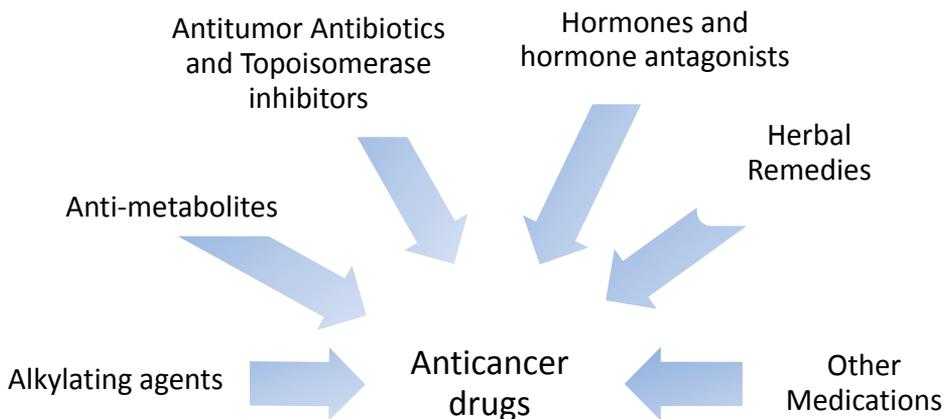


Figure 1. Classification of anticancer drugs.

2.1. Alkylating agents (Figure 2)

This class of drugs directly damages DNA by adding methyl or other alkyl groups onto nucleotide bases [6] and thereby inhibit their correct utilization by base pairing leading to mutation, DNA fragmentation as well as inhibition of DNA replication and transcription. They also disrupt cell respiration and intermediary metabolism by alkylation of proteins and enzymes. The anticancer drugs that contain alkylating agents are *cyclophosphamide*, *ifosfamide*, *melfhalan*, and *chlorambucil*.

2.2. Anti-metabolites (Figure 3)

Inhibitors of DNA synthesis inhibit essential biosynthetic processes or are incorporated into DNA, RNA, proteins and other macromolecules. These drugs (Figure 3) are either structural analogues for heterocyclic bases or agents interfering with folate metabolism. DNA building blocks include heterocyclic bases and folic acid. They inhibit main steps in the formation of purine and pyrimidine bases as well as nucleotides [7]. This class of drugs includes antifolates (*methotrexate*, *pemetrexed*) [8], antipyrimidines (*5-fluorouracil*, *capecitabine*, *eniluracile*, *hydroxyuracil*) [9] and antipurines (*6-mercaptopurine*, *6-thioguanine*).

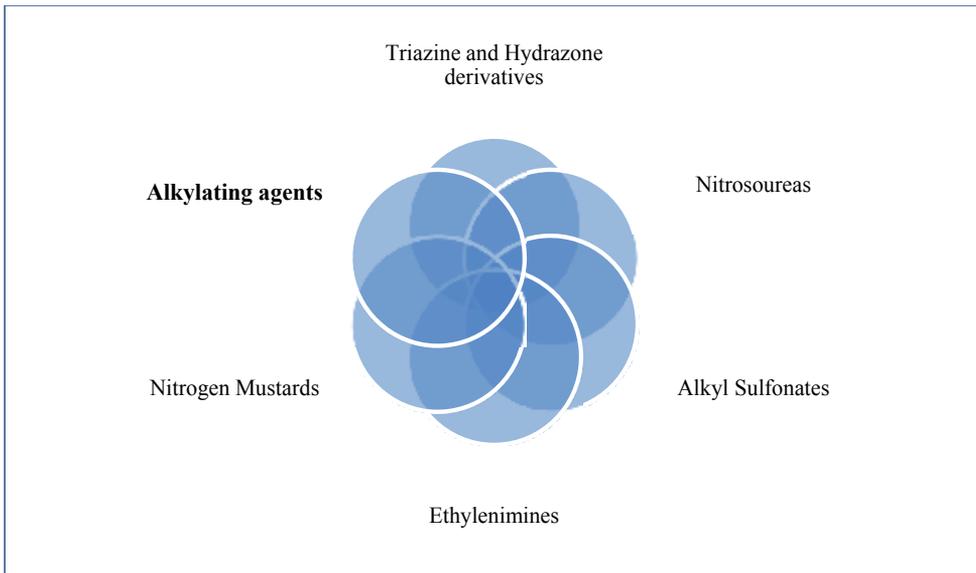


Figure 2. Family of alkylating agents.

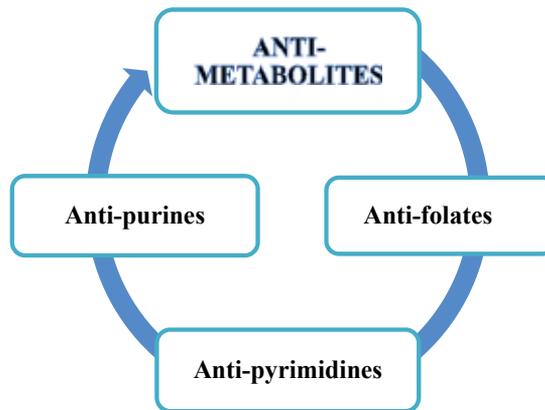


Figure 3. Anti-metabolites.

2.3. Antitumor Antibiotics and Topoisomerase Inhibitors

Antitumor antibiotics and topoisomerase inhibitors are obtained from the cultures of various microorganisms. Examples:

- *Doxorubicin (Adriablastina)*,
- *Daunorubicin (Remember Cerubi)*,
- *Bleomycin (Bleoc's)*,

- *Mitomycin*,
- *Mithramycin*,and
- *Epirubicin*.

Furthermore, topoisomerase inhibitors have been used to interfere with the action of topoisomerase I and II enzymes. These enzymes regulate the changes in DNA structure which includes DNA replication, transcription, recombination, and chromatin remodelling [10-15]. The important inhibitors are *camptothecin*, *irinotecan*, *topotecan* for Topoisomerase I; *Etoposide* (VP-16), *teniposide*, *doxorubicin*, *daunorubicin*, *ellipticine* etc, for *Topoisomerase II*. These drugs inhibit the ability of the topoisomerase to cleave nucleic acid molecules. Although these types of drugs have important clinical efficacy, they have undesired and/or adverse effects such as drug resistance, poor bioavailability problems and myelosuppression. Furthermore, some of them lead to disruption or stabilization of DNA, so that these are also called as topoisomerase poisons. The other inhibitors of topoisomerase bind to enzyme or DNA and interrupt the catalytic activity of the enzyme and prevent the enzyme binding actions.

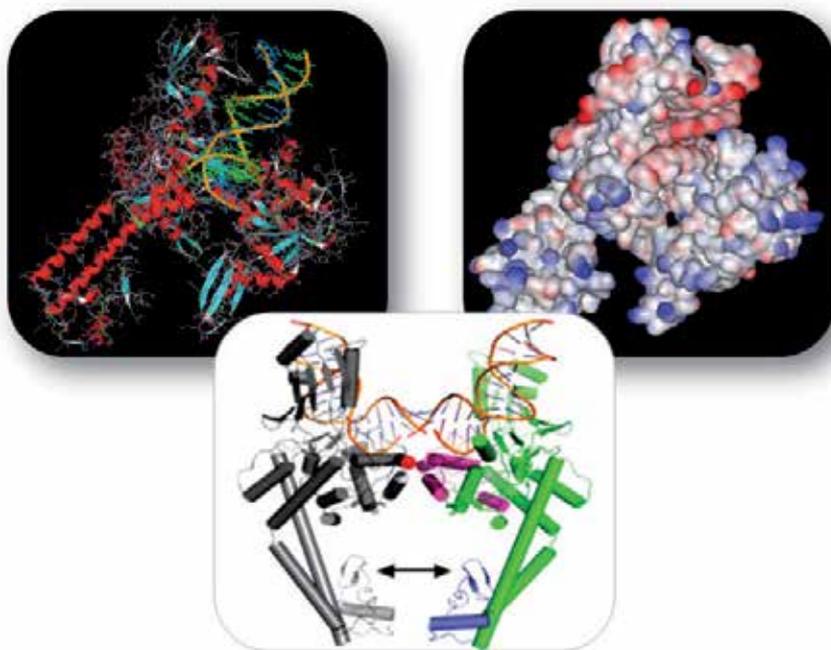


Figure 4. Structure of DNA-Topoisomerase II.

Bi- and ter-benzimidazole derivatives constitute a new class of DNA Topo I and II inhibitors [16-20]. In addition, a camptothecin derivative with a benzoxazole ring is shown significantly more potent than camptothecin as an inhibitor of DNA Topo I [21]. It is of the opinion that a fused ring system in the chemical structure is critical and important for the biological activity.

For example, 2-(4-aminophenyl)benzothiazoles, are observed by Shi et al. [22], exhibit potent anti-tumour activity against some cell lines (breast, ovarian, colon, and renal cell lines). Choi et al. [23] also synthesized a series of 2-(4-aminophenyl)benzothiazole and evaluated the Topo II inhibitory activity.

There are studies on the inhibitory effects of some novel fused heterocyclic compounds, (*benzimidazole, benzoxazole, benzothiazole, and oxazole(4,5-b)pyridine derivatives*) on eukaryotic DNA Topo II (Figure 4) in a cell-free system [24-26]. These compounds displayed more potent inhibitory activities than the reference drug etoposide (Table 1, Figure 5) [17-20]. Molecular modeling of the possible structural motifs of the fused heterocyclic compounds given in Table 1 have been studied to expose their binding mode to eukaryotic DNA topoisomerase II by molecular docking studies. The interactions involved in the anti-tumour activities of fused heterocyclic compounds lead to the rational design of novel eukaryotic DNA topoisomerase II-targeted drugs [27,28].

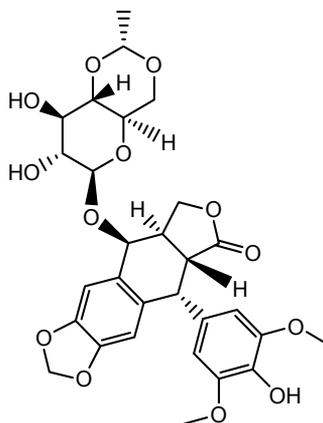


Figure 5. The structure of the reference drug-etoposide.

Compound	R	R ₁	R ₂	R ₃	Z	IC ₅₀ (μM) ^a
1a*	H	NO ₂	OCH ₃	H	CH	17
1b	H	CH ₃	F	H	CH	433.2
1c*	H	CH ₃	NO ₂	H	CH	18.8
1d	NH ₂	H	H	C ₂ H ₅	CH	115.5
1e	CH ₃	H	CH ₃	CH ₃	CH	44.4
1f	Cl	H	H	C ₂ H ₅	CH	NE
1g	CH ₃	H	OCH ₃	H	CH	433.0
1h	NO ₂	H	H	H	CH	32.4

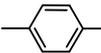
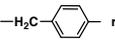
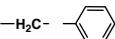
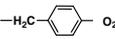
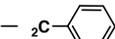
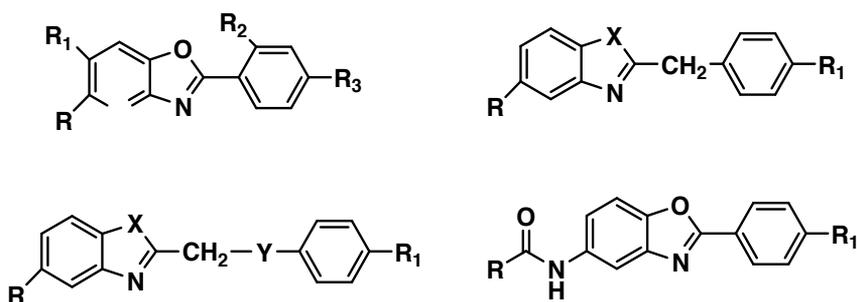
1i	Cl	H	H	Cl	CH	NE
1j	CH ₃	H	H	NHCH ₃	CH	128.4
1k*	NO ₂	H	H	OC ₂ H ₅	CH	22.4
1l	H	H	H	C ₂ H ₅	N	45.6
1m	H	H	H	Cl	N	119.5
1n	H	H	H	C(CH ₃) ₃	N	108.3
1p	H	H	H	CH ₃	N	91.2
Compound	R		R₁	X		IC₅₀ (μM)^a
2a	NO ₂		Br	O		NE
2b	H		OCH ₃	O		86.6
2c	CH ₃		NO ₂	NH		NE
2d	CH ₃		CH ₃	NH		101.9
2e	CH ₃		NH ₂	NH		46.8
Compound	R		R₁	X	Y	IC₅₀ (μM)^a
3a	H		Cl	S	O	NE
3b*	CH ₃		H	NH	S	27.4
3c*	COOCH ₃		H	NH	S	17
3d	H		H	NH	CH ₂	NE
3e*	NO ₂		H	NH	O	24.8
3f*	H		H	S	O	11.4
Compound	R		R₁			IC₅₀ (μM)^a
4a*				H		24.1
4b				C ₂ H ₅		315.1
4c				F		206.9
4d				H		420.1
4e				F		420.1
Etoposide						21.8

Table 1. Eukaryotic DNA topoisomerase II inhibitory activities of novel 2,5,6-substituted benzoxazole, benzimidazole and benzothiazole(4,5-b)pyridine derivatives. [The asterisk (*) refers to structures that are effective, according to the reference drug, *etoposide*. The small letter (a) implies that eukaryotic DNA topoisomerase II-50% inhibitory activity of the tested compounds and the reference drug, *etoposide* at the micromolar (μM) concentration of IC₅₀ values. NE: not effected].



2.4. Herbal remedies

These drugs show their effects on mitosis during metaphase by preventing the formation of the spindle.

Etoposide VP-16 (Vepesid), an effective anticancer drug, is applied to treat a broad spectrum of human cancers for more than two decades. Unfortunately, its wide therapeutic application is often hindered by multidrug resistance (MDR), low water solubility and toxicity. New derivatives of benzoxazoles, benzimidazoles and related fused heterocyclic compounds, exhibited significant eukaryotic DNA topoisomerase II inhibitory activity, were synthesized and exhibited better inhibitory activity even compared with the drug etoposide (Figure 5) [27].

Other examples are:

- *Vinblastine*(Velber A),
- *Vincristine*(Oncovin),
- *Podophyllotoxin*,
- *Teniposide*(VM26-Bristol),and
- *Vindesine*(Eldisine).

2.5. Hormones and hormone antagonists

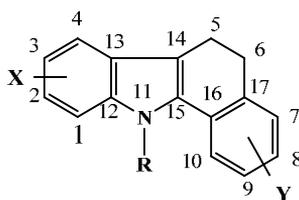
Hormone antagonists are used for tumors caused by hormones or hormonal imbalance. Examples:

- *Glucocorticoid hormones and*
- *Estrogens.*

The endogenous estrogens in women are steroid hormones. Possible consequences of the lack of estrogen in postmenopausal women are frequently reported, including postmenopausal symptoms, increased risks of osteoporosis, coronary heart disease and Alzheimer's disease [29-33]. On the other hand, the cumulative exposure to estrogen encourages development of female reproductive cancers. Such examples include breast cancer and uterus cancer, which are found associated with hormone replacement therapy, early menarche and late menopause [34]. The contribution of estrogens in various physiological and pathological pathways

depends on the binding to estrogen receptors. It also activates transcription of estrogen responsive genes [35-38].

The anti-cancer drug *benzodihydro [α]carbazole* (BDHC), which is widely used to treat breast cancer, and for which the primary target is the human estrogen receptor (hER) [39]. This study reveals a brief introduction of BDHC therapy for breast cancer and the related mechanistic pictures of small compounds signaling through hER by using QSAR and docking methods. They were applied to understand the nature of 5,6-dihydro-11-alkylbenzo [α]carbazole derivatives (Table 2) and to investigate the interactions of homolog series with binding sites on selected a-chains of human estrogen receptors (hER).



X, Y = OH

Compound	R	Position of		RBA ^a	logRBA*
		X	Y		
1	CH ₃	3	9	9,6	1,60
2	C ₂ H ₅	3	9	30	1,10
3	C ₃ H ₇	3	9	38	1,00
4	C ₂ H ₅	3	8	13	1,47
5	C ₂ H ₅	4	9	1,3	2,47
6	C ₂ H ₅	4	8	1,9	2,30
7	C ₂ H ₅	2	9	9,7	1,59
8	C ₂ H ₅	2	8	0,7	2,73
9	C ₂ H ₅	3		1,8	2,32
10	C ₂ H ₅		8	0,06	3,80
11	C ₂ H ₅		9	0,8	2,68

^aRelative binding affinities (RBA) for the calf uterine estrogen receptor = ratio of molar concentrations of 17β-estradiol (E₂) and inhibitor required to decrease the amount of bound [3H] E₂ by 50 %, x 100. [*logRBA (obs.) = log 1/RBA + 2.58].

Table 2. Relative Binding Activities (RBA) of 11-Alkyl-6,11-dihydrohydroxy-5H-benzo [α]carbazoles [39].

Furthermore, the X-ray structure of 17β -estradiol in hER was superimposed on compound **2** and **3** on the docked structure (Figure 6).

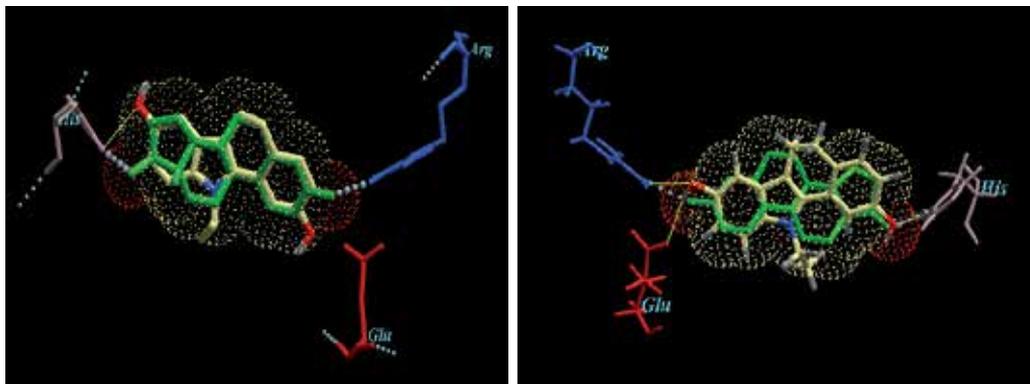


Figure 6. Estradiol (green) superimposed on compound **2** and **3** in the α -chain's binding site of hER. Key residues and H-bonds are shown by yellow lines and blue dotted lines.

Other hormonal antagonists are:

- *Progestins,*
- *GnRH (gonadotropin releasing hormone), and*
- *Antiandrogens.*

2.6. Other medicines

Medicines that are used as anticancer drugs are:

- *Platinum-based drugs (cisplatin, carboplatin),*
- *L-Asparaginase (Crasnit's),*
- *Hydroxyurea (Hydrea), and*
- *Amsacrine (Amsidyl).*

Many of the CTX drugs that are employed are naturally occurring compounds extracted from plants, while others are synthetic. Genotoxic agents bind to DNA and directly and/or indirectly affect the replication which induces the apoptosis. These agents are also divided into three important subunits-alkylating agents, intercalating agents and enzyme inhibitors. Etoposide is an enzyme inhibitor which inhibits topoisomerase II and prevents resealing of DNA that leads to cell death. However, this drug structure exhibits undesired effect for treatment of the disease. Due to this reason, different methods have been improved to evaluate the adverse effects of etoposide, the most effective and potent drug.

There are two approaches to evaluate the adverse effects of the potent drugs-first one, is synthetic way. Researchers try to find derivatives of this drug agent with the conventional

way. In the another approach, they use novel and rational drug design, discovery and development methods which are more economic and minimize time and labor by using computer models, compared with the usual conventional methods.

3. Harmful (or toxic) effects of anticancer drugs

An understanding of toxicity or adverse effects of anticancer compounds is important to design effective and potent drug combinations and also to interpret toxicological profile of new chemical entities. Most cytotoxic anticancer agents are evaluated at the maximum tolerated dose levels. The toxicity of these compounds is often a manifestation of their mechanism of action and their effect on growing normal cells such as *hair follicle cells*, *gastrointestinal surface epithelial cells*, and *stem cells*.

Toxicity or adverse effects of anticancer drugs include the following:

- Bone marrow depression due to damage for the growing stem cells causes a reduction in the white blood cell, platelet, and red cell counts. These, in turn, could cause susceptibility to infections, excessive bleeding, and anemia. In addition, certain drugs cause unique and severe bone damage, such as the osteonecrosis of the jaw associated with bisphosphonates [40].
- Damage to growing cells may cause temporary loss of hair (alopecia), skin rashes, changes in the color and texture, or loss of fingernails and toenails. These toxicities are usually reversible.
- Surface epithelial damage to the gastrointestinal tract may result in ulcers, stomatitis, difficulty in swallowing (dysphagia), vulnerability to oral infections such as candidiasis, and changes in saliva secretion. In addition, nausea, vomiting, diarrhea, or constipation occur commonly.
- Some drugs may cause kidney damage due to extensive cell destruction, purine catabolism, and deposition of urates in the renal tubules. The activity of drugs depends on the individual physiological system and the mode of renal handling of drugs.
- Cinnamaldehyde (an anticancer drug) at a dose level of 73.5 mg / kg body weight / day induced histopathological changes of kidney accompanied by increased activity of marker enzymes and an imbalance in the antioxidant status, in rats. Cinnamaldehyde induced renal damage, is due to the reactive oxygen species that formed while in the free radical scavenging reactions [41-43].
- In addition, liver damage may occur due to large blood supply. Metabolic conditions of the liver and the kidney are usually monitored for possible correlation to drug levels in the blood and dosage adjustments, since these are the major drug elimination sites or the target organs of toxicity.
- Certain symptoms and adverse effects associated with cancer could be secondary to disease progression. For example, cancer metastases to the bones could cause chronic pain due to

the proliferation of cancer cells in the bones and the associated bone remodeling and destruction. Also, tumors that compress veins, the use of central vein catheter, and relative immobility of the patient could lead to deep vein thrombosis with potential pulmonary embolism [44-46].

- Drugs such as paclitaxel and vincristine could cause peripheral neuropathy. Similarly, anthracyclines are known for rare but severe cardiotoxicity [47-49].

Thus, adverse effect and dose-limiting toxicity of anticancer compounds could be a manifestation of either their mechanisms of action or unrelated toxicities common to a given chemical entity of compounds (anthracyclines and etoposide). A close attention to monitor the emergence of known side effects of anticancer drugs, as well as those observed in the preclinical animal toxicology studies, ensures patient safety in early oncology drug clinical trials.

4. Conclusions and perspectives

Especially, chemotherapy has been integrated into treatment programs with surgery and radiation therapy. The major problem of the clinical efficacy in chemotherapy is because of toxicity of the anticancer drugs to the normal tissues of the body. Rapidly proliferating tissues such as bone marrow, gastrointestinal tract, hair follicle, etc are the major sites of acute toxicities. In addition, chronic and cumulative toxicities may also occur. There are measures and agents which can improve the toxicities of anticancer drugs. Furthermore, current challenges of anticancer drug development include the significant time and cost involvement, and the low success rates. These issues lead to increasing efforts of the pharmaceutical industry toward increasing the effectiveness of the drug discovery and development process to minimize failure of drug candidates at later stages of development. It also includes development of high throughput preclinical screening methods (computational molecular modeling techniques) and biological assays with greater specificity and predictability.

Increasing emphasis is being placed on developing a mechanistic understanding of the physicochemical and biological phenomena involved in drug development such as chemical structure and polymorph stability, and pharmacokinetics. The use of mathematical models to explain the mechanisms of drug degradation and predict the outcomes of formulation and process changes and scale-up is increasingly being adopted such as Quantitative Structure Activity Relationships (QSAR). This chapter summarises the beneficial and harmful (toxicity) effects of anticancer drugs and other measures adopted for its management. Proper handling of anticancer agents is the utmost importance at the earlier phase because it has an affiliation with the course of treatment and outcome of the patient in his physical, mental and social wellbeing. Because of these reasons, computer aided drug design and discovery are used to reduce the side effects of the anticancer drugs. These procedures result in effective therapeutic options for chemotherapy.

On general consideration, antioxidants (vitamins) play a significant role to ameliorate the toxicity. Thus, fruits and vegetables in the diet might protect human health from toxic effects

of drugs at certain extent [50]. While in chemotherapy, if the patients are given vitamin rich food (vegetables, fruits, etc), then toxicity of the chemotherapeutic drugs can be prevented at certain extent.

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Drugs and Drug Delivery System

Drug Carrier Systems Using Chitosan for Non Parenteral Routes

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Additional information is available at the end of the chapter

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1. Introduction

A safe and efficient drug carrier must offer protection to human tissues in which it is administered as well as protection to the drugs against degradation, improve therapeutic effect, prolong biological activity, control drug release rate, and decrease the frequency of administration. These characteristics could be achieved by using chitosan to prepare carriers of drugs. It is almost the only cationic polysaccharide in nature with a great innate medical potential. Chitosan, a cationic polymer of natural origin, is a remarkable example of an excipient which currently has enormous potential for using in pharmaceutical dosage forms because of its properties as polyelectrolyte with reactive functional groups, gel-forming capability, high adsorption capacity, biodegradability and biocompatible and non-toxic to living tissues as well as having antibacterial, antifungal and antitumor activity. These functional properties provide suitability and extensive pharmaceutical applications such as for the preparation of drug delivery systems (drug conjugate, micro/nanoparticles, hydrogels, emulsions, biodegradable release system, etc.) and for regenerative medicine for many components such as proteins/peptides, growth factors, anti-inflammatory drugs, antibiotics intended to be administered in non parenteral routes (oral, topical, intranasal, vaginal, rectal and ocular). Interesting applications of chitosan has been receiving considerable attention since it has been developed systems more versatile by the incorporation of chitosan and other components in novel systems. They have provided a strategy for the functionalization by modulating physico-

chemical properties (hydrophilicity, surface charge, etc.) improving the encapsulation, stability, and protection of drugs. The recent development of nanotechnology and the various processes of functionalization of chitosan have increased and improved its functionality as drug carrier.

This work reviews the drug carrier systems using chitosan to improve and increase the delivery of drugs for non parenteral routes. The most important properties of chitosan, the types of systems intended to be used for non parenteral routes, the strategies for the functionalization of chitosan systems by the incorporation of other components, advantages and limitations, and the relevance of pharmaceutical, pharmacological and toxicological experimental studies are reviewed in different drug carrier systems from chitosan.

2. General characteristics of chitosan

Chitosan is an abundant polysaccharide, it has the peculiarity of being the unique cationic biopolymer of natural origin [1, 2]. It is obtained from the chitin, the most second abundant polysaccharide in the nature [3, 4]. The wide variety of natural sources of chitosan include: structural components of the cell walls of certain fungi, algae and bacteria species, and in the egg shells of nematodes. Nevertheless, the principal source is the shells of mollusks and the exoskeleton of the phylum *Arthropoda* such as Crustacea, Insecta, and Myriapoda subphylums [3, 5-7]. The principal industrial source of chitosan is the chitin of the crustacean shell wastes such as crab (10% of chitin), shrimp (22% of chitin), lobster (17% of chitin) and crawfish (36% of chitin) [8, 9]. Although it is much less common than the natural occurrence of chitin, it is possible to obtain chitosan directly from a natural source, e. g, the structure of certain fungi [10]. However, chitosan is generally produced by chemical processes that involve the alkaline deacetylation of chitin aided by thermochemical conditions or by enzymatic hydrolysis in presence of a chitin deacetylase [2, 6, 11].

2.1. Chemical properties

The chemical structure of chitosan is ideally formed only by 2-amino-2-deoxy- β -D-glucopyranose, the deacetylated form of D-glucosamine, and chitin is ideally formed only by 2-acetamido-2-deoxy- β -D-glucopyranose, which is the acetylated form of D-glucosamine. However, the real found structure of chitosan, as well as the one of chitin, is a copolymer of 2-amino-2-deoxy- β -D-glucopyranose and 2-acetamido-2-deoxy- β -D-glucopyranose, in which both the deacetylated and the acetylated forms of D-glucosamine are randomly distributed along the whole copolymer chain. This is the reason why chitosan comprises a wide group of fully and partially deacetylated chitins. The two kind of D-glucosamine residues are linked by $\beta(1\rightarrow4)$ -glycosidic bonds. It is accepted chitosan is composed predominantly of the deacetylated form of D-glucosamine [1, 2, 4, 6, 7, 11-13]. The Figure 1 represents the chemical structure of chitin and chitosan.

Commercial chitosans have a deacetylation degree of 85%, and its elemental composition is 44. 11 % of carbon, 7. 97 % of nitrogen, and 6. 84 % hydrogen [6]. When chitosan is obtained

using an alkaline process, the degree of deacetylation can be controlled if the time, temperature and the concentration of alkali and chitin are also controlled. In addition to these processing factors, the degree of acetylation, the distribution of acetyl groups along the biopolymer chain, and the molecular size distribution of chitosans change depending of the source of chitosan. All these factors determine chitosan physicochemical and biological properties [13, 14].

Chitosan is distinguished by its solubility in dilute aqueous acid solutions derived from its polycationic character [9] and its insolubility in most solvents. In function of deacetylation degree, a chitosan polymer becomes soluble in dilute acidic medium; the minimum deacetylation level for solubilizing is 40-60%. The solubility of the polymer, the inter-chain interactions due to hydrogen bonds and the hydrophobic character of the acetyl group are affected by the distribution of N-acetyl groups along the biopolymer chain [13]. The solubility of chitosan is also affected by the formation of crystalline structures as a result of intra- and inter-macromolecular hydrogen bonds in the solid state; crystalline domains appear to be the main factor limiting for chitosan aqueous solubility [15]. Between acidic and neutral pH conditions, chitosan develops positive net charges because of its polycationic nature [9, 13]. Chitosan is positively charged due to primary amino groups, the magnitude of the charge density is dependent on the degree of deacetylation, pH, and ionic strength [9]. In weakly acidic aqueous solutions of inorganic acids (phosphoric and sulfuric acids) and organic acids (formic, acetic, tartaric, and citric acids), amino groups are partially protonated, while a total protonation is reached at pH 4.0 [15]. Another chemical characteristic of chitosan is its metal binding capacity attributed to its chelating ability of the amine groups [5]. Table 1 shows some intrinsic (structural characteristics of chitosan) and extrinsic parameters that affect some functionality parameters [16-18].

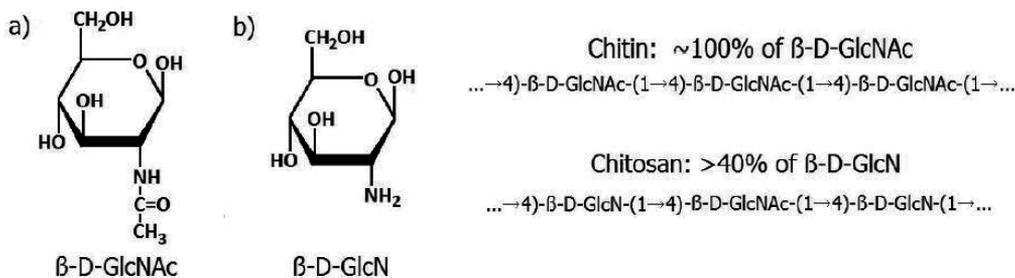


Figure 1. Chemical structures of chitin and chitosan and their monomers: a) 2-acetamido-2-deoxy-β-D-glucopyranose or β-D-GlcNAc and b) 2-amino-2-deoxy-β-D-glucopyranose or β-D-GlcN.

2.2. Physical properties

Physical properties of chitosan depend of factors as the molecular weight distribution, the acetylation degree and the source of chitosan. In Table 2 are listed some physical properties of reported in scientific literature and commercial chitosans from different sources [19-22]. The solution conformation of chitosan can occur as rod-like, spherical or random coil conformations in function of the molecular weight distribution and degree of acetylation; the molecular

mass affects the intrinsic viscosity and radius of gyration [23] while the degree of acetylation determines in turn the electric charge which is directly related to the solution conformation of chitosan [20]. When the charge on the chitosan chain increases, its conformation in solution expands and the viscosity increases substantially [24].

Functionality parameter	Intrinsic and extrinsic chitosan factors that affect its functionality	Correlation between chitosan functionality parameters and structural characteristics
Viscosity	<ul style="list-style-type: none"> •Molecular weight •Deacetylation degree •Presence of contraions •pH •Solvent 	<ul style="list-style-type: none"> •Viscosity of the chitosan solutions is affected by the presence of the amino groups with a pKa value of 6.3 (as a strong base its polyelectrolyte nature affects the hydrodynamic ratio in solution) [16].
Hydrogels formation	<ul style="list-style-type: none"> •Molecular weight •Deacetylation degree •Chitosan concentration •Presence of contraions •pH •Temperature 	<ul style="list-style-type: none"> •At pH above 7 and at low temperatures ($\approx 25^{\circ}\text{C}$) the hydrogels are greatly swollen [17]. •Hydrogels are more compact and irregular when the deacetylation degree (D.D.) of chitosan is increased [18].
Biodegradation	<ul style="list-style-type: none"> •Molecular weight •Deacetylation degree 	<ul style="list-style-type: none"> •Lower molecular weights leads to a faster biodegradation rates than higher molecular weights. •Deacetylation degree is determinant, increasing the presence of charged moieties leads to faster degradation rates.
Solubility	<ul style="list-style-type: none"> •Acid concentration •Ionic strength •Deacetylation degree 	<ul style="list-style-type: none"> •Chitosan is soluble at a higher concentration of hydrogen ions (below at a pH of 6) [16].

Table 1. Correlation of intrinsic properties and extrinsic factors that affect its functionality and structural conformation

Source	Housefly larvae	SeaCure+210	Crawfish	Shrimp shells
Color	White		White	White to beige
Appearance	Powder		Powder	Powder/chips
Granularity (mm)	0.245			
Ph	6.96			
D. D. (%)	83.1	89	73	>75
Viscosity	347 mPa.s	540 ml/g $[\eta]$	563.7 cP	
Density (g/ml)		1.72	0.23 (tapped)	0.15-0.3
Reference	[19]	[15]	[21]	[22]

Table 2. Some physical properties of reported in scientific literature and commercial chitosans

Rheological properties of chitosan solutions are independent of the ionic strength and the pH, this behavior can be related to the fact that the ionic strength within certain range does not affect the conformation in solution [23, 25]. In organic acid solutions as acetic acid ones, the viscosity curves for chitosan solutions consist of two distinct viscosimetric behaviors, the first one is a Newtonian zero-shear viscosity region and second one is a shear rate dependent apparent viscosity region. An increase in chitosan concentration produces a pseudoplastic behavior, i. e., a higher shear rate dependence of viscosity [9] while at a lower concentration exhibits a shear thinning behavior [25]. The pseudoplastic behavior is explained because as the polymer concentration increased, the freedom of movement of the individual chains becomes more restricted due to the correspondingly increased number of entanglements between chitosan chains [9]. Table 3 shows viscosities of various chitosan samples [21, 26-29]. Because chitosan undergoes acid-catalyzed hydrolysis, chitosan presents an irreversible decrease in viscosity in dilute acid medium [28], the degree of hydrolysis depends on the molecular weight and degree of acetylation: the higher the values of both parameters, the quicker decrease in the viscosity and, consequently, in the viscosity-average molecular weight [29].

Due to its degradation in acidic media, chitosan dissolved quickly after swollen in gastric juice and could not achieve sustained release in the gastrointestinal tract [30]. In order to obtain chitosan derivatives with new functionality and to extend its uses in a greater number of pharmaceutical applications, chitosan has been subjected to various chemical modifications. The chitosan structure has been modified through trimethylation, N-succinylation, thiolation, azidation, sugar-modified chitosan, chitosan-dendrimer hybrids, cyclodextrin-linked chitosans, crown-ether-bound chitosan, chemical grafting of chitosan, enzymatic modification of chitosan. These reactions yield chitosan-based derivatives that retain the biodegradability and non-toxicity characteristic of chitosan [1].

Chitosan is able to form physical gels, these gels retain the main properties of this polysaccharide, in particular, biocompatibility. The process of gel formation depends on the initial concentration of the polymer and charge density, which is determined by the degree of deacetylation [31]. The degree of hydration of chitosan hydrogels are affected by the molecular weight of chitosan [32]. Chitosan hydrogels have been investigated as potential vehicles for targeted drug delivery [15] specially seems to be suitable for sustained-release drug [32, 33].

Polycationic character of chitosan opens the possibility for interactions with negatively charged molecules (anions and polyanions) [34] and allows the forming of polyanion-chitosan complexes with polyanions such as heparin, carboxymethylcellulose, carrageenan, alginate, Pluronic, dextran sulfate, and xanthan have been produced [35-51]. The complexes formed by chitosan with other polymers can be divided into hydrogen bonding complexes, polyelectrolytes complexes, coordination complexes and self-assembly complexes based on dominant intermolecular interactions [52]. Some chitosan-based non-stoichiometric polyelectrolyte complexes are soluble at physiological pH and ionic strength [53], because polyelectrolyte complexes are formed in aqueous solutions, it should be taken into account molecular information as the molecular weight of chitosan, its mass distribution, the degree of deacety-

lation, the location of free and acetylated amino groups in the polymer chain, chain length, and conformation of molecules in solution [34]. The synthesis mechanism of chitosan-polyanions complexes can be the result of changing the chemical structure of component polymers, such as molecular weight, flexibility, functional group structure, charge density, stereoregularity, and compatibility, as well as synthesis conditions: pH, ionic strength, concentration, mixing ratio, and temperature [30].

Preparation method	D. D (%)	M. W. (kDa)	Viscosity	Reference
Oxidative fragmentation (1% NaNO ₂) of chitosan	91.3	659.4	1.05 dL/g (intrinsic, in 0.25 mM acetate buffer, 0.05-0.3 g/dl of chitosan, 25°C)	[26]
Oxidative fragmentation (4% NaNO ₂) of chitosan	90.19	864.2	0.21 dL/g (intrinsic, in 0.25 mM acetate buffer, 0.05-0.3 g/dl of chitosan, 25°C)	[26]
1% NaOH, 21 hr, 4% HCl, 2 hr	75.9	Not determined	830 cP (1% acetic acid, 1% chitosan)	[27]
4% HCl, 2 hr, 1% NaOH, 21 hr	76.3	Not determined	2919 cP (1% acetic acid, 1% chitosan)	[27]
Demineralization 1N HCl, 30 min, room temperature after decolorization 0.315% NaOCl, 5min, room temperature	73	10.59	563.7cP(1% acetic acid, 1% chitosan, 25°C)	[21]
Deproteinization 3.5%NaOH, 2hr, 65°C after decolorization 0.315% NaOCl, 5min, room temperature	70	9.63	444.9 cP1% (1% acetic acid, 1% chitosan, 25°C)	[21]

Table 3. Viscosity of chitosan related to deacetylation degree (D. D.), molecular weight (M. W.) of chitosans produced by different methods.

In addition to the electrical charge, dipole-dipole interactions, as well as hydrogen and hydrophobic bonds are determinant for the formation of complexes with polyanions [34]. As a matrix for releasing drugs, chitosan complexes must allow the controlled release either modulating tissue drug levels or spatially-placing (or temporarily-placing) a drug in some region of the body to maintain efficacy and stability of drugs within the matrix. For drug delivery systems for gastrointestinal, respiratory, ophthalmologic, cervical, and vaginal routes, the mucins are hydrophilic saline gels that are thickened by natural anionic glycoproteins. Here, a cationic polymer as chitosan is ideal [52].

2.3. Biological properties

Chitosan has excellent properties such as hydrophilicity, biocompatibility, biodegradability, antibacterial and adsorption applications, and a very low toxicity [54-57]. The biocompatibility

of chitosan is generally regarded as the ability of the newly developed material to interact with living cells, tissues, or organs by not being toxic or injurious and not triggering immunological reactions or rejections while functioning appropriately *in vitro* and *in vivo* [57]. Accordingly the features mentioned above, besides the chitosan being used for drug delivery, is used in tissue engineering, gene delivery, nasal drug and vaccine delivery [58]. The formulation of chitosan with a drug may alter the pharmacokinetic and biodistribution profiles, and for pharmaceutical applications it is necessary to take in account the route of administration, its concentration, contact time and cell types that enter in contact with chitosan or chitosan complexes [58-63]. In Table 4 are listed some biological and toxicological properties of chitosan in several biological systems [64-75].

Due to its good biocompatibility and biodegradability properties, chitosan provides a useful excipient for mucoadhesive drug delivery systems in order to prolong the mucosal residence time. An inconvenient issue about chitosan, it is that glucosamine from shellfish may not be suitable for allergic people to shellfishes [10]. Although chitosan has not yet been related directly to cases of allergic reactions, some cosmetics or nutraceuticals products prepared with chitosan are related to skin irritation or even anaphylaxis [21, 76, 77]. It is possible that proteins from shellfish such as tropomyosin and arginine kinase remain as residues on the chitosan and chitin, being these substances responsible for such allergic reactions [78]. As a result of this review, no allergenic reactions produced to nasal membranes have been found reported. Arai et al. found that chitosan has an LD50 comparable to sucrose of >16 g/kg in oral administration to mice [79]. No oral toxicity was found in mice treated with 100 mg/kg chitosan nanoparticles (80 kDa, 80% DD) [80]. Exposure of rat nasal mucosa to chitosan solutions at 0.5% (w/v) over 1 h caused no significant changes in mucosal cell morphology compared to control [81]. From most studies reported it appears that chitosan shows minimal toxic effects and this approves its adoption as a safe material in drug delivery. Others authors studied the safety of a chitosan bandage in shellfish allergic patients (shellfish allergy prevalence of 2.8% in adults) showing that all subjects tolerated the bandage without reaction. Although larger cohort studies should be considered, the results from this study are encouraging and consistent with two previous studies demonstrating the safety of other chitin-derived products in patients allergic to shellfish [82].

An important aspect in the use of polymers as drug delivery systems is their metabolic fate in the body or biodegradation [54]. Degradation of chitosan by thermal, acidic, enzymatic, and irradiation process have been reported [28, 29, 60-63, 83-85]. In general, both rate and extent of chitosan biodegradability in living organisms are dependent on the degree of deacetylation [54]. Enzymatic degradation of chitosan is sensitive to the supermolecular structure of the polymer [83], in human body lysozyme produces the hydrolysis of chitosan [86]. Aminosugars released as a result of its biodegradation can be used in the metabolic pathways of glycosaminoglycans and glycoproteins in the body [87, 88]. In the stomach, some chemical degradation is wide catalysed by gastric acid [54], but it is not hydrolyzed by human digestive enzymes [69], its lack of absorption in the human body provides chitosan a function as dietary fiber [56]. In the case of the systemic absorption of hydrophilic polymers such as chitosan, they should have a suitable Mw for renal clearance [54].

	D. D (%)	M. W.	Concentration of chitosan	Reference
Biological activity				
Antitumor activity against HepG2, A549, and PC3 model tumor cell	100 87.5	Pentamers Octamers	IC50 < 50 µg·mL ⁻¹	[64]
Prevent leakage and bleeding from lung punctures	99 98	230 kDa 300 kDa	2.3% (w/v) 2.3% (w/v)	[65]
Reduction of 25-30% in plasmatic cholesterol in rats			5% of the diet	[66]
Decreasing of fat digestibility, mineral absorption and vitamin E level in rats fed with high-fat diets	90	Not specified	50g/100g of solids	[67]
Prevention the symptoms of isoprenaline-induced myocardial infarction in rats	85-87	750kDa	2% of the diet for 60 days	[68]
Reduction of body weight and plasma triacylglycerol concentration in mice	Not specified	46 kDa	300 mg/Kg daily administrated	[69]
Antibacterial activity				
Against E. coli	20% 11%	55, 155 5, 300	50-100 ppm 2500-10000 ppm	[70, 71]
Against L. monocytogenes	10 (56 kDa)	5, 150	1000 ppm	[72]
Against S. typhimurium	15-25% (150 kDa)	5, 150	1000 ppm	[72]
Maximum inhibition of absorption of Streptococcus sobrinus by hydroxyapatite (anti-plaque activity)	50-60%	5-6	250 ppm	[73]
Toxicity				
Aspartic acid salt of chitosan in B16F10 cells	78%	<50	(IC50)2.50 mg/mL	[74]
Aspartic acid salt of chitosan in Caco-2 cells	87%	20, 45, 200, 460	(IC50)670, 650, 720 mg/mL	[75]

Table 4. Some biological and toxicological properties of chitosan related to deacetylation degree (D. D.), molecular weight (M. W.) and its concentration in several biological systems.

The administration of chitosan in humans has been extensively studied (Table 5) [89-94]. The chitosan exhibits a hypocholesterolemic effect, the administration of chitosan, regardless of their molecular weight, coupled with ascorbic acid, produce a decreased fat absorption. When chitosan enters in contact with gastric fluids, forms a gel that traps lipids preventing their absorption in the intestine. Sodium ascorbate enhances the gelling and flexibility of chitosan, increasing the amount of fat that is stuck [95]. By blocking the absorption of fat is inhibited the atherosclerotic plaque formation, reducing the risk of atherosclerosis in hypercholesterolemic persons [88], besides that no deterioration occurs in the intestinal mucosa causing anti-hypercholesterolemians as cholestyramine [96].

Fat absorption depends on the degree of deacetylation and the viscosity of chitosan. The greater degree of deacetylation and a high viscosity of the chitosan cause a higher absorption of dietary fat [66]. Chitosan also reduces cholesterol, urea and creatinine, and increases of hemoglobin levels, which may be used as coadyuvate in the treatment of patients with kidney failure [89]. Chitosan can cause wound healing and fibrosis decreasing mortality genitourinary surgery, and produce a hemostatic effect, which is attributed to the interaction between the cell membrane of the erythrocytes and chitosan, being this interaction independent of the classical cascade of coagulation [97].

Health problem	Treatment	Effect	Reference
Renal failure	45 mg/tablet per 12 weeks	Reduction of urea, cholesterol, creatinine levels in serum.	[89]
Obesity	Four tablets, 400 mg/tablet, per day with low calorie diet for 4 weeks	Variable adverse effects in 5% of treated subjects	[90]
Overweight	0, 4, 5, 6, 75 g of chitosan per day for eight weeks, no dietary restrictions	No effect on serum content of vitamins A, E, D, alpha carotene, beta carotene. Modest reduction in plasma cholesterol concentrations	[91]
Hepatocellular carcinoma	Local percutaneous Ho-166/ chitosan complex injections	Complete tumor necrosis in 77. 5% of treated patients	[92]
No health problems	5 g of chitosan	Prevention of blood pressure after a high in salt meal	[93]
Hypercholesterolemia	1. 2 g of chitosan per day, no dietary restrictions	Mild reduction in total and LDL cholesterol, few adverse effects observed	[94]

Table 5. Effects of chitosan and complexes containing chitosan administrated on humans with different health problems.

2.4. Chitosan and chitin biodegradability

Biodegradation of chitosan and chitin implies the cleaving of the $\beta(1\rightarrow4)$ -glycosidic bond between the two kind of D-glucosamine residues that form its chemical structure. Biodegradation of chitosan has been assessed by enzymatic methods using enzymes from different sources. The lysozyme (EC 3. 2. 1. 17) is an enzyme which degrades chitosan and chitosan-conjugates by cleaving the $\beta(1\rightarrow4)$ bonds between N-acetyl-D-glucosamine residues; lysozyme also degrades $\beta(1\rightarrow4)$ -glycosidic bonds between the N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan [98]. Lysozyme exists in various human body fluids and tissues with concentrations from 4 to 13 mg/L in serum and from 450 to 1230 mg/L in human tears [99]. The rate of biodegradation of chitosan by the lysozyme is affected by the degree of deacetylation; initial degradation rates increase with a decreasing degree of deacetylation.

Some chitosan derivatives exhibit a faster degradation rate than chitosan, for example with the increasing of the molar ratio of glycolic acid to chitosan of poly(glycolic acid) grafted chitosan the rate of degradation gradually increased [100].

Chitin polyphorms exhibit different rates of degradation. Lysozyme degrades β -chitin more readily than α -chitin due to the weak intermolecular forces of the latter. In β -chitin the degree of deacetylation is decisive for the degradation behavior of chitin [101].

Chitinases are enzymes present in fungi, insects, and bacteria, these enzymes degrades chitin to oligosaccharides. In general, microbial endo-chitinases hydrolyze $\beta(1\rightarrow4)$ -glycosidic bonds randomly. Chitinases isolated from different organisms have widely different characteristics. In human the presence of chitinases is associated to allergic reactions. Human chitinases with enzymatic activity have been identified, but they have not been investigated with regards to the degradation of chitosan and/or its derivatives [52].

2.5. Toxicological properties

Biodistribution, in vivo and in vitro toxicity using various chitosans of different molecular weights and degrees of deacetylation and derivatives would provide data that could help correlate chitosan's structure and safety profile [48]. Some derivatives increase in toxicity and any residual reactants must be carefully removed [49]. In laboratory mice, the LD₅₀ of chitosan is similar to that of salt or sugar (16 g/kg of body weight) [50].

3. Drug carrier systems using chitosan intended to be used for non parenteral routes

A considerable amount of work has been published on chitosan and its potential use in drug delivery systems. In recent years considerable research has been focused on noninvasive routes, such as mucosal (oral, buccal, nasal, pulmonary and vaginal) and (trans)dermal. Chitosan has a cationic character because of its primary amino groups. These primary amino

groups are responsible for properties such as controlled drug release, mucoadhesion, in situ gellation, transfection, permeation enhancement, and efflux pump inhibitory properties.

The mucoadhesive properties are also based on its cationic character. The mucus gel layer exhibits anionic substructures in the form of sialic acid and sulfonic acid substructures. Based on ionic interactions between the cationic primary amino groups of chitosan and these anionic substructures of the mucus, mucoadhesion can be achieved. In addition, hydrophobic interactions might contribute to its mucoadhesive properties. Moreover, several studies have shown the effects of chitosan systems for the drug delivery. The molecule has been widely used in a variety of pharmaceutical multipurpose excipients capable of increasing aqueous solubility and drug stability [102].

3.1. Nasal delivery systems

Owing to nasal obstacles such as low membrane permeability, a short local residence time, and high turnover rate of a secretion in nasal cavities, the bioavailability of nasally administered drugs is often comparatively poor [103]. In order to overcome those problems, chitosan particles or polyelectrolyte complexes have been studied for nasal delivery of therapeutic proteins [104-106]. It was found that insulin loaded chitosan nanoparticles enhance nasal drug absorption to a greater extent than relevant chitosan solutions.

Chitosan has been also used recently via intranasal in many studies particularly in vaccines as a potent mucosal adjuvant. In a study, the matrix protein 1, which is highly conserved in all influenza A strains, was purified and used for immunization (twice at an interval of 3 weeks) of BALB/c mice by intranasal drip using chitosan as adjuvant to test the efficacy as vaccine. The results showed that nasal administration of 100 µg of the matrix protein 1 in combination with chitosan could not only completely protect the mice effectively against the homologous virus (H9N2) but also protect 70% and 30% of the mice against the heterologous H1N1 and H5N1 viruses, respectively, indicating that the matrix protein 1 is a candidate antigen for a broad-spectrum influenza virus vaccine and the adjuvant chitosan significantly improved the efficacy of the vaccine. This vaccine could provide effective protection against unknown influenza virus infection in future [107].

Microparticles (with suitable range for vaccine delivery) and gels using chitosans with different molecular weight and solubility as adjuvant/delivery system for mucosal (nasal) immunization against bovine herpes virus 1(BHV-1) showed that when the virus was incorporated into microparticles, the particle size was increased ($p < 0.05$). Narrower particle size distribution was obtained with water soluble chitosan compared to that of base chitosan particles at different molecular weights ($p < 0.05$). This difference can be attributed to difference in solubility of chitosans [108]. Similar results were reported in other studies [109-112]. With blank microparticles prepared with base chitosan at different molecular weights, the surface appearance and morphology were observed to be similar (with a smooth surface and spheroids). When the particles were loaded with the antigen; the surface appearance of the microparticles was changed with the increasing molecular weight of chitosan (Figure 2). These results suggest that antigen is entrapped within the microparticles, as well as associated with the surface of the microparticles [108]. Similarly, increased particle size and surface roughness

with antigen loading has been reported by other groups [113, 114]. These systems are promising adjuvant/delivery systems for non-invasive delivery of antigen tested as well as for other antigens.

Moreover, the nasal administration of vaccines can induce specific IgA antibody responses at distant mucosal sites, including the upper and lower airway mucosa and the small and large intestines, as well as the nasopharynx, salivary glands, genital tract, and tonsils, because of the dissemination of antigen-specific lymphocytes in the common mucosal immune system (immunocompetent cells in the body, such as M-cells, T-cell, B-cell, dendritic cells, and macrophages) [114-116].

3.2. Oral delivery systems

Chitosan-based formulations have been used for the delivery of drugs to specific sites of the body such as oral cavity, stomach, small intestine and colon. The site-specific delivery of the drug to the oral cavity can be used to treat a number of diseases of the mouth, such as stomatitis, periodontal disease, fungal and viral infections, and oral cavity cancers, thereby avoiding the first pass metabolism effect. In this sense, some points should be considered for buccal administration of drugs, such as maintain the device in its position for many hours against buccal motion and salivary flow, which could reduce the mucosal absorption. Consequently, the dosage form must have good adhesive properties and show an efficient control of drug delivery. Investigations have shown in several studies that drug release is influenced by swelling and erosion of the matrix, whereas matrix adhesiveness can be modulated using different mixtures of polymers, both adhesive and not. Here, investigations have reported that the chitosan has good mucoadhesiveness and a significant enhancing effect on the permeation of drugs across the buccal mucosa [117, 118]. The applications using chitosan include chlorhexidine loaded chitosan microparticles, which showed a determination effective of antibacterial activity of chitosan from thermosensitive hydrogel (with or without drugs) or as activator for the antibacterial process [119]. Chitosan microspheres based drug delivery is applicable for systemic as well as for local therapy. In case of oral drug delivery, the use of microspheres loaded with antibiotics would be beneficial for gastric diseases such as peptic ulcer [21], *Helicobacter pylori*, and intestinal infections, ulcerative colitis and carcinomas [20]. On the other hand, in many studies it has been demonstrated that chitosan-based formulations were superior in enhancing absorption of therapeutic proteins as well as induction of antibodies after mucosal vaccination [34-36].

With respect to the colon drug delivery, some microcrystalline cellulose core beads containing 5-aminosalicylic acid produced by extrusion-spheronization were coated with chitosan and Aquacoat® ECD mixtures. An adequate selection of the coating thickness and the chitosan level in the coat could minimize drug release in simulated intestinal fluid and provide zero order release. These products could also be used to achieve controlled release of drugs in the small intestine. Beads coated with chitosan/ Aquacoat® showed to be susceptible to the action of rat cecal and colonic enzymes and demonstrated their potential for colon specific drug delivery [120].

3.3. Ocular delivery systems

Various ophthalmic vehicles, such as inserts, ointment, suspensions, and aqueous gels, have been developed in order to lengthen the resident time of instilled dose and enhance the ophthalmic bioavailability. Chitosan based systems have the potential for improving the retention and biodistribution of drugs applied topically onto the eye. One of the pharmaceutical forms most investigated are the *in situ* gels, which have been developed to prolong the precorneal resident time of the drug and to improve ocular bioavailability [121-126]. The interaction and prolonged residence time of fluorescence-labelled nanoparticles prepared with chitosan was reported showing, these colloidal drug carriers remained attached to the cornea and the conjunctiva for at least 24 h [127]. Therefore, mucoadhesive chitosan nanoparticles may have potential as colloidal drug delivery systems for the ocular mucosa. It was demonstrated the potential of chitosan nanoparticles with cyclosporine A to improve the delivery of drugs to the ocular mucosa. Furthermore, chitosan-based colloidal systems were found to work as transmucosal drug carriers, either facilitating the transport of drugs to the inner eye or their accumulation into the corneal epithelia. The use of chitosan-based colloidal suspensions *in vivo* showed a significant increase in ocular drug bioavailability [128]. Additionally, bioadhesive chitosan microspheres have been also studied for ophthalmic administration, in which a high concentration of acyclovir for an extended period of time was obtained from *in vivo* ocular studies on rabbits. According to the authors, in addition to its mucoadhesive properties, chitosan is effective in retarding the rate of drug release [128].

Another interesting study reported chitosan microparticles to be administered via ophthalmic. The *in vitro* release kinetics of chitosan microparticles and their *in vitro* and *in vivo* biocompatibility and cytotoxicity on retinal cells were examined. The results showed that chitosan microparticles were effective to obtain long-term protein or drug delivery agents to the outer segment of the retina. Chitosan microparticles exhibited enhanced encapsulation capacity and a better release profile than the Polyethylene glycol-Polylactic acid (PEG-PLA) microparticles investigated previously. However, the concentration of chitosan microparticles may be critical to determine the extent of toxicity, and the concentration required will depend on the encapsulation efficiency and on the amount of protein (or drug) required to obtain a therapeutic dose [129].

3.4. Topical/transdermal delivery systems

Transdermal drug delivery systems can deliver drugs for systemic effects through skin at controlled rate (can be interrupted if it necessary), with the advantage of avoiding the first pass metabolism effect [130-134]. An example of this could be obtained with the lidocaine hydrochloride-loaded transdermal chitosan patches as a drug reservoir, which released the drug in a manner prolonged at 95% chitosan degree of deacetylation [135].

In a recent study, warfarin- β -cyclodextrin loaded chitosan nanoparticles for transdermal delivery were successfully prepared by ionic gelation method. Chitosan nanoparticles were found to be spherical, smooth and with narrow size distribution. They showed high drug entrapment efficiency and well accepted yield. The release profile from nanoparticles showed an initial burst effect followed by a slow and continuous release phase. The nanoparticle

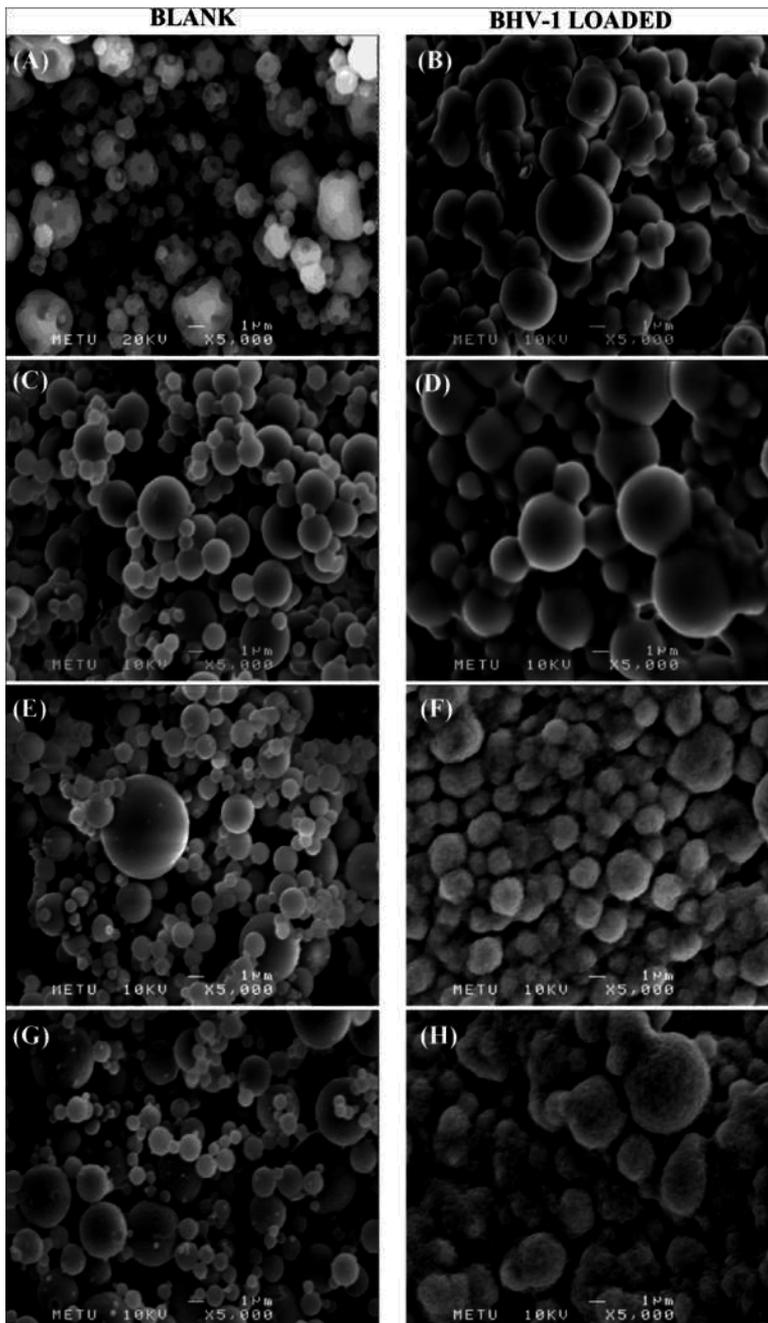


Figure 2. SEM micrographs of blank (A) and BHV-1 loaded microparticles (B) prepared with water soluble chitosan; chitosan base-low molecular weight (50-1000 kDa): blank (C), BHV-1 loaded microparticles (D); chitosan base-medium molecular weight (300-2000 kDa): blank (E), BHV-1 loaded microparticles (F); chitosan base-high molecular weight (500-5000): blank (G), and BHV-1 loaded microparticles (H). This figure is reproduced with the permission [108].

formulation enhanced the permeation of warfarin through excised rat skin in a constant and continuous profile. Therefore, it could be concluded that this formulation enhanced the controlled release and the permeation of warfarin, offering a promising system for the transdermal delivery [136].

3.5. Regenerative systems

In order to regenerate some loss or damaged tissue and organ, *in vitro* seeding and attachment of human cells onto a scaffold, followed by the culturing of the cells to form the new organ or tissue must to be performance to avoid some transplantation of them. Chitosan is one of the most exploited polymers for this application due to its known biocompatibility, biodegradability, antibacterial and wound-healing activities. Its use in tissue engineering stems from the capacity of chitosan (unmodified or as a derivative) to produce efficient scaffolds having desirable characteristics such as porosity, gel forming ability and high affinity to *in vivo* macromolecules [137]. Additionally, it has a structural similarity to glucosaminoglycans which are the major component of the extracellular matrix. Recent studies, attempted to address this issue through the conjugation of cell adhesion moieties to chitosan based materials, hence aiming to produce more physiologically relevant scaffolds that mimic of the extracellular matrix functions. In addition to provision of correct environment for cell growth and support, biodegradability of the scaffold material is also important. It is preferred that the materials are absorbed or excreted from the body without the necessity of surgical removal. The *in vitro* investigation of the responsiveness of articular chondrocyte-like cells using a multimembrane hydrogel of chitosan showed a large amount of cartilage-type matrix proteins were produced [138]. Chitosan has been extensively used in bone tissue engineering, since it was shown to promote cell growth and mineral rich matrix deposition by osteoblasts cells in culture [139]. Studies in order treat acute and chronic liver disease are also been investigated. Hepatoma HepG2 cells were seeded onto the microfluidic-based pure chitosan microfibers for liver tissue engineering applications without the use of any chemical additives. The results showed an aggregation of these cells forming spheroids, which also had higher liver function that was confirmed by albumin secretion and urea synthesis. This method represents a potentially useful tool for liver tissue engineering applications [140].

4. Strategies for the functionalization of chitosan systems using other components

Chitosan is a non-toxic, biocompatible and biodegradable polymer and has attracted considerable interest in a wide range of biomedical and pharmaceutical applications including drug delivery, cosmetics, and tissue engineering. The primary hydroxyl and amine groups located on the backbone of chitosan are responsible for the reactivity of the polymer and also act as sites for chemical modification. However, chitosan has certain limitations for use in controlled drug delivery and tissue engineering. These limitations can be overcome by chemical modification. In order to achieve high mucoadhesive properties, this polymer needs to exhibit also

high cohesive properties as the adhesive bond otherwise fails within the mucoadhesive polymer rather than between the mucus gel layer and the polymer. In case of chitosans, however, these cohesive properties are also comparatively weak. Although they can be strongly improved by the formation of complexes with multivalent anionic drugs, multivalent anionic polymeric excipients, and multivalent inorganic anions, this strategy is only to a quite limited extent effective, as the cationic substructures of chitosan being responsible for mucoadhesion via ionic interactions with the mucus are in this way blocked. The combination of chitosan with other materials appears to be a common theme in various reports. Blending with other polymers is widely investigated. Blends with synthetic and natural polymers can imbibe the wide range of physicochemical properties and processing techniques of synthetic polymers as well as the biocompatibility and biological interactions of natural polymers.

A significantly improved oral bioavailability of buserelin was demonstrated with mucoadhesive polymers such as chitosan and carbomer to rats. This effect, however, could not be observed anymore when chitosan was combined with the polyanionic carbomer in the same formulation [141]. Trimethylation of the primary amino group of chitosan provides an even more cationic character of the polymer. When trimethylated chitosan is additionally PEGylated, its mucoadhesive properties are even up to 3.4-fold improved [142]. Due to the immobilization of thiol groups on chitosan, its mucoadhesive properties can also be strongly improved, as the thiolated polymer is capable of forming disulfide bonds with mucus glycoproteins of the mucus gel layer, placing it among the most mucoadhesive polymers known so far [143]. In addition, as inter- and intrachain disulfide bonds are also formed within chitosan itself, thiolated chitosan exhibits substantially improved cohesive properties. Recently, the mucoadhesive properties of thiolated chitosans were even significantly further improved by the preactivation of thiol groups on chitosan via the formation of disulfide bonds with mercaptanocotinamide.

4.1. Oral drug delivery

Chitosan, the second most abundant polysaccharide next to cellulose, has been adopted as having great potential application as a protein drug carrier for oral administration due to its outstanding properties of non-toxicity, biocompatibility, biodegradability and low cost [144, 145]. Oral administration of drugs represents the easiest and the most convenient route of drug delivery. Therefore, the enhancement of oral bioavailability of some drugs particularly those with poor aqueous solubility, is gaining increasing attention for successful development of oral treatment. Chitosan based hydrogel systems can be designed to deliver drugs locally to the stomach or the upper part of tract to improve bioavailability. It was tested by confocal laser scanning microscopy that amoxicillin loaded pH-sensitive hydrogels composed of chitosan and poly(γ -glutamic acid) could be infiltrated in the cell-cell junctions and interact with *Helicobacter pylori* infection sites for the treatment of peptic ulcer [146]. Hydrogels of chitosan and polyacrylic acids containing amoxicillin and clarithromycin showed similar results. Modified chitosan hydrogels loaded with metronidazole, tetracycline and theophylline could bypass the acidic environment of the stomach and release the loaded drug into the intestine [147].

Another investigation reported that the oral bioavailability of acyclovir could be improved 3-fold and 4-fold due to the incorporation of this drug in chitosan and thiolated chitosan, respectively. Within this study, a prolonged residence time in particular of thiolated chitosan microparticles in duodenal and jejunum regions was observed. These data need to be confirmed in human volunteers. So far, an improved oral bioavailability of various model drugs could be shown in human volunteers for mucoadhesive formulations likely because of an intimate contact of the delivery system with the absorption membrane and a prolonged mucosal residence time of the delivery systems [148].

Several systems have been proposed to encapsulate insulin to improve oral insulin bioavailability, including polymeric hydrogels polymeric solid nano-particles and liposome-based carriers [149-153]. However, limitations related to the enzymatic degradation in the gastrointestinal tract and the low permeability across the intestinal epithelium are common problems in those systems [154, 155].

Contrarily, it was reported that insulin analog can be successfully encapsulated in chitosan microspheres with a high loading content. The quaternized groups on N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) can protect the insulin analog from the cross-linking reaction and maintain its activity. The positive charged chitosan microspheres also showed improved bioadhesion to the intestinal tract due to a strong interaction with the mucus. Evaluation using an *in vivo* diabetic model showed an optimal reduction in blood glucose level and compelling therapeutic effects after treatment with insulin analog loaded chitosan microspheres, which further confirmed the feasibility of using quaternized chitosan microspheres as insulin carriers for oral administration [156].

In case of oral drug delivery, the use of microspheres loaded with antibiotics would be beneficial for several diseases [157]. A particular problem related to a low molecular weight compound like ampicillin is the high permeability of the chitosan microbead matrix material. In order to overcome these restrictions, chitosan gel beads and microspheres are generally crosslinked chemically using glutaraldehyde or ethylene glycol diglycidyl ether [157]. However, residual glutaraldehyde and ethylene glycol diglycidyl ether in the chitosan microspheres give rise to health concerns and can cause undesirable effects including irritation to mucosal membranes. To solve these disadvantages of chemical crosslinking, researchers have proposed to apply chitosan microspheres reacylated with acetic anhydride. The reacylated chitosan microspheres were able to interact closely with the gastric mucosa and to exhibit sustained delivery of entrapped antibiotic [158].

The factors affecting drug encapsulation efficiency, particle size, surface charge, surface hydrophilicity, pharmacokinetics and biodistribution were studied in clozapine-loaded nanoparticles coated with chitosan, pluronic F-68, polyethylene glycol (PEG) 4000 and polysorbate 80. The results proved that although a similarity in surface hydrophilicity, chitosan-stealth nanoparticles showed different pharmacokinetic profile and biodistribution behavior compared to polysorbate-stealth nanoparticles [159]. A great improvement in surface hydrophilicity was brought by chitosan and polysorbate 80 coatings. However, the *in vivo*

particle uptake by the reticuloendothelial system was less pronounced with positively charged chitosan-stealth nanoparticles than with polysorbate 80. In another study, the thermal amide conjugation of COO⁻ group of EDTA with NH₂ group of chitosan was employed to prepare microparticles, which showed higher amphotericin B loading capacity, enhancement in the in vitro dissolution performance 12-fold and a nanoemulsion was produced in the size range of 70–90 nm [160]. On the other hand, microparticles were prepared entrapping ovalbumin as a model antigen following oral vaccination. In another investigation, methylated N-(4-N, N-dimethylaminocinnamyl) chitosan was used to coat microparticles, which demonstrated a greater swelling, mucoadhesive properties and a more sustained release than uncoated microparticles. Thus, this formulation represent a useful carrier to improve the immunogenicity of oral vaccines [161].

Galactosylated trimethyl chitosan-cysteine nanoparticles were developed for oral delivery of a *mitogen-activated protein kinase kinase kinase kinase 4*, siRNA to the activated macrophages. This formulation was effective in protecting mice from dextran sulfate sodium induced ulcerative colitis at a relatively low therapeutic dose by attenuating colonic TNF- α production. Additionally, a stability enhanced, cell binding and cellular uptake in activated macrophages, low cytotoxicity, high transfection efficiency in vitro, and direct delivery to the focus of disease were showed [162].

4.2. Buccal drug delivery

The buccal route is an alternative choice to deliver drugs to the application site. In addition, this route shows high acceptance by patients. An ideal buccal delivery system should stay in the oral cavity for hours and release the drug in a controlled way. Mucoadhesive polymers prolong the residence time of the drug in the oral cavity [163]. Based on its mucoadhesive as well as absorption enhancement properties, chitosan is a promising polymer to be used for buccal delivery, such as chitosan mixed with sodium alginate, which was studied as a vehicle in buccal tablets; while chitosan glutamate, interacted with polycarbophil and other anionic polymers and was proposed for bilaminated films and bilayered tablets [164, 165].

Chitosan salts have different physical properties and can have different effects on mucosa permeability [166, 167]. A combination of chitosan and Pluronic F-127 was investigated in one study, showing that the drug release systems for via buccal was improved and demonstrating that independently of chitosan salt type (citrate, acetate and lactate), mucoadhesion was significantly favoured when the concentration of Pluronic F-127 in the matrix was about 30% (w/w). Chitosan lactate gave good sustained release, controlled swelling, and higher mucoadhesion when combined with Pluronic F-127 present in the matrix at the above concentration (Figure 3). These results indicate that such a matrix could find useful application in buccal drug delivery systems [168].

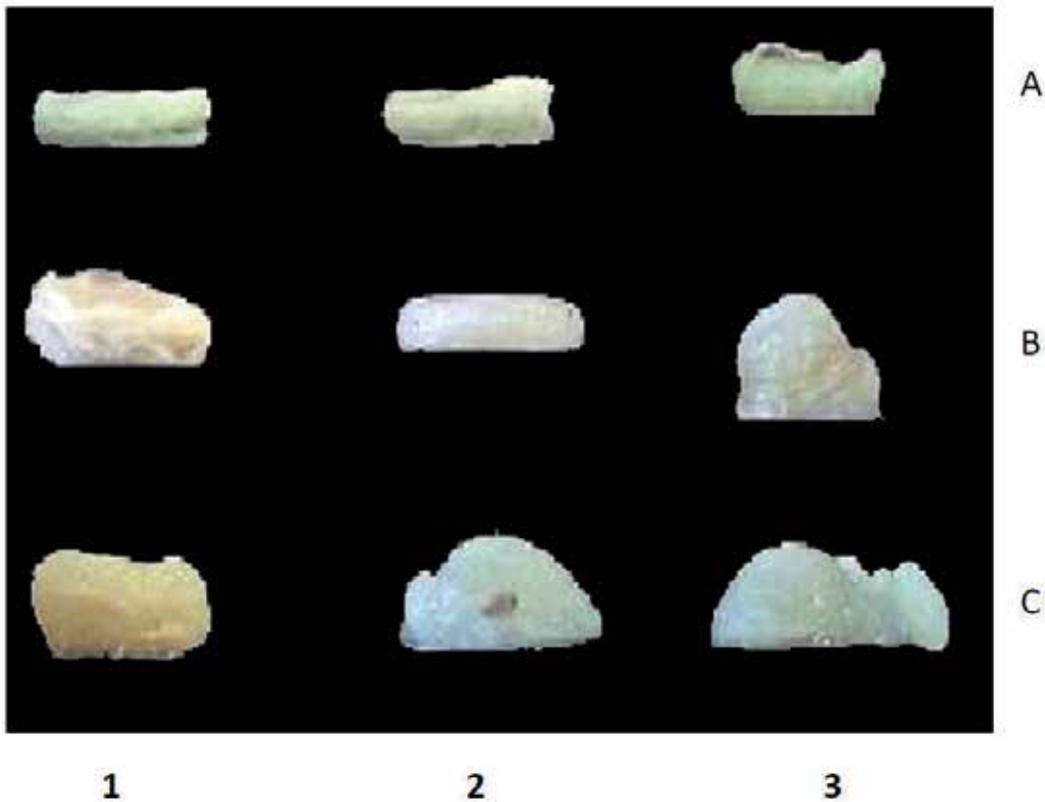


Figure 3. Pictures of swollen matrices, containing different chitosan salt type (1—chitosan acetate; 2—chitosan citrate; 3—chitosan lactate) and different amounts of P407 [A—at about 50% (w/w); B—at about 30% (w/w); C—at about 12% (w/w)]. The images were taken 4 h after starting the swelling study. This figure is reproduced with the permission [168].

Other modifications such as, trimethylated chitosans seem to be promising excipients for drug delivery systems intended for buccal mucosa applications to enhance the absorption of hydrophilic macromolecules [169]. In another study, the potential of thiolated chitosan for peptide delivery systems via the buccal mucosa was investigated in pigs [170]. The therapeutic peptide PACAP was applied to pigs, and its bioavailability was determined in order to facilitate the treatment of type 2 diabetes. Due to its strong permeation enhancing properties, tablets based on thiolated chitosan raised continuously the plasma level of this peptide drug, allowing for therapeutic range levels to be maintained over the whole period of application. Furthermore, buccal bilayered devices with a mixture of nifedipine and propranolol as well as chitosan displayed promising potential for use in controlled delivery in the oral cavity [171].

Chitosan (derivatives) can interact with mucus and epithelial cells and induced a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1 resulting in opening of cellular tight junctions and increasing the paracellular permeability of the epithelium [172, 173]. Besides their charge, other structural elements of these polymers likely contribute to their penetration-

enhancing activity, since cationic polysaccharides such as quaternized diethyl aminoethyl-dextran were ineffective as an enhancer.

4.3. Colon drug delivery

Colon specific drug delivery systems are gaining importance for use in the treatment of chronic diseases, such as irritable bowel syndrome, inflammatory bowel disease, ulcerative colitis, and also for the systemic delivery of protein and peptide drugs. By making use of this colon-specific degradation, chitosan has been discovered as useful coating in order to guarantee a site specific delivery. Radiolabelled (^{99m}Tc) tablets coated with a combination of pectin/chitosan/hydroxypropyl methylcellulose (3 + 1 + 1), for instance, were administered orally to human volunteers [174]. Within this study, gamma scintigraphy was used to evaluate the gastrointestinal transit of these tablets, showing that they remain intact through the stomach and small intestine. In the colon, the bacteria degraded the coat, and thus, the tablets disintegrated. In another study, it was developed a sustained dosage form for alpha-lipoic acid making use of ionic interactions between this anionic drug and chitosan used as carrier matrix. Studies in human volunteers showed a release maximum once the formulation had reached the colon [175].

Chitosan hydrogel beads coated with enteric polymer Eudragit S 100 were also investigated to be targeted to the colon; they prevented premature drug release in simulated gastric fluid, but delivered in the colon, because chitosan was degraded by the bacterial enzymes. Prednisolone, 5-aminosalicylic acid, metronidazole, 5-fluorouracil and indomethacin are being investigated in chitosan formulations for the same purposes [176].

Mixes are being used to obtain some characteristics on the formulations. Here, blended chitosan with gelatin was used to improve the biological activity since (i) gelatin contains Arg-Gly-Asp (RGD)-like sequence that promotes cell adhesion and migration, and (ii) forms a polyelectrolyte complex [177]. Addition of gelatin affected the stiffness of 2D and 3D scaffolds, facilitated the degradation rate and maintained the dimension in the presence of lysozyme. The effect of blending chitosan with poly(ϵ -caprolactone) (PCL) also improved mechanical properties as well as cellular support [178]. The γ -poly (glutamic acid), a hydrophilic and biodegradable polymer, was also used to modify chitosan matrices and the γ -poly (glutamic acid)/chitosan composite matrix was found to enhance hydrophilicity and serum proteins adsorption, and to increase the maximum strength through addition of γ -poly (glutamic acid) in tissue engineering applications [179]. The mechanical properties and biocompatibility also were improved with galactosylated chitosan-based scaffolds by combining them with alginate. The scaffolds exhibited the usual pore configurations, and the pore sizes were dependent on the freezing pre-treatments, the molecular weight of chitosan and amount of galactosylated chitosan [180, 181].

4.4. Pulmonary drug delivery

Powder formulations of protein-loaded chitosan nanoparticles suitable for pulmonary delivery have been prepared by spray drying [182-184]. Moreover, insulin-loaded nanoparticles chitosan have been reported obtaining a good loading capacity (65–80%) and were fully

recovered from the powder formulations after contact with an aqueous medium, and showed a fast release of insulin. The biocompatibility and penetration-enhancing effects of their chitosan powder formulations were examined *in vitro* using A549 and Calu-3 cells as models for alveolar and respiratory epithelial cells, respectively [185]. The formulations exhibited a very low cytotoxicity in both cell lines, but no effects on opening of tight junctions of the cells were reported. Further, CLSM studies did not reveal internalization of nanoparticles which contrasts previously reported studies [183]. The authors speculated that the total amount of chitosan used in their study was lower than that used in other publications. Moreover, the chitosan salt (glutamate) probably did not lose its charge after dispersing the particles in buffer [183].

An inhalable chitosan-based powder formulation of salmon calcitonin-containing mannitol as a cryoprotecting agent using a spray drying process was prepared. The effect of chitosan on the physicochemical stability of the protein was examined with chromatographic and spectrometric techniques [186]. The dissolution rate of the protein decreased when it was formulated with chitosan, which might be due to an irreversible complex formation between the protein and chitosan during the drying process [183]. On the other hand, chitosan-coated PLGA nanoparticle suspensions improved the absorption of calcitonin after pulmonary administration aerosolized with a nebulizer. The elimination of the chitosan-coated nanoparticles from the lungs was retarded as compared to non-coated particles, most likely due to the mucoadhesive properties of chitosan. It was shown that after pulmonary administration of the chitosan-coated particles the pharmacological action of calcitonin was prolonged as compared to that of the protein loaded in the non-coated nanoparticles [187]. In another study, the potential of chitosan oligomers and polymers for pulmonary delivery of proteins was studied. The absorption of interferon- α in rats was improved after pulmonary administration of aqueous solutions of the oligomers and the interferon- α . Among various oligomers, glucoseamine hexamers at a concentration of 0.5% (w/v) showed the highest efficacy. Chitosan polymers were less efficient than the studied oligomers in increasing the systemic level of the interferon- α , likely due to their lower solubility in lung fluids [188].

Interesting results were obtained with N-trimethyl chitosan powder formulations of insulin using a drying process for pulmonary delivery [189]. The particles had an average volume aerodynamic diameter of 4 μm suitable for peripherally pulmonary deposition. After one-year storage at 4 $^{\circ}\text{C}$, the particle characteristics were maintained and the insulin structure was largely preserved [189]. Pulmonary administration of N-trimethyl chitosan-insulin microparticles significantly enhanced the systemic absorption of insulin, with a bioavailability of about 95% relative to subcutaneously administered insulin.

It has been studied the development of a novel nanocarrier consisting of Lipoid S100 and chitosan or glycol-chitosan for the systemic delivery of low molecular weight heparin upon pulmonary administration. These nanosystems, formed by ionic gelation technique, provided both sufficient entrapment efficiency and mucoadhesive properties. Aerosolization of these formulations indicated that heparin could be delivered to the lung. Overall, these nanocarriers might have a use potential for systemic delivery of low molecular weight heparin as compared to free drug with a therapeutic potential effect for the treatment of pulmonary embolism and

other thromboembolic disorders [190]. The potential to deliver ofloxacin directly to alveolar macrophages via the respiratory route was enhanced from loaded glutaraldehyde-crosslinked chitosan microspheres in comparison with the ofloxacin powder. It suggests that chitosan microspheres are efficient delivery system of ofloxacin to cure tuberculosis [191].

4.5. Intranasal drug delivery

Chitosan-coated gold nanoparticles have been investigated for mucosal protein delivery [192]. Chitosan was used as a reducing agent in the synthesis of gold nanoparticles and also as a mucoadhesive and penetration enhancer. Insulin was efficiently adsorbed (~50%) through electrostatic interaction onto the surface of the coated nanoparticles and they were colloiddally stable for 6 months. Intranasal administration of these nanoparticles in diabetic rats showed an improved pharmacodynamic effect as evidenced by higher reduction in blood glucose levels as compared to insulin-loaded sodium borohydride gold nanoparticles [192].

In another study nanoparticles consisting of chitosan and negatively charged cyclodextrin sulfobutylether- β -CD or carboxymethyl- β -CD derivatives were prepared and characterized [104]. It was demonstrated that chitosan-sulfobutylether- β -CD-insulin nanoparticles induced lower TEER values of Calu-3 cells than chitosan-carboxymethyl- β -CD-insulin nanoparticles. However, both insulin-loaded nanoparticles showed similar effects on reduction of rats' plasma glucose levels upon intranasal administrations. It should be noted that, the plasma insulin concentrations of the treated animals which may give better indications in absorption enhancement properties of the formulations, were not determined [104]. Chitosan has been investigated as auxiliary agent in nasal drug delivery systems [193] due to mucoadhesive properties improving significantly nasal uptake of isosorbide dinitrate due to the co-administration of chitosan in rats [194]. In the same study, they showed a minor cilio-inhibiting effect of the polymer. In addition, fentanyl nasal spray formulations with pectin, chitosan, and chitosan-poloxamer 188 were developed for clinical evaluation to provide rapid absorption and subsequently increased bioavailability. The study was conducted in 18 healthy adult volunteers and revealed significantly increased systemic exposure as well as reduced times to peak plasma values for all formulations compared with oral transmucosal fentanyl citrate lozenge [195].

Recently, formulations prepared with chitosan and Pluronic F-127 as nasal delivery vehicles of vaccines have been reported [116, 196, 197]. In a study, some mice Balb/c mice were intranasally immunized with the antigen tetanus toxoid in the presence of chitosan, Pluronic F-127/chitosan or lysophosphatidylcholine (LPC) showing that the antigen specific IgA response in the nasal and lung washes of these animals had a significant increase in anti-tetanus toxoid mucosal IgA response in the group of mice immunized and boosted intranasally with Pluronic F-127/chitosan, enhancing the systemic and mucosal immune responses compared with those in the control groups. Successful mucosal vaccination is therefore largely dependent on the development of effective mucosal adjuvants. As it is well known, the adaptive humoral immune defense at the mucosa is mediated by the antibodies IgA that in mucosal secretions binds to the microbes and toxins present in the lumen and neutralize them by blocking their entry into the host [198, 199]. This nasal vaccine could induce not only

systemic IgG antibody responses but also mucosal IgA antibody responses, which results in two layers of immune defense against infectious diseases. So, this study showed that the system represents a novel nasal vaccine delivery system to enhance immune response [200]. Important effects in the nasal delivery of vaccines were found, when the *Bordetella bronchiseptica* antigen was included in the chitosan microspheres prepared with Pluronic F-127, which was used as a stabilizing and immunomodulating agent [115]. In the in-vitro release study, a greater amount of the antigen was released from the chitosan microspheres prepared in the presence of Pluronic F-127 than from only chitosan microspheres, due to the hydrophilic property of poloxamer. The mice intranasally immunized with the microspheres using Pluronic F-127, showed higher IgA antibody titers against the antigen in their mucosal secretions (nasal washes and saliva) than the mice intranasally immunized with microspheres containing only the antigen. This study suggested that chitosan microspheres prepared in the presence of Pluronic F-127, could enhance the nasal delivery of a variety of clinically useful antigens in vaccination schemes. Besides the enhanced immune responses, advantages of this system include the easy preparation method, which only involved simple mixing by the ionic gelation method with tripolyphosphate or by the emulsion-crosslinking method employing glutaraldehyde avoiding extreme conditions such as heating and organic solvent which might result in the denaturation of protein antigens [115, 201].

Another objective was obtained with leucine-enkephalin loaded N-trimethyl chitosan nanoparticles, which were evaluated as a brain delivery vehicle via nasal route and prepared by ionic gelation method. The permeability of Leucine-enkephalin released from nanoparticles was 35 fold improved from the nasal mucosa as compared to Leucine-enkephalin solution. Fluorescent microscopy studies of brain sections of mice showed higher accumulation of fluorescent marker NBD-F labelled Leucine-enkephalin, when administered nasally by N-trimethyl chitosan nanoparticles, while low brain uptake of marker solution was observed. It was concluded that N-trimethyl chitosan nanoparticles could generate a significant improvement of bioactive Leucine-enkephalin levels in the brain when is intranasally administered [203].

4.6. Ocular drug delivery

Chitosan is a suitable material for the design of ocular drug delivery systems due to its nontoxic character, permeation enhancing properties, and physicochemical characteristics. Chitosan-based formulations used for ophthalmic drug delivery are hydrogels [203], nanoparticles [127], and coated colloidal systems [204]. Chitosan as well as Pluronic F-127, has recently been proposed as a material with a good potential for ocular drug delivery, since their solutions were found to prolong the corneal residence time of antibiotic drugs and nanocapsules coated with chitosan were more efficient to enhance the intraocular penetration of some specific drugs [205]. Making use of their in situ gelling properties, the formulations can be applied and distributed on the ocular surface in almost liquid form thereafter transforming into the gel status [203]. A combination of polycaprolactone nanocapsules as ocular carriers with the advantages of the cationic mucoadhesive chitosan and poly-L-lysine as coating was performed. Even though poly-L-lysine and chitosan displayed a similar positive surface charge,

only chitosan-coated nanocapsules enhanced the ocular penetration of indomethacin with respect to uncoated nanocapsules. The authors suggested that an undetermined property of chitosan was responsible for this enhanced uptake [204].

In other studies chitosan-alginate microspheres or beads were investigated for the encapsulation of several drugs, proteins, cells and oligonucleotides, with promising results [206-211]. The complex has biocompatible and biodegradable characteristics, and limits the release of encapsulated materials more effectively than either alginate or chitosan alone [212]. A further advantage of this delivery system is its non-toxicity permitting the repeated administration of therapeutic agents. In another study, chitosan-sodium alginate nanoparticles entrapping gatifloxacin (a broad-spectrum antibacterial agent used in the treatment of ocular infections) were successfully formulated. The results showed that the drug was released over a period of 24 hours in a sustained release manner, primarily by non-Fickian diffusion. This new formulation is a viable alternative to conventional eye drops by virtue of its ability to sustain the drug release, for its ease of administration because of reduced dosing frequency resulting in better patient compliance [213].

4.7. Topical/transdermal delivery systems

Some mixes of polymers using chitosan could be used to prepare thermosensitive hydrogels which were a good choice to reduce local irritation in the skin caused by conventional transdermal patches (40 % of application-site skin reaction) due to components of the patch (acrylic adhesive, polyester, polyurethane, and silicone). Therefore, these formulations can also provide advantages for particular applications as it is transformed from a liquid to a gel when administered topically [214]. Additionally, modifications in the delivery of drugs could be achieved, such as that obtained for a hydrogel patch composed of chitosan and starch developed for cosmetic applications, in which a rapid curcumin release rate was observed [215].

4.8. Regenerative systems

Amino acid grafted chitosans possess a great potential for application in these biomedical fields, whereby a combination of the properties of chitosan and those belonging to different amino acid moieties could produce materials with synergetic properties. Moreover, this conjugation can enhance some properties of chitosan, such as its antimicrobial activity, which are important in the area of tissue engineering such as wound healing [216]. Many tissue analogs including cartilage, bone, liver, and nerve have been prepared using this engineering technology. Systems of blood clots based in chitosan-glycerol phosphate disodium salt were purposed since bleeding has been identified as an initiating event in post-surgical repair and it was hypothesized that microfracture-based repair could be improved by stabilizing the clot formed in the lesion with chitosan that is thrombogenic and actively stimulates the wound-healing process [217]. These systems were applied as implants to marrow-stimulated chondral defects in rabbit cartilage repair models, where they induced greater fill of chondral defects with repair of tissue compared to marrow-stimulation alone [218]. In another investigation, a chitosan-hydroxyapatite multilayer nanocomposite with high strength and bending modulus rendering the material suitable was prepared for possible application as an internal fixation of long bone

fractures [219]. A series of chitosan-tricalcium phosphate composite scaffolds were developed for the same purpose using freeze-drying process, which provided macroporous composite scaffolds with different pore structures. The biocompatibility, evaluated subcutaneously on rabbits indicated that these scaffolds can be utilized in non-loading bone regeneration [220]. The use of biomimetic hydroxyapatite/chitosan–gelatin network composites in the form of 3D-porous scaffolds improved adhesion, proliferation and expression of rat calvaria osteoblasts on these systems [221]. Recently, a scaffold with calcium phosphate cement and chitosan fibers with improved resistance to fatigue and fracture was used to harvest human umbilical cord mesenchymal stem cells without an invasive procedure that is commonly required when studying bone marrow mesenchymal stem cells. This system had flexural strength of 26 MPa, while calcium phosphate cement control was 10 MPa. In addition, an excellent and higher viability of human umbilical cord mesenchymal stem cells was obtained with scaffolds using chitosan fibers than those controls without fibers. Human umbilical cord mesenchymal stem cells had excellent proliferation (300 and 700 cells/mm² on 1 and 4 day, respectively) and viability on the scaffolds [222]. A study showed that the chitosan surface modified with fructose (ligand of asialo-glycoprotein receptor in hepatocyte) on porous chitosan scaffolds induced the formation of cellular aggregates and enhanced liver specific metabolic activities and cell density to a satisfactory level [223, 224]. Chitosan microfibers were also developed and coated with collagen. Schwann and fibroblast cells were cultured on the chitosan microfibers to be adhered to the surface of the systems. After 72 h, the Schwann cells had proliferated linearly while the fibroblast cells covered the surface of the chitosan microfibers. The chitosan microfibers provide very good scaffolds for many tissue engineering applications with the advantages of ease of fabrication, simplicity and cost effectiveness [225].

5. Uptake and endocytic pathway of chitosan

One of the most important routes to drug delivery is the oral pathway. The uptake of chitosan into the bloodstream is generally not investigated in oral administration studies. Chitosan's systemic absorption and distribution from this route of delivery may be largely dependent on the Mw. It is very likely that oligomers could show some absorption whereas larger Mw chitosans are excreted without being absorbed. This effect was seen with FITC-labeled chitosans with 3.8 kDa (88.4% degree of deacetylation) chitosan having the greatest plasma concentration after oral administration vs 230 kDa (84.9% degree of deacetylation) having almost no uptake. Increasing Mw was seen to decrease the plasma concentration in this, one of the only studies investigating plasma concentration after oral administration [226]. Trimethyl chitosan oligomers/DNA nanoparticles were taken up in the gastric and duodenal mucosa and to some extent in the jejunum mucosa, ileal mucosa and large intestinal mucosal cells as shown by green fluorescent protein (GFP) expression [227]. Chitosan polymers are not absorbed by the gastrointestinal way and are unlikely to show biodistribution. Chitosan oligosaccharides however may be absorbed to some extent.

Although native chitosan has not been investigated, the intracellular uptake and distribution of chitosan/DNA complexes have been studied *in vitro* [228–230]. Chitosan polyplex uptake

at 37 °C was 3-fold higher than at 4 °C [228] but this could be due to increased interaction and not an ATP dependent endocytic mechanism. The authors suggested nuclear localization and they also stated little dissociation of the DNA from the chitosan. In a more comprehensive study, Leong et al. stained for lysosomes and found some co-localization with chitosan DNA nanoparticles. However, the majority of the polyplexes were found in the cytosol [229]. A complex of doxorubicin with chitosan has also been studied; complexes enter cells through an endocytic mechanism which was not further elucidated [231]. Hydrophobic (5- β -cholanic acid) modified glycol chitosan nanoparticles were internalized into HeLa cells through all the endocytic mechanisms studied: clathrin coated vesicles, caveolae and macropinocytosis. This study agrees with that of Leong, in that some particles were lysosomal but most were not [232]. Unfortunately, these studies all involve nanoparticle uptake of relatively large (>100 nm) nanoparticles or aggregates of complexes and not just labeled chitosan. Dodane and Vilivalem reported that chitosan has membrane perturbing properties that do not decrease cell viability [233]. It is likely that chitosan and chitosan nanoparticles enter the cell via cell membrane perturbation due to the cationic charge. It is important to understand chitosan's cell trafficking and investigate both endocytosis and exocytosis. Such study should shed some light on chitosan's biocompatibility.

It is important to mention that cellular uptake kinetics may be altered due to the charge interaction (e. g. in the case of DNA complexes). This balancing, or reduction, of the positive charges on the chitosan molecule has effects on its interaction with cells and the microenvironment, often leading to decreased uptake and a decrease in toxicity. In the case of a covalent drug conjugate, the polymer's physicochemical properties (hydrophilicity) and conformation are altered (i. e. micelle formation) with a consequent effect on distribution and cell uptake [52]. Similar results were found for poly(dl-lactic-co-glycolic acid) nanospheres surface modified by adsorption of chitosan for pulmonary administration, which were preferentially taken up by human lung adenocarcinoma cells (A549) in a temperature dependent manner [234]. Moreover, cellular uptake of these nanocarriers increased with decreasing diameter to the submicron level and the cellular uptake of nanospheres were promoted through electrostatic interactions between the surface due to chitosan adsorbed and the negatively charged cell membrane without showing cytotoxicity. Internalization of nanospheres (200-nm) by A549 cells appears to occur predominantly through adsorptive endocytosis initiated by nonspecific interactions between nanospheres and cell membranes, and is partially mediated by a clathrin-mediated process. Thus, chitosan is suitable as a material for surface modification of systems for intracellular targeting because it could increase the interaction between the cell membrane and the systems [234]. The effect on the charge of the systems was also reported for a large array of N-(2-hydroxypropyl)methacrylamide (HPMA) based copolymers, which were internalized into the prostate cancer cells through multiple endocytic pathways: positively charged copolymers robustly engaged clathrin-mediated endocytosis, macropinocytosis and dynamin-dependent endocytosis, while weakly negatively charged copolymers weakly employed these pathways; strongly negatively charged copolymers only mobilized macropinocytosis [235].

The hydrophobicity of the systems as drug delivery vehicles for therapeutic applications is another physicochemical property which has been investigated on the cellular uptake. Here, a hydrophobic glycol chitosan system (5 β -cholanic-acid conjugated glycol chitosan) was reported to show several distinct uptake pathways involved in their internalization with a single degree of substitution [232]. Moreover, different degree of substitution (or hydrophobicity) could affect the endocytosis of hydrophobically-modified polymers. The cellular uptake of nanoparticles prepared by a hydrophobically-modified chitosan (N-palmitoyl chitosan), was significantly enhanced with increasing the degree of substitution. The internalization of these systems was clearly related with the lipid raft-mediated routes. With increasing the hydrophobicity on polymer, the caveolae-mediated endocytosis became more important. The internalized nanoparticles transiently associate with CAV1 at cell membranes and at a peripheral CAV1- positive structure coupled with caveosomes before trafficking to the endosomal pathway [236].

In addition, the cell entry and subsequent intracellular trafficking of drug carriers using chitosan or a combination with another polymer are strongly dependent on their physicochemical characteristics, such as charge and molecular weight. In addition, the route of administration determines the uptake, concentration, contact time and cell types affected [52].

6. Advantages and limitations over other pharmaceutical systems for non parenteral routes

6.1. Advantages

Chitosan is one of the most promising polymers because of its nontoxic, polycationic, biocompatible, and biodegradable nature and particularly due to its mucoadhesive and permeation-enhancing properties [237-239]. Moreover, the strong mucoadhesive property of chitosan is most important for drug delivery through the mucosal routes. In addition, the interaction of the positively charged chitosan with the negatively charged mucin layer and the tight junctions facilitates the paracellular transport of hydrophilic macromolecules by opening the tight junctions of the mucosal barriers [111, 239-242]. Additionally, chitosan is cheap [243]. Practical use of chitosan has been widely investigated due to its ability to form hydrogels, to its biocompatibility in physiological environments (enzymes chitosanase and lysozyme degrade chitosan and form harmless products), enhancing it with deacetylation reactions. Moreover, its biocompatibility was demonstrated with viable cartilage producing any untoward effect [216].

One of many investigations using chitosan is as vaccines vehicles, which have showed better efficacy than the approved injectables (induce strong systemic immune responses and represent a pharmaceutical form painful). Diverse chitosan microspheres have been evaluated for controlled drug release and to enhance the protection and permeation of the antigens in the nasal mucosa, inducing antigen specific immune responses in both the nasal mucosa and the systemic compartment [114, 115, 196, 197]. Besides, the induction site of the antigen-specific

mucosal immune responses were found contained in a broad range of mucosal surfaces (nasal and vaginal routes and the salivary gland).

Several studies have been conducted on chemically modified chitosan systems through their concomitant use with adjuvants for a synergistic effect, and through the mannosylation of chitosan for target the receptor-mediated. The chemically modified chitosan systems combined with other adjuvants have showed to have an increased immunostimulatory in nasal vaccine delivery [114, 115]. Another advantage of chitosan formulations is its cationic property, which has been also exploited to deliver a tissue plasminogen activator to substrates of the fibrin network or insulin to mucosa surfaces [244, 245]. Nasal delivery of insulin from solutions and gels based of chitosan incremented greatly the permeability and transmucosal absorption, which could avoid the pain and inconvenience of injections of insulin in patients [246, 247]. Moreover, *in vitro* insulin delivery using gels of chitosan, glutaraldehyde, Pluronic F-127/F-68 and glycine significantly reduced the burst release of insulin and sustained the release of it for up to 20 h, suggesting that such gels are potentially effective carriers of insulin for nasal delivery [247].

Systems based in chitosan can provide advantages by their mucoadhesive properties overcome to the conventional formulations such as those for via ophthalmic, that are eliminated from the precorneal area immediately upon instillation because of lacrimal secretion and nasolacrimal drainage, needing a frequent instillation of concentrated solutions to achieve the desired therapeutic effects [248, 249]. In order to lengthen the resident time of instilled dose and enhance the ophthalmic bioavailability various conventional and non conventional (colloidal drug delivery systems, such as liposomes, biodegradable nanoparticles and nanocapsules) ophthalmic vehicles based in chitosan have been developed, but their use is reduced due to some adverse effects such as blurred vision from ointment or low patient compliance from inserts [250]. In order to avoid the blurred vision, a combination of chitosan and Pluronic F-127 could be used for the preparation of *in situ* forming gels with improved mechanical and mucoadhesive characteristics for prolonged precorneal residence time *in vivo* [251].

Mucoadhesive properties are also important for the buccal administration, which involve the direct entry of the drug into the systemic circulation avoiding the first pass hepatic metabolism. This route is easily accessible for self medication where the drug can be easily administered or if necessary, removed from the site of application. In order to maintain the device in its position for many hours against buccal motion and salivary flow, which could reduce the mucosal absorption, the dosage form must have good adhesive properties and show an efficient control of drug delivery. These characteristics have been obtained with systems using chitosan alone, modified chitosan, chitosan mixed with other components such as, sodium alginate or using chitosan glutamate proposed for bilaminated films and bilayered tablets [117, 118, 164]. In addition, chitosan has the potential to be a safe pharmaceutical excipient for non-parenteral drugs. Although it was approved for dietary applications in Japan, Italy and Finland and it has been approved by the FDA for use in wound dressings, more studies must be performance to ensure its safety. In Table 6 summarizes mains advantages in applications of chitosan systems by use other components.

Materials	Applications	References
Chitosan and carmomer	Improved oral bioavailability of buserelin.	[141]
Chitosan and PEGylated	3. 4-fold was improved its mucoadhesive properties of chitosan.	[142]
Preactivation of thiol groups on chitosan via the formation of disulfide bonds with mercaptanocotinamide	Mucoadhesive properties of thiolated chitosans were even significantly improved.	[143]
Chitosan and poly(g-glutamic acid) and chitosan and polyacrylic acids	This combination could be infiltrated in the cell-cell junctions and interact with <i>Helicobacter pylori</i> infection sites for the treatment of peptic ulcer.	[252]
Modified chitosan hydrogels loaded with metronidazole, tetracycline and theophylline	Modified chitosan hydrogels loaded with metronidazole, tetracycline and theophylline could bypass the acidic environment of the stomach and release the loaded drug into the intestine	[252]
Chitosan and thiolated chitosan	Chitosan and thiolated chitosan are used to improve the oral bioavailability of acyclovir 3-fold and 4-fold.	[148]
Chitosan with glutaraldehyde or ethylene glycol diglycidyl	Chitosan with glutaraldehyde or ethylene glycol diglycidyl are used to load antibiotics.	[174]
Chitosan microspheres reacylated with acetic anhydride	The re-acetylated chitosan microspheres were able to interact closely with the gastric mucosa and to exhibit sustained delivery of entrapped antibiotic	[175]
Clozapine-loaded nanoparticles with chitosan and polysorbate 80	A great improvement in surface hydrophilicity was brought by chitosan and polysorbate 80 coatings	[176]
Pectin/chitosan/hydroxypropyl methylcellulose (3 : 1: 1)	This formulation had reached the colon	[174]
Chitosan and pluronic F-127	Drug release systems for via buccal was improved and demonstrating that independently of chitosan salt type (citrate, acetate and lactate), mucoadhesion was significantly favoured when the concentration of Pluronic F-127 in the matrix was about 30% (w/w).	[175]
Trimethylated chitosans	Sandri et al. (2005) showed that trimethylated chitosans seem to be promising expipients for drug delivery systems intended for buccal mucosa applications to enhance the absorption of hydrophilic macromolecules.	[169]
Chitosan hydrogel beads coated with enteric polymer Eudragit S 100	Chitosan hydrogel beads coated with enteric polymer Eudragit S 100 to be targeted to the colon, prevented premature drug release in simulated gastric fluid, but delivered in the colon, because chitosan was degraded by the bacterial enzymes.	[159]
Chitosan with gelatin	Huang et al. (2005) blended chitosan with gelatin to improve the biological activity.	[177]
Chitosan with poly(ϵ -caprolactone)	These blending membranes improved mechanical properties as well as cellular support.	[178]
The γ -poly (glutamic acid) (γ -PGA) a hydrophilic and biodegradable polymer	This formulation was also used to modify chitosan matrices and the γ -PGA/ chitosan composite matrix was found to enhance hydrophilicity and serum proteins adsorption, and to increase the maximum strength through addition of γ -PGA in tissue engineering applications.	[179]
Chitosan and PLGA	Yamamoto et al. showed that chitosan-coated PLGA nanoparticle suspensions improved the absorption of calcitonin after pulmonary administration.	[187]
Chitosan-coated gold nanoparticles	Chitosan-coated gold nanoparticles have been investigated for mucosal protein delivery.	[253]
Chitosan nanoparticles with cyclosporine A	Chitosan nanoparticles with incorporated cyclosporine A in improving the delivery of drugs to the ocular mucosa.	[127]

Table 6. Main advantages in applications of using of chitosan with different materials.

6.2. Disadvantages

Several limitations of the systems using chitosan alone, modified chitosan, or chitosan mixed with other components could be mentioned. One of the most important is the general rapid clearance of the formulations in the mucosal surface owing to the mucociliary clearance and the presence of a variety of metabolic enzymes (cytochrome P-450 enzymes, conjugative Phase II enzymes, nonoxidative enzymes, and proteolytic enzymes that could reduce the absorption) of drugs administered via nasal using conventional chitosan formulations [254]. Nonetheless, these limitations are reduced when the drugs are entrapped in micro or nanoparticles using chitosan and other components.

Chitosan, has shown a hypoglycemic effect in streptozotocin (STZ)-induced diabetic animals [255- 257]. Other studies also found that low molecular weight chitosan (average MW about 2.0×10^4 Da) as well as chitosan oligosaccharides can reduce plasma glucose level in diabetic animals [258, 259]. On the other hand, when chitosan is used as a coat in liposomes, it helps in delaying intestinal transit time so as to increase absorption of insulin. An increase in chitosan molecular weight caused increase in the hypoglycemic efficacy of chitosan-coated insulin liposomes. The hypoglycemic efficacy of the liposomes coated by chitosan 1000 kDa was markedly superior to that of the liposomes coated by other chitosans. Both increasing and decreasing chitosan concentration, the hypoglycemic efficacies of chitosan-coated insulin liposomes were decreased and the systems were not able to protect insulin from enzymatic digestion [260]. Thus, diabetic patients should be careful when administering chitosan if they not eating above.

Chitosan systems have limited applications in drug delivery and tissue engineering due to their hydrophilic nature and insolubility in certain physiological conditions (eg. blood-brain barrier), due to chitosan is soluble at pH values below 6.5 [261]. Besides its mucoadhesive and controlled release properties chitosan is also able to increase the paracellular permeability due to the opening of tight junctions which has been shown for various routes of delivery such as for nasal or intestinal drug delivery. However, no effect on the paracellular permeability could be observed at pH 7.4, which is the physiological pH of blood [262]. This indicates that chitosan solutions are not effective as permeation enhancer at neutral pH values, because of the missing solubility of chitosan at neutral and alkaline pH values. Chitosan in form of particles can overcome this problem, because particles need not to be dissolved.

It is important to mention that the modification of the polymer can change the properties of the systems. Chitosan can be readily modified by reactions at the amino and hydroxyl groups present in the molecule, but adequate optimizations of the formulations need to test, increasing costs, time, and toxicological studies. It is important to mention, that some derivatives increase in toxicity and any residual reactants must be carefully removed [51]. Additionally, studies of *in vivo* and *in vitro* biodistribution and toxicity, using various chitosans of different molecular weights and degrees of deacetylation and derivatives would provide data that could help correlate chitosan's structure and safety profile [50]. Further studies needs to be conducted in this sense in order to approved based chitosan systems.

7. Conclusion

Potential applications of chitosan as weight supplement in the market as well as drug carrier in pharmaceutical formulations and as an important material able to prepare regenerative structures for bones and cartilages have been investigated. Thus, this work summarizes recent pharmaceutical developments using chitosan, modified chitosan or mixes with other components as drug carriers for the most non parenteral routes of administration including oral, topical, intranasal, and ocular, etc. Various therapeutic agents, such as anticancer, anti-inflammatory, antibiotics, antithrombotic, steroids, proteins, amino acids, antidiabetic and diuretics have been incorporated in chitosan-based systems as carriers to improve the dissolution rate of poorly soluble drugs and to achieve controlled release. Although, chitosan systems have limited applications in drug delivery and tissue engineering due to their hydrophilic nature and insolubility in certain physiological conditions (eg. blood-brain barrier) due to chitosan is soluble at pH values below 6.5, chitosan particulate systems can be used as carriers for encapsulate drugs and to enhance their bioavailability and delivery. The primary amines of chitosan impart these valuable physicochemical properties including particular interactions with cells, proteins and living organisms. On the other hand, the safety of chitosan could also be achieved in shellfish allergic patients since it was shown that the subjects have tolerated the polymer without reaction demonstrating the safety of other chitin-derived products in patients allergic to shellfish. Since chitosan has the hydroxyl and amino functional groups, important results have been obtained by reacting chitosan with controlled amounts of multivalent anions (functional groups) to control hydrophobic, cationic and anionic properties enhancing the vectorization, the stability, and the mucoadhesively (to prolong the drug residence time) of the drug carrier systems. These properties of chitosan, especially their intrinsic antibacterial activity, their ability to bind anionic molecules such as growth factors, glucosamine glycans and DNA and their ability to be processed into a variety forms are also used to generate suitable structures for bone and cartilage regeneration. However, more studies to improve their mechanical properties are essential for this type of application. Although many successful systems of chitosan have been developed, more toxicity tests must be conducted in order to ensure their safety when it is incorporated in other systems.

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Addiction Pharmacology

Drug Abuse, Addiction and Dependence

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Additional information is available at the end of the chapter

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1. Introduction

Before the dawn of civilization, they are natural substances that were discovered e.g euphoria's, narcotics, hallucinogens, excitants. Some of these were used by farmers. In fact, there were dope addict long before they were farmers. There are certain drives that persuade or compel somebody to resort to drug to obtain vacation from intolerable selfhood. One of the principal appetite of the soul is the urge to escape if for a few seconds the painful aspect of life, acquisition of wealth which may not be forthcoming. The distinguishing facts between legitimate use of drug for social purpose and their abuse are not certain if not indistinct. It is not a matter of which drug but the amount taken and if directed anti-socially or not. For instance normal people use alcohol for their occasional purpose without harm but, here there is appropriate degree of mental abnormality to the individual and the society as well. These people may then depend on it physically or emotionally [1]

Drug abuse is not a pharmacological problem but a social problem with pharmacological aspect. In connection with the use of drugs, such terms as 'abuse' 'misuse', 'addiction', 'habit-forming', 'tolerance' and 'dependence', which are often used interchangeably, should be properly defined [2] officially adopted the following usages:

Abuse: Drug use without medical supervision.

Misuse: Wrong use of drug under medical supervision.

Addiction: Specific side or adverse effect of drugs caused by prolonged use. In the case of addiction WHO recommend the use of the term dependence, subdivided into psychological or physical. Further, such terms as 'habit-forming' and 'tolerance' should also be properly defined in advance of dealing with problems of drug use.

Drug abuse is defined as 'the drug use that is not generally accepted on medical ground. In other words it is non-prescribed or social drug use. This means a continuous or occasional use of drug by the individual either of his own choice or under feeling of compulsion, to achieve his own well being or what he conceives as his own well being'. Drug addiction on the other hand is to devote or apply habitually these simple medicinal organic or inorganic ingredients in medicine. There is much-belated attempt by the scientific community to sever its conceptual apparatus from the vocabulary of politics and emotion. "Addiction," like "narcotics" and "drug abuse," has a general connotation of evil, suggesting illicit ecstasy, guilt and sin because the public image is conditioned more by cultural perceptions than by medical ones. Medically-precise meanings simply cannot be harmonized with common parlance." This may have come because of ambiguity in meaning as in relating drug abuse to a cluster of symptoms previously called "Substance Abuse" [3]

Drug abuse needs some clarifications and that the term is really convenient, but not very precise way of indicating that an unspecified drug is being used in an unspecified manner and amount ... and such use has been judged by some person or group to be wrong (illegal or immoral) and/or harmful to the user or society, or both. What might be called "drug abuse" by some would not necessarily be considered so by others. ... For these reasons, the term "drug abuse" is avoided here" [4] in Wikipedia). It has come to fore why World Health Organization presently prefers to use the terms "harmful use" and "hazardous use" of drugs. This may be to distinguish between the health's effects of drug abuse rather than the social consequences. According to [2], the term "Misuse" may be less judgmental hence preferred

Poly drug abuse is used to refer to:

- a. Simultaneous or sequential non-medical use of more than one psychoactive drug [14]
- b. The abuse of any psychoactive drug singly, in combination or sequentially which does not include heroin or alcohol as the primary drug. This was defined by the Federal Government in USA. One that is associated with therapeutic settings. Therefore, clinicians have a responsibility to avoid prescribing potentially habituating drugs for longer periods than is absolutely necessary, especially in the case of individuals who are known to abuse any agent e.g chronic alcoholics.

In drug abuse, the drug is obtained illicitly and the prescribed drugs are used in dosages beyond that prescribed medically. Over the counter drugs used is beyond the amount recommended on the package. Drug abuse is associated with urban crime. Most heroine abuse also abuse a variety of psychoactive substances, either in combination or in succession.

Drug abuse is a complicated phenomenon which is related to inter personal need, psychic and physical problems, and social adaptation. No one substance could be targeted as "brand switching" commonly occurred among individuals. The choice of a particular drug abuse and addiction appeared to be dictated primarily by availability. Differences in the pattern of abuse between geographic areas and among different population became apparent.

Drug addiction is a chronic, often brain disease that causes compulsive drug seeking and use, despite harmful consequences to the addicted individual and to those around him or her. Although the initial decision to take drugs is voluntary for most people, the brain changes that occur over time challenges an addicted person's self control and hamper his or her ability to resist intense impulses to take drugs [5]

1.1. Scope of the problem

The abuse of drugs has reached an epidemic proportion during the 1960's and early 1980's. The actual number of drug abusers is difficult to ascertain because of illegality of its use and some of the drugs are prescribed by physicians. The rate of first-time heroine use has reached a peak in the major cities in the USA in 1968. In smaller cities in 1980 it continues to rise. The exact number of people on heroine in the USA is unknown but according to [10] on drug abuse 2 million people in the United States have abused heroine. Those who have used Marihuana is around 43 million; hallucinogens 10 million and simulates 13 million.

1.2. The undesirability of compulsive drug abuse

There are a number of medical problems that are detrimental to the individual life or health and need to be suppressed e.g anxiety, fear, and sufferings. These can be relieved by using sedatives, hypnotics, anti anxiety and analgesics. On the other hand, their suppression it was though may decrease the adaptability of the individual and thus become undesirable. Drugs that produce physical dependence are powerful determinant of behavior. In order for the addict to satisfy his artificial appetite, life sustaining need are partially denied and social obligations are not met, with resulting harm to both the individual and the society [5]

1.3. Social cost

Non opiate abusers and addicts are seldom forced to steal or commit other crimes to pay for drugs, the social cost of non opiate abuse is less obvious than that of heroine abuse and receives less attention. The ultimate cost of non-opiate abuse to society is substantial when all its ramifications are considered. The cost associated with non opiate abuse can be measured in terms of productivity, industrial disruption, and unnecessary use of emergency rooms and psychiatric services. The treatment of physical dependence also expends considerable medical measures.

The dangers of drug and alcohol abuse are many. They are not confined to the toxic or the lethal effects on the individual. Drug and alcohol abusers often become un-productive, dissipating their available resources, neglecting their family and community obligations, becoming dangerous and threatening the safety of others while intoxicated as well as endangering their own lives because of drug and alcohol induced toxic effects. These dangers are magnified by the physical and psychological dependence produced by many drugs and by alcohol [1].

2. The phenomenon of abuse

Drug dependence is a complex phenomenon involving social, personal and pharmacological factors. It is at the same time a disease, a result of other diseases and a cause of criminal act. Fundamentally, it is an interaction of human being, their environment and a variety of drugs or chemical substances. The term drug abuse is difficult to define since it is based on social and cultural norms. In our culture the use of nicotine is not considered to be an abuse but it is considered to be a deviation from the dogmas of certain religious sect. Alcohol is a socially accepted drug usage in our society, yet certain degree and pattern of alcohol ingestion considered to be deviant or abnormal.

Drug abuse usually involves a persistent and excessive, non medical or non prescription use of chemical substances. Those drugs that are abused in our society are those that produce mind or mood altering effects. Drugs may be misused rather than abused if they are taken occasionally but in an indiscriminate or inappropriate way. The haphazard use of laxatives or antibiotics for example can constitute drug misuse by physician or lay-person [3].

2.1. When does it start?

Most drug addicts began experimenting with drugs when they are teens or early twenties. Data from Bureau of narcotics indicate that more than half the known addicts are less than 30 years of age. Most addicts have a history of delinquency and have experimented with other drugs such as tobacco, marihuana, alcohol, barbiturates and amphetamines before using narcotics.

2.2. Hard and soft drug

Non medical or non prescription use of drug can be classified into two (2) groups:

- a. Hard Drug
- b. Soft Drug

2.2.1. *Hard Drugs*

These are those drug that are liable seriously to cause disability to the individual as a functioning member of a society by inducing severe emotional and in case of cerebral depressants, physical dependence. Among these groups are heroin, morphine and analogue.

2.2.2. *Soft Drugs*

Soft drugs are less depending. They may produce emotional dependence but little or no physical dependence except with large doses of depressants e.g alcohol, barbiturates. The group includes sedatives, tranquilizers, amphetamines, cannabis, hallucinogen, alcohol and tobacco. Attempt to distinguish between the two terms fail because it does not seem to recognize individual variation in drug use. Barbiturate can be used in heavy, often in doses

that are gravely disabling and induce severe physical dependence but upon withdrawal, it could result in serious convulsion. To such individual it is a hard drug. Some other people (middle aged) can use it as a mild hypnotics and sedative but still retain their position in the home and society. Amphetamines on the other hand can be regarded as hard or 'soft'. What is of assistance in distinguishing these two terms is that while 'hard' use is central to the individual life, the 'soft' use is mere individual. What is really or classified here is not the drug but the effect it produces or the way it is used by the individual. This also depends on the dose. As Paracelsus in the 16th century, once wrote, "The right dose differentiates a poison from a remedy".

Non-medical use of drug has two principal forms:

- Continuous use
- Intermittent or occasional use

Continuous use-Here there is a true dependence based on repeated usage. E.g Alcohol, Barbiturates, Opioids e.t.c.

Intermittent use-This is the occasional use of obtain an experience or relieve from stress; to obtain an experience, e.g (Lysergic acid Diethylamide) LSD, cannabis, amphetamine, cocaine, solvents or to relieve stress e.g alcohol. Some of the drugs can be used in both ways e.g alcohol while LSD; cannabis are confined to occasional use alone [7]

3. Purpose for non-prescription use of drug

- Relief from anxiety, stress, tension, depression or from personal psychological problems or detachment from harsh reality.
- Rebellion against orthodox social values and the environment.
- Fear of conforming with own social sub-group or of missing something.
- Search for self knowledge and for meaning in life and in religion, also in interpersonal relationship to have a sense of belonging.

3.1. Culture

Culture defined simply as the way of life. Here it was stated that drug provides spiritual, emotional and intellectual experience that are the basis of life. It is really not certain if chemical could be central to a constructive culture. It is another for a chemical to be destructive to one's way of life. Drug use is a secondary phenomenon and not primary issues when sub-group are for the individual and social value of experience must be tested on the basis of its fruitfulness to the individual and to the society. The judgment by the individual alone is not conclusive but attested to by majority of people. In fact, the result of illegal use of drug is not encouraging let alone its damaging effect [8]

A good human quality is to love his neighbor in a practical and effective way. This cannot be promoted or diminished by drug. Obviously love of a neighbor is incompatible with driving a car over him. To believe drug e.g alcohol provide a vague benevolence is and of spirituality is doubted. What matters here is that it is not how a person feels but what he does in response to the feeling. The other claim by addict or abusers is that drug provides mystical experience; mystical experience has the following characteristics:

- a. Unity-This is a sense of oneness with nature and with God too.
- b. Joy, soundness, and peace.
- c. That the experience is beyond human power to express or describe (i.e ineffability).
- d. Transcendence of space and time.
- e. An insight i.e. knowledge into the truth of life, values, illumination, revelations of enormous significance mystical experience is transient and passive. When drug provide this experience, I do not think the individual gain an insight into the truth of life or experience outside his own self.

Mysticism is of the following forms, Nature's mysticism, soul's mysticism and God's mysticism.

3.2. What is mysticism?

Mysticism has the following characteristics.

- **Unity**-It has a sense of oneness with God and/or nature.
- **Ineffability**-That the experience is beyond ones power to experience or describe.
- **It provides joy, peace, soundness**
- **Knowledge**-This is an insight into the truth of life illumination, revelation of enormous significance.

3.3. Transcendence of space and time

It shows that in mysticism the person's will is in abeyance, Transient and passive. When it occurs it tells us nothing about the real truth about the person's mind or reality outside the person's life or about the mind of the person having the experience.

At this juncture we will like to see the various forms of mysticism

- a. Natures Mysticism
- b. Soul's mysticism
- c. God's mysticism

3.3. Nature's mysticism

This is an institution as it appears so vivid to be a vision of reality in the world outside the mind. It is concerned with natural beauty and sublimity or a quasi personified nature as its object.

3.4. Soul's mysticism

The soul of the spirit strives to enter not into communion with God or nature but into isolation from everything other than itself. That is the quest for self and of knowledge about it.

3.5. God's mysticism

Here the spirit is absorbed into the God and there is complete union with God. There is also an inexpressible knowledge or love of God and religious youth. This type of experience is so attractive that the subject looks for easy way to deliver it. Apparently, they can be no mystical experience with drug. Mystical experience is not a normal dose-related pharmacodynamic effect of any drug. Its occurrence depends on the subject (mood, personality) and environment and any preparation he may have undergone. The drug can facilitate the experience but cannot induce it. For instance, drugs can facilitate pleasant or unpleasant experience. It is noted that drug can provide mystical experience if it alters consciousness. A good example of these drugs is chloroform. Quoting somebody's experience with this drug "I seem at first a state of utter blankness with a keen vision of what was going on in the room around me, but not sensation of touch. I thought that I was near death; who was manifestly dealing with me, handling me, so to speak in an intense personal, present reality... I cannot describe the ecstasy I felt". In this place we can see that there is no good evidence that drug can produce experience that passes the test of result i.e fruitfulness.

Reliance on repeated drug experience as in drug abuse or non-medical use of drug even inhibits the subject's complete isolation from the material things of this world which is to be described as freedom of spirit [8]. Whether a single administration of drug can trigger or initiate experience which is beneficial to the individual is still to be proven. For instance, if emotional shock is acceptable in religious conversation, there seems so obvious reason why a drug should not also be used after careful preparation. There is always risk of the experience becoming an end rather than a means of development. It has been found out that psilocybin (a hallucinogen) facilitates mystical experience. It has to be noted again that a religious man is not a man with experience but takes the whole life in a religious way.

4. Tissue toxicity

All drugs that produce dependency in individual are liable to cause tissue toxicity with excessive use. It is not surprising to see users of narcotic to die of respiratory depression. Barbiturates and alcohol produce the same effect. Opiates produce chronic toxicity while alcohol produces many, mainly liver damage [7]

4.1. Mechanisms of overdose and toxicity in opioids

There has been an increasing recognition of the problems of fatal opioids overdose. The pharmacological basis of respiratory depression following opioids administration will be stated here. Respiration is controlled principally through medullary respiratory centres with peripheral input from chemoreceptors and other sources. Opioids, produce inhibition at the chemoreceptors via mu (μ) opioids receptors and in the medulla via mu (μ) and delta (δ) receptors. While there are a number of neurotransmitters mediating the control of respiration, glutamate and GABA are the major excitatory and inhibitory neurotransmitters respectively. This explains the potential for interaction of opioids with benzodiazepines and alcohol: both benzodiazepines and alcohol facilitates the inhibitory effects of GABA at the GABA receptors, while alcohol also decreases the excitation of glutamate at NMDA receptors. Heroin and methadone are the major opioids implicated in fatal overdose. Heroin has three metabolites with opioids activity. Variation in the formation of these metabolites due to genetic factors and the use of other drugs could explain differential sensitivity to overdose. Metabolites of methadone contribute little to its action. However, variation in the rate of metabolism due to genetic factors and other drugs used can modify methadone concentration and hence overdose risk. The degree of tolerance also determines risks. Tolerance to respiratory depression is less than complete, and may be slower than tolerance to euphoric and other effects. One consequence of this may be a relatively high risk of overdose among experienced opioids users. While agonist administration modifies receptor function, changes (usually in the opposite direction) also result from use of antagonists. The potential for supersensitivity to opioids following a period of administration of antagonists such as naltrexone warrants further investigation [9]

5. Social effect

We are all aware of the social and moral havoc produced by narcotic addict in order to obtain their drugs. Drugs have been linked to criminality because of the dubious means employed to get them compulsorily and not necessarily that drug induced the criminal act. Other social aspect of drug dependence is the inability of the addict to be productive or economic independence as well as member of his or her family. There is always a breakdown of interpersonal relationship and emotional support because the individual is busy or is not concerned with his drug and so isolate himself. He then avoids both personal and social obligations [5]

6. Factors determining the abuse potentiality of a drug

Some of these factors have the capacity to induce compulsory drug-seeking behavior, its toxicity and social attitudes towards drug effects and use. Since antiquity, drugs that produce dependency problem have been known. It is not clear when their dependency producing nature was first recognized. Opium preparations were used as soporific agents, analgesics anti-diarrheic antipyretics in ancient civilizations. These drugs were used to an extent even in

those times that some of them were physically dependent on them. In the middle of 19th century, opiate abuse was first recognized on a world wide scale especially in China. It became a problem because of the introduction of opium smoking and the commercial exploitation of opium grown in India and sold by the East Indian Company. At this point the consumption of morphine and opium was increasing in the United State. The ready availability of these products the supply by the Chinese labourers and the use of morphine as a pure salt for hypodermic injection makes the addiction a problem [10]

In 1909, the effective narcotic law was passed which prohibit the trafficking of opium and morphine (narcotics) except on medical ground. This prohibits the sale not only in U.S.A but also by the USA Commission on trafficking in narcotics to suppress the sale and consumption of narcotics in the far east. Because of the high profit from the illegal sale of narcotics, effort to arrest this act is still not successful.

7. Approaches to the drug abuse problems

There are varieties of approach to the incidence of drug abuse. The first is to prevent its occurrence by introducing a course on drug and drug abuse in the elementary, secondary and tertiary institution. The success of this needs further research. Other ways to prevent the abuse of drug is to alleviate the social and economic factor that is associated with drug abuse. Advice should be given to individuals on how to cope with anxiety and other stressful condition.

The legal approach could be by making the drug illegal and presumably unavailable. This approach is so primitive than other approaches. By making it illegal will drive the drug to the black market and high prices will be attached to them. In this case the individual will seek a more expensive and more criminal method in having the drug by all means to satisfy his urge or satisfaction. Other way is to introduce an alternative to the abuse drug e.g the use of nicotine gum in cigarette smokes so that others do not partake in sharing some percentages of nicotine while it's been smoked publicly. Rehabilitating the subject could be of advantage e.g "let them know about the cause of drug dependence, they could be also rehabilitated by complete withdrawal from the drug, or a modified drug use, vocational training, improvement of self image and development of changes in life style and attitude" [10]

8. Legalization of drug abuse or non-medical use of drug

A drug can be acceptable in medical practice if it is safe and its efficacy is guaranteed. These same principles should be used for non-medical use of drug. But the usual critical factor for judging efficacy against a disease or discomfort should not or hardly apply here. Some reasons why people abuse drug or non-medical use have been highlighted but not one carry weight if the drug is found to be a health-risk. These are some of the reasons why people abuse cannabis, for example. Medical prudence dictates that such risk be defined before legalization is to be effected. Indirectly, drug abuse can result in loss of education or employment. If laws are

implemented autocratically, it can lead to corruption among people, police and even alienation of important persons from the society who could have been part of decision making. Lack of discrimination by the law may lead to progression by the association from less to a more harmful drug since similar illegal behavior is needed to obtain all. But though written laws are so often inflexible and grouped together what would best be separated. Informal judicial discretion under the present law may be permitting more experimentation than would recurrent. Legislative debate leading to substitution of one written law for another written law may be encouraged. This untidy approach which may be best for the time being cannot certify the extravagant advocate either of reliance or of repression. What normally happens is that penalty for possession of small amount be removed (which is discrimination) whilst retaining penalties for suppliers [11]

9. Drug abuse and sport

In competitive sport, the goal for self or personal, national, financial prestiges are the cause of determination to win at all cost. Most of the drugs abused are those that enhance performance e.g anabolic steroids. The efficacy of this is largely not documented.

Drugs are abused in sports for the following reasons:

- a. For events in which body weight, brute, strength are the principal determinants (e.g weigh lifters, shot put, rowing etc). A good example here is anabolic steroids. Whether this has effect on performance is doubted apart from increasing the lean body weight.
- b. For event in which output of energy is explosive. The stimulants are used e.g Fencam-famin. These are used in bicycling, marathon racing e.t.c
- c. For events in which steadiness is essential e.g pistol, rifle shooting e.t.c subjects may use adrenoceptor blockers.
- d. For events in which body phancy is essential e.g in gymnastics, delaying puberty in child gymnasts by endocrine techniques.

In case of minor injuries in athletes, the use of non steroidal anti inflammatory drug is rampant and also corticosteroids so that training will proceed maximally. In some cases females have been virilised to outperform their sisters by the administration of androgen. These are administered for immediate gain of fame [8]

10. Drugs abuse liability

Drug most likely to be misused if not compulsively abused are those that can alter mood or behavior in ways that satisfy the emotional needs of certain individual. The classes of therapeutic central nervous system agents that are known to be misused for non therapeutic purposes include the sedative – hypnotics, alcohol, narcotic, analgesics, antagonists, sympha-

thomimetic stimulants such as metamphitamine, and certain general anesthetics such as ether and nitrous oxide. Other misused compound that have notable central nervous system effect but without proven therapeutic usefulness includes marihuana and lysergic acid diethylamide (LSD). The first gratifying drug experience is frequently, but certainly not necessarily [3]

11. Drug dependence

Drug dependence may be defined as a state resulting from an interaction of a person and a drug in which there is a compulsion to continue taking the drug to experience a pleasurable psychological effect and sometimes avoid discomfort due to its withdrawal. There are several groups of drug of dependence: Opioids, cocaine, Amphetamine, and Ecstasy, Barbiturates, Nicotine, Alcohol, Hallucinogens, Caffeine e.t.c. Drug dependence may also be defined as a state which arises from a repeated, periodic or continuous use or administration of drug. This could result in harm to the individual and sometimes to the society. The individual feels a desire or a need or compulsion to continue taking the drug. This is referred to as withdrawal or abstinence syndromes. The term drug dependence according to the World Health Organization (WHO) could be substituted for addiction and habit [12]

Dependence is characterized by the following phenomenon:

- a. Emotional or psychic dependence
- b. Physical dependence
- c. Tolerance

11.1. Emotional dependence

This is the first to occur if the drug is discontinued. The person become emotional or may be in distress.

11.2. Physical dependence

There is physical illness if the subject discontinues the drug called withdrawal syndrome. In physical dependence, repeated administration produces biochemical changes in the subject taking the drug. If the drug is withdrawn, very unpleasant symptoms and signs of physical nature develops which may last for a varying period, but will finally disappear. During this period, there is intense craving for the drug, which, if given, will temporarily relieve the unpleasant symptoms. Thus, after the establishment of physical dependence, the patient's drug-seeking behavior is motivated chiefly by fear of the withdrawal symptoms [12]

11.3. Tolerance

Tolerance is a phenomenon whereby more of the drug is needed to produce the same response. This often develops with drugs causing dependence, especially morphine and heroin.

Tolerance usually (but not always) develops to the central but not peripheral effects of a drug. Morphine and heroin causes euphoria (central) and constipation (peripheral). Thus, with heroin or morphine, tolerance to the central effects develops invariably, and the user will have to keep increasing the dose to get the euphoria, but will not develop tolerance to the drug's effect in causing constipation and will be severely and chronically constipated. This even occurs with many drugs that do not induce dependence e.g Lysergic acid diethylamide (LSD). This results from compensatory biochemical cell response to continued exposure to the drug e.g opioid. Both physical dependence and tolerance could result from homeostatic adaptation to continued occupancy of the receptors e.g opioids. It could also occur with Gamma amino Butyric Acid (GABA) receptors [12]

Physical dependence develops to a substantial degree with cerebral antidepressants but less with stimulants e.g amphetamines. There is usually cross tolerance between drugs of similar or dissimilar chemical group e.g Barbiturates, Benzodiazepine, alcohol. No drug possesses any mysterious powers to subjugate any person. It is said that "the first exposure to any drug could be an index of addictive proneness".

Emotional dependence occurs to any drug that alters consciousness however bizarre e.g muscarine. To some in ordinary doses do not e.g non narcotic analgesics, diuretics or purgatives. Dependence also depends on the patient's belief about the drug. For instance those depending on purgative and diuretics are obsessed with the dread of obesity. Emotional dependence can occur by taking either tablet or injection and it also depends on the content. We are all physically dependent on food but some with a stronger emotional dependence eat too much [5]

11.4. Psychological dependence

In psychological dependence, the patient exhibits a compulsive drug-seeking behavior. The drug often produces a pleasant feeling, often relaxation, freedom from worry, or heightened awareness and increased energy and sexual drive. The patients suffer mental anguish when it is withdrawn. It was customary to divide regular continuous drug abuse into two categories: Addiction, Habit [7, 12]

12. Addiction, habit

Addiction here shows that there is a compulsion to continue taking the drug whereas in habit is a mere desire to continue taking the drug. In addition, the harmful effect is to the individual and the society at large while in habit the harm is to the individual alone. This difference cannot be quantified in the sense that what is considered to be addiction in one subject could be a mere habit in another. In some other cases in addiction, the individual could have both emotional and physical dependence. In habit the subject is confined to only emotional dependence.

Even with these differences, there is still fault. For instance alcohol is a drug of addiction to many in which the individual becomes physically dependent on it and without it could become

ill. In some other people, it is a case of just solace or just pleasure and some take it on a regular basis. Of course, these may be the reason why the term 'addiction' and 'habit' are replaced by dependence. This term removes irrelevant argument or difference between the two terms which are never true differences and also remove the arguments that tobacco is addictive or habit-forming.

Dependence on a drug depends on some factors among which are the availability of the drug itself, the subject personality disorders and the socio-economic factor. The speed of onset of dependence and its tendency to induce emotional, physical dependence depends on the pharmacological actions of the drugs, dose and the frequency with which it is administered [1, 12]

12.1. Reasons for drug abuse and tolerance

Drugs may be used intermittently for social or emotional reasons – for example, to relieve a stressful situation. Those who are truly dependent take drugs continually and may reach a state in which their whole life centres on obtaining and using drugs. Dependence may not be confined to one drug or groups of drugs. It is common to find dependent subjects who have escalated from minor drugs (for example, Cannabis) to hard drugs (for example, Heroin) and some subjects may alternate or combine drugs; for example, Cocaine and morphine would produce alternating stimulation and relaxation. It is a difficult question to know why people depend on drugs. The answer is still incomplete. People may depend on drugs for the following reasons; curiosity and wanting to belong, chemical props and escapism, biological make-up, availability, pressure at work [12]

13. The mechanism of physical dependence

This could be related to tolerance but is poorly understood. The physical dependence and tolerance implies that the adaptive changes have taken place in the body tissues and that changes are lost and in consequence, there is rebound over activity. It was discovered that there is morphine-like substances called endorphins and that the CNS employs this as a neurotransmitter. This allows speculation that exogenous administration of morphine could suppress endorphins and that when the drug is withdrawn, the lowered endorphin cannot immediately compensate this because the suppression was due to a feedback mechanism. Because of this immediate deficiency, there is a withdrawal syndrome [8]

14. Pattern of drug abuse or non-medical use of drug

Any age: Alcoholism – mild dependence on hypnotics and tranquilizers: Occasional use of LSD and cannabis.

20 – 35 years of Age: Hard use drugs e.g heroine, morphine (imported illicitly) and synthetic analogue – dipipanone, methadone (diverted prescription) or amphetamine injected.

Under 16 years: Volatile inhalants: solvent of glue, aerosol sprays, glue sniffing, paints, inhalation of excreta from toilets e.t.c [8]

15. Multiple drug abuse

These are the following; Hypnotics, sedatives, amphetamines, cocaine, opioids e.t.c. The pattern of use of these drugs changes frequently [7]

16. Drug supply to addicts

In the UK the supply of drugs to addicts especially heroine and opioid is by specially licensed doctors. The subjects are either on treatment or fail to respond to treatment. This is done to maintain the patient as total withdrawal can lead to withdrawal syndrome. To avoid infection, sterilized syringes and the pure drug could be supplied and this pure drug may discourage them from taking the drug which may be adulterated. Oral methadone is convenient and may occupy the opioid receptor which reduces the kick produced by the injectable opioids. This procedure is very good to some extent because as the price of these drugs fluctuates (high), the subject uses more illicit way to obtaining the drug and can result to crime. Prescription of this drug ensures a measured drug is provided for the patient and not him requesting for an xmg of drug. He or she can sell it to others as well as initiating other people [5]

17. Withdrawal syndrome in opioids dependence or addiction

No doubt, withdrawal can cause unpleasant feelings in addict. To say addicts continuously seek the drug primarily to avoid the unpleasantness is false. There is the capacity to relapse after complete withdrawal and it has been recorded. Addicts seek super normally rather than normally to get their drugs and also seek intense pleasurable 'kick' or 'high' so that after complete withdrawal, the psychopathic or neurotic addict still have tendency to revert to drug [8]

18. Treatment of severe pain

This may present special problem when high efficacy opioid is used, they may either be tolerance or toxicity could result. When low efficacy opioid is used, not only will it be ineffective but there could be withdrawal syndrome especially if they are agonist/antagonist e.g

pentazocine. This leaves aspirin as a drug of choice. Aspirin could be given in combination to ensure efficacy. For instance, Aspirin 600mg and aminoacetic acid 300mg. Nefopam is a non-opioid analgesics which could be used to relieve pain [8]

19. Route of administration of drug

Highest plasma concentration occurs with intravenous route as compared to oral route. This is responsible for the 'kick' of 'flash' reported by addict. Addict who seeks illegal means of drug use could be supplied with diluted drug which if supplied with pure drug could result in toxicity [12]

20. Mortality

Especially young people that abuse and are addicted to heroin, barbiturates, amphetamines by intravenous route die of septicemias, Acquired immune Deficiency Syndrome (AIDS), tetanus gas gangrene, endocarditis, pulmonary embolus e.t.c [10]

21. Escalation

There is possibility that subjects progress from soft to hard drugs. For instance, individual on cannabis and amphetamine could progress to a harder one i.e heroin and even be addicted to it. This brings the idea of cross tolerance [1]

21.1. De-escalation

As the subject progress or advances in age, he or she is disillusioned with taking these drugs. Any drug that alters ones consciousness is liable to be abused, even with anti-parkinsonism e.g levodopa, anticholinergics [8]

22. Substance abuse

A number of substances are abused. This is attributed to seekers of self gratifying 'high' who often inhale volatile substances that affect the central nervous system. Substances like glue sniffing, aerosol, petrol, paint scraping, nail-vanish, lacquer paint solvents (snuffing), butane gas which later with continuous use can paralyze the larynx and in consequence, food materials, gastric contents, drinks could enter the respiratory tract and could even flood the lungs and eventually death will occur. Some of these aerosols could be sprayed in a plastic bag or in confined enclosure and the subject will begin to inhale. This has serious consequences

which ultimately lead to death. Some drugs are deliberately designed to suite chemist needs which could be of high efficacy. This is by molecular modification by skillful and criminally minded chemists.

Pethidine production could be by short cut leading to a substance closely related to a by-product. Example is methylphenyltetrahydropyridine (MPTP). This is known to have parkinsonian syndrome which may respond to levodopa. MPTP selectively destroys the melanin containing cells in the substantia nigra [8]

23. Treatment of drug dependence

A step toward social and psychological rehabilitation is to withdraw the drug. This is a long and often disappointing journey to psychological and social rehabilitation. In the case of physical dependence, the drug is withdrawn gradually over a long period of time. It may be done for about 10 days and steps should be taken to control abstinence syndrome. In some of the chemicals, a different chemical should be substituted. E.g for heroin, methadone can be given. For alcohol, it is better to use diazepam, chlormethiazole or chlordiazepoxide could be used. A barbiturate is substituted with 30mg phenobarbitone up to a maximum of 400mg phenobarbitone per day for every 100mg barbiturates. Some patients are in a very poor physical state. In such a case effort should be made to wait for sometimes until the patient is a little normal.

In some cases of sympathetic autonomic over activity, B-adrenoceptor blocking drugs should be used e.g clonidine.

Maintenance should be by supervision by medical personnel where there is hope of cure. In this case the addict should be supplied with the same or alternative drugs but by less harmful routes e.g patient who prefers to use intravenous (I.V) route for heroine should be given oral methadone. It is so because the patient prefer to be excited as quickly as possible and that the I.V produce the immediate high.

Measurement of blood or urine level of some of the drug is important for effective management of drug abuse. Testing of some of the drug to know their power as to induce abstinence syndrome should be done. This is by giving animals' e.g monkeys. Some percentage of the drug regularly and abruptly is withdrawn to see the withdrawal syndrome. The power of a drug to control abstinence syndrome could be another serious problem because it could cause serious dependence.

The World Health Organization (WHO) recommends drug dependence should be classified by types.

Tobacco Type:

- Emotional dependence is high
- Physical dependence is slight

Alcohol Type

- Emotional dependence is severe
- Physical dependence with prolong use
- Cross tolerance with similar sedatives

Cocaine Type

- Psychic dependence is severe
- Physical dependence is slight or absent
- Tolerance is absent.

Canabis Type

- Emotional dependence in some
- Physical dependence is slight or dubious.
- There is no characteristics abstinence syndrome.
- Tolerance is slight.

Amphetamine

- Emotional dependence is severe
- Physical dependence is slight
- Tolerance exists
- Psychoses occur during use

Barbiturates Type

- Psychic dependence is severe
- Physical dependence is severe. It develops slowly with continuous use.
- Cross tolerance occur with alcohol, diazepam, chlordiazepoxide, meprobamate, glutethimide, chloral, paralaldehyde

Morphine Types

- Emotional dependence is severe
- Physical dependence is severe, develops rapidly.
- Tolerance occur with related drugs
- Naloxone induce abstinence syndrome.

Drug Mixtures

In this case there is alteration of mood.

- Psychic dependence is severe
- Physical dependence is strong
- Tolerance exists

Heroin, Cocaine

These combinations have similar characteristics [8]

24. Prevention of drug dependence after medical use of drug

If drugs are used or handled properly, there is no need of drug addiction but when drugs are used excessively especially analgesics, and then dependence will occur. Prevention could be brought about by spacing the drug dosages as much as Possible. Effort should also be made by withdrawing the drug of addiction from the patients or conceal the nature of the drug. In some patient whose expectation of life is little, there is no need to bother much on drug dependence when planning therapy

25. Tobacco

Tobacco was introduced to Europe from South America in the 16th century. Tobacco is habit forming. In the USA, the Federal Government spends her subsidies to tobacco farmer's larger sums than it does on telling people not to smoke. The highest production of tobacco is from USA, India, USSR and china

25.1. Composition of tobacco smoke

There are about 500 compounds in tobacco smoke. The chief pharmacologically active substance is *nicotine*-acute effect and rare-chronic effect. The smoke of cigars and pipes is alkaline (PH 8.5) and nicotine is un-ionized and lipid soluble and is absorbed in the mouth. Smokers of cigars and pipe have lower death rate from lung cancer because they do not need to inhale to obtain nicotine as it is alkaline and lipid soluble. Smoke of cigarettes is acidic and the nicotine is lipid insoluble and ionized. In this case the smoker needs to inhale to obtain and coupled with large surface area for absorption in the lung will absorb a substantial quantity of nicotine and the individual will suffer lung cancer. The amount of nicotine by smokers is about 90% and in non-smokers by about 10%. The tobacco contains about 1 – 5% carbon monoxide. In habitual smokers it's about 3 – 7%. In heavy smokers, about 15% of their blood is carboxy-haemoglobin. This cannot carry oxygen. This is enough to induce angina pectoris.

Chronic carboxy-haemoglobinaemia causes polycythaemia. Tobacco is known to contain polycyclic hydrocarbon and N-nitroso compound. These compounds are carcinogenic and are responsible for the microsomal enzyme inducers in smokers [8]

25.2. Tobacco dependence

The reason why people smoke is a complex phenomenon. The purported benefits on mental health are so intangible and elusive, so intricately woven into the whole fabric of human behavior, so subject to moral interpretation and censure, so difficult of medical evaluation and so controversial in nature that few scientific groups have attempted to study the subject.

Satisfaction of smoking is due to nicotine and also tars which provide the flavour. There is no clear cut personality difference between smokers and non-smokers. Cigarette smokers tend to be more extraverted, less rigid and more prone to antisocial behavior than non smokers. Cigars and pipes smokers are introverted.

Again, smoking is not associated with neurotism or is they liable to psychiatric problem. To this problem, psychoanalysts have made characteristics contribution to this. "Getting something orally" one asserts "is the great libidinous experience in life"; first, the breast, then the bottle, then the comforter, food and finally, the cigarette. Those who give up smoking substitute it with other oral activities e.g nail biting, gum chewing and eating.

Sigmud Freud was a life-long tobacco addict. He suggested that some children may be victims of a "constitutional intensification of the erotogenic significance of the labial region" which if it persist will provide a powerful motive for smoking. Beginning to smoke is related to status need, personality recognition, and self esteem and is not due to rebellion. "*That which hath made them drunk hath made me bold*" [13]. There is no difference in intelligence between smokers and non smokers but non-smokers are more academically favoured or successful then smokers. Learning to smoke occurs in adolescent and mostly in the age of 20 years. The factor that facilitates smoking initially is psychosocial with pharmacodynamic factor unpleasant. With the psycho-social pressures especially from members of the same age group, one get used to the benefits of nicotine, learning to avoid and coping with the various pharmacodynamic unpleasantness. As he progresses, the psychosocial factors diminish and are replaced with the desire and need for nicotine. The subject with the fear of withdrawal syndrome and maintenance of nicotine level in the blood, he then develops high drive for nicotine consumption and tendency to be a chain smoker. People who want to smoke in order to become 'tough' or grown up, outgrown this immature drive and smoke because of the pleasure they derive from the nicotine. Offering and accepting to smoke is another factor. There is also development of personal relations in business and private life. People do smoke because, to them it relief them of the stressful situation and it may be that smoking is an expression of stress [8]

25.3. Types of smoking

Smoking could be pharmacological or non-pharmacological.

25.3.1. Non-pharmacological

This includes:

- i. Psychosocial: This is done to increase self esteem, status need, personality.
- ii. Sensorimotor: To achieve oral, sensory or manipulative satisfaction.

25.3.2. Pharmacological

By Changes in puffing and inhalation rate, the plasma concentration of nicotine is adjusted automatically.

Indulgent: To obtain pleasure or to enhance already pleasurable condition.

Sedative: to ease unpleasant conduction.

Stimulant: Help performance of monotonous task, to escape stressful condition or to get a 'life' in order to perform.

Another case is where the subject smokes automatically, being unaware of the act. He becomes aware of the act only when the cigarette is not at hand [5]

25.4. Acute effect of smoking tobacco

Airway resistance: Accumulation of carbon particles can result in bronchial narrowing sufficient to double airway resistance. This is insufficient to cause dyspnoea but four to five fold increase cause noticeable dyspnoea and in about 20 fold can cause severe dyspnoea as occur in asthma. Nicotine does not increase airway resistance.

Colliery Activity: Develops after the drug is gradually withdrawn or after transient stimulation in depressed.

Carbon monoxide Absorption: Is less in healthy young adult but become significant in coronary heart disease.

Nicotine is well absorbed with a plasma half-life ($t_{1/2}$) of 2 hours. Nicotine can stimulate or depress nervous tissue depending on doses and dosage interval and the psychological state of the patient. It can relief boredom or stressful situation. Smokers who become more alert tend to take a lower dose of nicotine than smokers who become tranquil. Nicotine causes the release of catecholamines from the hypothalamus and anti-diuretic hormones from the posterior pituitary. In large doses it stimulates the end of peripheral cholinergic nerve whose cell bodies lie in the central nervous system. It acts at autonomic ganglia and neuromuscular junction. This is called the nicotine-like or nicotine effect. Higher doses paralyse the same point. The central nervous system is stimulated with the vomiting centre. Tremors, convulsion could result. Depression follows stimulation in case of peripheral actions. The nicotinic effect can be blocked by the canuylamine which antagonize the nicotinic acetylcholine receptors.

"Fatal nicotine poisoning has been reported from smoking, from swallowing tobacco, from tobacco traumas, from topical application to the skin and from accidental drinking of nicotine insecticide preparation. In 1992 a florist sat down on a chair, on the minutes later he felt ill (vomiting, sweating, faintness and respiration difficulty, following by lose of consciousness and cardiac irregularity). He recovered in hospital over 24 hours. On the 4th day he was deemed well enough to leave hospital and was given his clothes which damp. Within one hour of leaving hospital he had to be readmitted suffering once again from nicotine poisoning. He recovered over three weeks, but for persistent ventricular extra systole" [8]

26. Conclusion

Conclusively, the above brief discussions do little more than raise issues that deserve considerations. Drug induced experience can only be discussed in terms of attitudes and beliefs held by the individual as to the nature of man, his purpose (if any), his obligations (if any) and his relationship to a transcendent being or God (if any) and there is no good evidence that drugs can produce experience that passes the test of result, e.g fruitfulness. Indeed, reliance on repeated drug experience may even inhibit the development of independence from the material things of this world, which is vital for anything that deserves to be described as freedom of spirit.

Whether a single administration of a drug can be used to initiate or trigger experience that may result in an individual gaining beneficial insight is unproved. Finally, there is a risk of the experience becoming an end in itself rather than a means of development.

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Edited by Sivakumar Joghi Thatha Gowder

The book “Pharmacology and Therapeutics” targets every aspect of the mechanisms for the chemical actions of both traditional and novel drugs. This book covers six sections: Molecular Modeling and Bio-molecular Pharmacology, Immunopharmacology, Environmental Pharmacology and Toxicology, Nanotechnology and Chemotherapy, Drugs and Drug Delivery System and Addiction Pharmacology. Each of these sections is interwoven with the theoretical aspects and experimental techniques of physiology, biochemistry, nutrition, cellular and molecular biology, microbiology, immunology, genetics, and pathology. This book will be a significant source to scientists, physicians, health care professionals and students who are interested to explore the effect of chemical agents on human life.

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