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Notice

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Meet the editor

Dr Varaprasad Bobbarala is working as a senior researcher at Adhya Biosciences Pvt. Ltd., Visakhapatnam, Andhra Pradesh, India. He received his doctoral degree from the Faculty of Science, Andhra University, in 2008. He specialized in biochemistry, medicinal chemistry, and microbiology. Dr Varaprasad has published outstanding articles on alternatives to antimicrobials, medicinal chemistry, and drug discovery. He currently serves as editor, associate editor, and peer reviewer for several international journals. He has published more than 90 papers and several book chapters on microbiology, biochemistry, and medicinal chemistry. His recent research has also focused on drug discovery, bioefficacy studies, and isolation of bioactive metabolites from natural resources.

Contents

Preface

Drug discovery is a process through which potential new medicines are identified. It encompasses different scientific steps from the validation of the target and characterization of the hits. Drug discovery is arduous, exhaustive, exciting, and extremely challenging and it involves a wide range of scientific disciplines, including biology, chemistry, and pharmacology. Drug candidate selection is an iterative process between chemistry and biology, refining the molecular properties until a compound suitable for human use is found. The developed product must not only be safe and efficacious, but its efficacy also has to be proven across racial and ethnic groups as well as across different age groups. Every drug has to pass a global regulatory review in what is currently the most regulated industry in the world. Once this is done, approved products must appeal to global markets across different cultures, healthcare systems, and distribution systems. There is an urgent need to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. This book provides few important concepts in recent drug developments.

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Chapter 1

In Vitro Biotransformation in Drug Discovery

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Abstract

In vitro Biotransformation studies play a crucial role in drug discovery program that determine the fate of the new chemical entities (NCE's). Enzyme rich matrices such as microsomes, hepatocytes, liver fractions and S9 fractions transform the new chemical entities to different metabolites. Metabolites could be pharmacologically important or toxic. Newly formed metabolites are identified using liquid chromatography interfaced with mass spectrometry. Identification of the biotransformation sites in the new chemical entity helps the medicinal chemists to optimize its structure and develop the NCE as a pharmaceutical drug. Screening pharmaceutical drugs using *in vitro* biotransformation studies assist in selecting the right new chemical entity for further *in vivo* studies in animal systems and later in human clinical trials.

Keywords: *In vitro* biotransformation, metabolism, drug discovery, chromatography, mass spectrometry

1. Introduction

Organisms such as Enzymes, Bacteria and Fungi play a crucial role in the conversion of organic compounds to different products. This process of transformation is termed as biotransformation. In different fields of science, biotransformation has a significant impact. In the case of drug discovery and development, metabolism of drug to many different compounds is catalyzed by the enzymes in the liver [1, 2]. Similarly microorganisms in the gut are responsible for microbial biotransformation of organic compounds [3]. Most of the pharmaceutical drugs are organic moieties and undergo enzymatic biotransformation or microbial biotransformation. Therefore, *in vitro* biotransformation studies that mimics the actual *in vivo* system gains importance.

In vivo studies on various species are not economical and have restrictions; therefore *In vitro* biotransformation studies are vital in drug discovery programs [4]. Screening pharmaceutically important compounds using *in vitro* matrices such as microsomes, hepatocytes, liver slices and S9 fractions from different species directs the drug discovery team to make an appropriate decision to advance the molecules. Incubation of new chemical entities as well as pharmaceutical compounds with different *in vitro* matrices [5–8] results in metabolites. Metabolites could be pharmacologically important or it could be toxic [9].

Metabolites are identified and characterized using liquid chromatography and mass spectrometry (LC–MS/MS) [10]. *In vitro* biotransformation studies performed on various species guides the researchers to choose the right animal model to validate the developed molecule [11, 12]. Thus the *in vitro* biotransformation studies assist to identify and eliminate the false positives and make it possible to choose the relevant molecule for further *In Vivo* studies and later for clinical trials. Biotransformation of pharmaceutical drugs with different structural moieties results in different metabolite structures. Liquid chromatography assists in separation of the metabolites and drugs according to the polarity of the molecules. Liquid chromatography also provides a vital information to purify the drugs, metabolites and nano materials that serves as delivery systems for various biomedical applications [13, 14]. Mass Spectrometry resort to identify the mass of the drugs, metabolites and thus helpful to assign the biotransformation sites in the metabolites [10, 12].

2. *In vitro* metabolism of drugs by Microsomes

The important site of the body for drug metabolism is liver. Membrane bound drug metabolizing enzymes were present in liver microsomes as subcellular fractions. Hepatic CYP-450 enzymes were the reason for metabolism and clearance of more than 60% of marketed compounds [5, 15]. *In Vito* intrinsic clearance of a compound is determined using microsomes and interspecies differences in drug metabolism using species-specific microsomes. Commercially available microsomes for rat, mouse, dog and cyanomolgus monkey were used to understand the interspecies differences [11, 12].

Microsomal stability assays performed for a drug is used to calculate *in vitro* half-life ($T_{1/2}$) for a particular drug and also intrinsic clearance (CLint). Thus microsomes are ideal model systems to determine the metabolic stability, phase-I metabolism and intrinsic clearance of a compound that can be scaled to *in vivo* situation to predict human clearance [5]. Control compounds such as Verapamil, Diazepam, Diphenylhydramine, Quinidine and Dextromethorphan are used as matrices for human, rat, mouse, monkey and dog respectively to compare the deviation of test compounds. Both the control and test incubations were performed in the absence of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) to identify the chemical stability of compounds or non enzyme mediated pathway and in the presence of NADPH to determine the enzyme mediated metabolism.

Microsomes were prepared, stored and used conveniently in comparison to whole cell models. Microsomes are generally used to screen hundreds of compounds in drug discovery and development and rank order the compounds. Compounds are rank ordered as high,

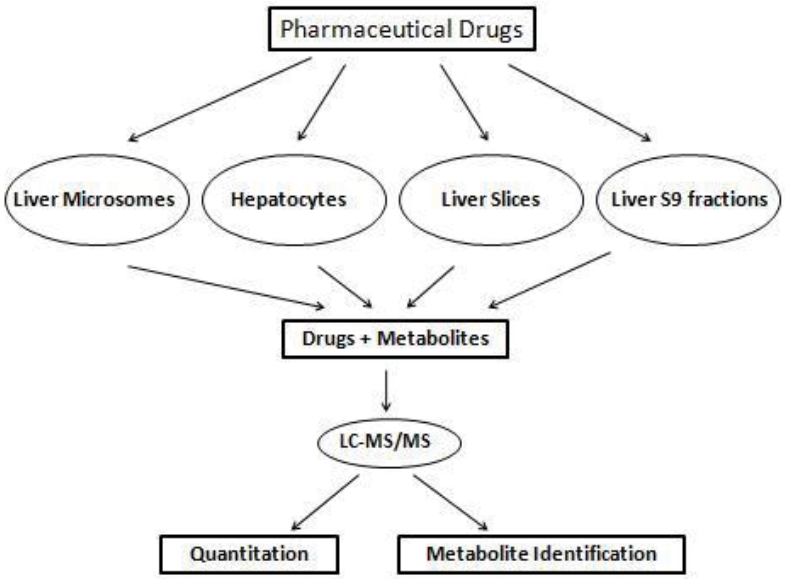

Figure 1. *In vitro* biotransformation scheme to screen pharmaceutical drugs.

medium and low clearance compounds. For example intrinsic clearance value of >47 μL/min/mg protein for a particular drug in human liver microsomes is considered to be high and intrinsic clearance value of <8.6 μL/min/mg protein is considered as low clearance. Intrinsic clearance value between 47 μL/min/mg protein and 8.6 μL/min/mg protein is considered to be moderate intrinsic clearance. Similarly for monkey, dog, rat and mouse high, medium and low clearance values were assigned based on the extensive studies done for many compounds [15, 16].

Predominantly microsomal stability studies are performed to assure the Phase-I metabolism in the presence of NADPH as cofactor (**Figure 1**). Besides this microsomes can also be utilized to understand the Phase-II metabolic pathway of a compound. Incubating the test compound in the presence of microsomes, NADPH and UDPGA (Uridine Diphosphate Glucoronic Acid) would reveal the possible glucoronide pathway. Similarly incubation of a parent drug with NADPH and Glutathione helps to identify the conjugation site for glutathione and its pathway.

3. *In vitro* metabolism of drugs by hepatocytes

Intact cell of hepatocytes consists of both Phase-I and Phase-II drug metabolizing enzymes. Hence, hepatocytes based studies to identify the intrinsic clearance of compound mimics *In Vivo* system to a greater extent than microsomes [16]. While, microsomes are rich in Phase-I enzymes, hepatocytes consists of both Phase-I and Phase-II enzymes. Quantity of Phase-I enzymes were abundant in microsomes and helps to detect the Phase-I metabolites with accuracy, but lacks Phase-II enzymes. Hepatocytes derived from various species such as rat, mouse, dog, monkey and human were imperative to understand the impact of species

differences in metabolism. Hepatocytes studies help to identify the species akin to that of humans and choose the model system for further studies of any drug [17]. Thus Human hepatocytes serve as a gold standard to understand the metabolism and toxicity of drugs.

Hepatocytes are cryopreserved and stored for longer duration of time. Cell viability and activity are better with cryopreserved hepatocytes and best alternatives for fresh cells. Presence of both phase-I and phase-II enzymes result in better assessment for clearance of drugs [18, 19]. Based on intrinsic clearance values compounds can be rank ordered as low, medium and high using hepatocytes for different species. Intrinsic clearance for humans range from <3.5 to >19.0 μL/min/10^6 cells, in the case of monkey <5.2 and >28.3 μL/min/10^6 cells; for dog <1.9 and >10.5 μL/min/10^6 cells; for rat <5.1 and >27.5 μL/min/10^6 cells and mouse <3.3 and 17.8 μL/min/10^6 cells.

4. Microsomes versus hepatocytes

Compounds that are metabolized by phase-II enzymes are best studied by hepataocytes, compounds whose primary metabolic pathway is through phase-I enzymes is best understood by microsomes (**Figure 2**). Microsomes are rich in CYP450 enzymes and therefore metabolic turnover is very high compared to that of hepatocytes. Compounds with the property of poor permeability through cell membranes are more stable in hepatocytes than microsomes. Identification of phase-I metabolites using microsomes are confirmatory while phase-II metabolite identification can be authenticated using hepatocytes. Quantities of enzymes are less in hepatocytes compared to microsomes resulting in less quantity of metabolites for hepatocytes. Hence, in order to confirm few of the phase-II metabolites such as glucuronidation and glutathione conjugation, pharmaceutical drugs are incubated with microsomes along with cofactors such as UDPGA or Glutathione. Incubation of microsomes with drugs and cofactors result in more quantity of metabolites and can be quantified accurately.

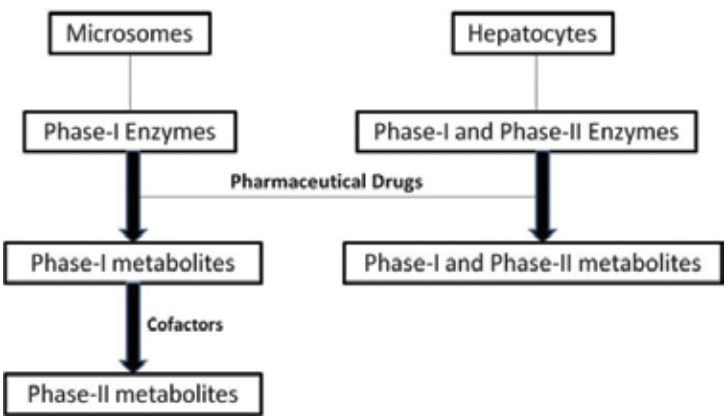

Figure 2. Comparison of outcome of microsomes and hepatocytes upon incubation with pharmaceutical drugs.

Thus both microsomes and hepatocytes are very much essential as a matrix to study the xenobiotics of pharmaceutical drugs. Prediction of clearance using hepatocytes is better and results are similar to that of *in vivo* systems [20, 21].

5. *In vitro* metabolism of drugs by liver slices

Metabolism plays a key role in detoxification of xenobiotics. Metabolic enzymes as well as transporter proteins that transfer the metabolites and parent drugs through the cells are essential for *in vitro* metabolism to mimic the *in vivo* metabolic systems [7]. Liver slices from human and various species are precious and valuable to conduct drug metabolism studies and acute toxicity studies [22, 23]. Metabolic enzyme activity of liver slices declines rapidly and restricts its prolonged use for drug metabolism studies. In spite of this inherent problem of liver slices pharmacological and toxicological studies have been performed and liver slices were proved to be one of the efficient model systems [23]. Precision cut liver slices consists of all liver cell types that are present in natural cells with cell to matrix and cell to cell interactions, thus representing actual liver functions [24]. Thus precision cut liver slices helps to recognize and identify the mechanisms for exposure in humans. Most of the precision cut liver slices with active enzyme components and cells are viable for not more than 2 days [25, 26]. Therefore modifications of culture conditions such as medium composition were attempted in recent years [27] to extend the viability of precision cut liver slices to 5 days with promising results.

6. *In vitro* metabolism of drugs by liver S9 fractions

Liver S9 fractions are rich in both microsomal and cytosolic fractions. Optimum metabolic information about a compound is obtained from liver S9 fractions than microsomes. Liver S9 fractions consist of both microsomal and cytosolic fractions and corresponding enzymes, whereas microsomes have only microsomal proteins. Isolation of liver S9 fractions is uncomplicated and obtained during the initial stages of microsomal preparations [28, 29]. Major components of cytosolic S9 fractions are cytochrome P450's, Uridine 5′-diphospho-glucuronosyltransferase, aldehyde oxidase, xanthine oxidase, sulfotransferases, methyl transferases, N-acetyl transferases, glutathione transferases and represents the *in vivo* system to a greater extend. Microsomes consists of CYP 450's and Uridine 5′-diphospho-glucuronosyltransferase and lack other enzymes that are present in S9 fractions [30]. Similar to microsomes liver S9 fractions need cofactors such as β-Nicotinamide adenine dinucleotide phosphate-regenerating system (for oxidation), Uridine 5′-diphospho-α-D-glucuronic acid (glucuronidation), 3′-phosphoadenosine-5′-phosphosulphate (Sulfate conjugation) and glutathione (glutathione conjugation) for phase-I and phase-II assays. Hepatocytes do not require any of these cofactors that are used during microsomal or liver S9 fractions based assays [31]. Human hepatocytes are considered to be golden standard and 7-ethoxy coumarin has been used as an appropriate substrate to understand the phase-I and phase-II metabolism [31] in different matrices. Recently liver S9 fractions were shown as a matrix comparable to that

of hepatocytes to screen compounds for metabolic stability assays [32]. Researchers have used 7-ethoxy coumarin and performed a comparative study on three different matrices i.e. microsomes, hepatocytes and liver S9 fractions. Phase-I metabolite 7-hyroxy coumarin was observed in all the three matrices, whereas glucuronide and sulfate conjugates were observed for hepatocyte and liver S9 fractions [33]. Therefore activities of liver S9 fractions are more akin to hepatocytes than that of microsomes.

7. Purified CYP enzymes for reaction phenotyping

Compounds that exhibit medium or high clearance in microsomes and hepatocytes assays are subjected to reaction phenotyping studies.

Expressed enzymes or purified CYP enzymes are used to identify the enzyme responsible for the metabolism of a particular compound and is termed as reaction phenotyping [34]. CYP enzymes CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are primarily responsible for phase-I metabolism of most of the drugs. If phase-II metabolite glucuronide conjugate is expected, enzymes UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15 need to screened. Thus the above mentioned purified enzymes need to be incubated with a particular drug to identify the percentage contribution of each enzyme for the metabolism of a drug. During the reaction phenotyping assay positive controls are tested for each purified enzyme. Ethoxycoumarin is a positive control for CYP1A2; Amodiaquine for CYP2C8; Diclofenac for CYP2C9; Diazepam for CYP2C19, Dextromethorphan for CYP2D6; Testosterone for CYP3A4 and Efavirenz for CYP2B6. There are instances where a particular drug can result in different metabolites. Formation of each metabolite can be from same enzyme or due to different enzymes or due to the contribution of mixture of enzymes. Hence reaction phenotyping is essential to identify and confirm the enzyme responsible for the formation of particular metabolite [35]. Knowledge from reaction phenotyping helps to suppress or enhance the formation of a particular metabolite using inhibitors or enhancers. In case a particular metabolite is expected to be toxic in expected quantity, then the formation of that metabolite can be suppressed using inhibitors. Reaction phenotyping studies will also help the medicinal chemist to design a synthetic strategy to avoid the formation of a toxic metabolite.

Thus the information derived from reaction phenotyping studies can be utilized to (1) predict drug–drug interactions with the coadministered drugs and (2) metabolism of a drug by an isoform can lead to increase or decrease in the concentration of drugs, metabolites in plasma and therefore difficult to determine the therapeutic range of a compound (3) helps medicinal chemist to modify the structure of drug or pharmacologist to use an inhibitor or enhancer to address the safety of the drug (4) Generally compounds which exhibit high or medium clearance in the microsomal or hepatocyte stability assays are subjected to reaction phenotyping assays.

Food and Drug Administration also highly recommends *in vitro* studies to understand the systemic clearance and also to determine the responsible CYP450 or UDPGA enzyme for the metabolism of new investigational drug.

8. CYP450 time dependent inhibition assay

Drug-drug interactions is caused by the inhibition of CYP450 enzymes. Inhibition is of three types (1) irreversible (2) Quasi reversible and (3) reversible. Irreversible CYP450 inhibition reactions are of major concern compared to that of reversible CYP450 inhibition, because synthesis of inactivated enzyme is essential to restore the activity of deactivated enzymes. Hence it is mandatory to understand the mechanism of new chemical entities at an early stage of the drug discovery and development [36]. Two types of time dependent inhibition are generally referred as mechanism based inhibition (MBI) and time dependent inhibition (TDI). Mechanism based inhibition is due to the inhibition of enzyme by chemically derived metabolite. Mechanism based inhibition is classified as a subset of time dependent inhibition. Time dependent inhibition arises when there is an increased inhibition during the incubation of test compound with metabolizing system prior to the addition of the substrate. Available CYP isoforms such as CYP3A4, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP2D6 are generally tested for CYP inhibition. The substrate used for CYP3A4 inhibition is midazolam and control compound is mifepristone; substrate for CYP1A2 is ethoxyresorufin and positive control compound is furafylline; substrate for CYP2B6 is bupropion and positive control compound is ticlopidine; substrate for CYP2C8 is paclitaxel and control compound is gemfibrozil; substrate for CYP2C9 is diclofenac and control compounds is tienilic acid; substrate for CYP2C19 is S-mephenytoin and control compound is ticlopidine and substrate for CYP2D6 is dextromethorphan and control compound is paraxetine. These control compounds are inhibitors which are selected by screening few time dependent inhibitors. For example probe substrate of CYP3A4 midazolam can be subjected to inhibition studies using time dependent inhibitors like clarithromycin, verapamil, troglitazone, mfepristone and mibefradil. The inhibitor that exhibits maximum inhibition is chosen as the inhibitor for a CYP isoform. During the CYP inhibition experiments quantity of substrate, inhibitor and formation of metabolites is analyzed using liquid chromatography interfaced with mass spectrometry [37].

9. LC-MS/MS and *In vitro* biotransformation studies

Accurate bioanalytical methods are essential in drug discovery to assess the concentrations of drugs and metabolites. Pharmacokinetic investigations, toxicokinetic analysis and several *in vitro* studies such as metabolite identification, metabolic stability, caco-2 permeability studies, drug–drug interactions and protein binding studies are extensively studied using liquid chromatography hyphenated with mass spectrometry. Therefore, high throughput screening to develop methods is challenging and time dependent.

Chromatography and Mass Spectrometry plays a major role in determining the concentrations of drugs and metabolites at the levels of attomole or femtomole [38]. The science of separation achieved through chromatography is very important to separate the drugs and metabolites based on the polarity of the molecules. Advent of high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) resulted in analyzing samples

in shorter period of chromatography run time (1–5 min). Sensitive mass spectrometers such as triple quadrupole mass spectrometers, quadrupole ion trap mass spectrometers, orbi trap mass spectrometers, time-of-flight mass spectrometers have accelerated the detection of drugs and metabolites at very low quantity such as attomole levels and to identify the structure of drugs and metabolites. Thus chromatography hyphenated with mass spectrometers were supportive to address the LADMET (liberation, absorption, distribution, metabolism, excretion and toxicity) related issues in drug discovery programs [39]. Quantitation by chromatography and mass spectrometry is important to assess the liberation, absorption, distribution, excretion and toxicity of a drug molecule and its metabolites.

10. Quantitation of drugs and metabolites

Efficient sample preparation methods are essential for effective quantitative analysis of drugs and metabolites. Recovery of drugs and metabolites in high amount is an important factor in this process. Several methods such as liquid–liquid extraction, solid–liquid extraction, use of sep-pak cartridges, elution of drugs and metabolites through small columns packed with silica or C-18 were used to purify the samples. Purification of samples removes the matrices and other unwanted impurities resulting in ideal samples for bioanalysis by chromatography and mass spectrometry. Precipitation methods using centrifuges, precipitation using solvents were also employed to purify the samples. Depending on the nature of the drugs and metabolites one or more of the above process has to be practiced for samples with high purity [40]. Highly pure samples result in reproducible quantification results. Along with purification of samples, choosing an appropriate internal standard is also essential for quantification of drugs and metabolites. The chosen internal standard should have structure similar to that of parent drug and should not interfere with parent drug during analysis. For ex internal standards should have different retention time and molecular weight compared to the drugs and metabolites. Thus chosen internal standards can be distinguished from parent drugs by LC–MS/MS during the analysis. Different retention time in chromatography and different multiple reaction monitoring (MRM) transitions in mass spectrometry for internal standards with respect to that of parent drugs is essential for accurate quantitation of drugs and metabolites. Thus the separation power of chromatography and sensitivity of mass spectrometers are an added advantage for a pharmacokineticist and toxicokineticist to determine the fate of new chemical entities (NCE's) [41].

11. Identification of metabolites

Biotransformation of pharmaceutical drugs results in metabolites. Structural identification of metabolites by liquid chromatography and mass spectrometry confirm the metabolic softspots and hotspots [42, 9]. Metabolic softspots involve usual biotransformation pathways such as oxidation, reduction, hydrogenation, dehydrogenation, hydroxylation, dehydroxylation, loss of a functional group, oxidative dehalogenation, epoxidation, decarboxylation, hydration etc. [4].

These are the major phase-I biotransformation along with few other unusual metabolic pathways. Apart from phase-I metabolites, conjugation reactions leads to phase-II metabolites such as glucuronidation, sulfation, glutathione formation, methylation, glycine conjugation, and taurine conjugation [4]. Identification of the structure of phase-I and phase-II metabolites and its quantity is essential to understand the elimination of drugs, metabolites and residence time of drugs, metabolites in human system. LC–MS/MS plays a crucial role in rapid identification of the metabolic soft spots and hot spots and directs synthetic chemists to make appropriate modifications in the drug moiety [43, 44].

12. Conclusions

In vitro biotransformation of pharmaceutical drugs utilizing matrices viz. microsomes, hepatocytes, liver slices or liver S9 fractions results in various metabolites. Each matrix has its advantages and shortcomings. Therefore, all the matrices are essential to identify, confirm and quantify the metabolites. During the preclinical pharmaceutical candidate optimization, screening the pharmaceutical drugs using various matrices helps bioanalytical and biotransformation scientists to identify the metabolic spots of the molecules with the aid of chromatography and mass spectrometry. Identification of metabolic spots at the early stage is beneficial for medicinal chemists to design and alter the structure of pharmaceutical drug for better potency and accelerate the drug discovery and development process.

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Conflict of interest

None declared.

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Chapter 2

On the Organization of a Drug Discovery Platform

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Additional information is available at the end of the chapter

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Abstract

Some of the most exciting parts of work in the pharmaceutical industry are the steps leading up to drug discovery. This process can be oversimplified by describing it as a screening campaign involving the systematic testing of many compounds in a test relevant to a given pathology. This naïve description takes place without taking into consideration the numerous key steps that led up to the screening or the steps that might follow. The present chapter describes this whole process as it was conducted in our company during our early drug discovery activities. First, the purpose of the procedures is described and rationalized. Next follows a series of mostly published examples from our own work illustrating the various steps of the process from cloning to biophysics, including expression systems and membrane-bound protein purifications. We believe that what is described here presents an example of how pharmaceutical industry research can organize its platform(s) when the goal is to find and qualify a new preclinical drug candidate using cutting-edge technologies and a lot of hard work.

Keywords: drug discovery, validation, cloning & expression, biophysics, structural biology, organization

1. Introduction

Drug discovery involves a suite of processes as part of a program aimed at finding drug therapies for diseases. These programs encompass many different scientific steps from validation of the target (or attempts to do so) and characterization of the hits until the selection of candidates for medicinal chemistry programs. We felt that an accurate description of those steps has not previously been available and that such a description could be interesting.

In PubMed®, several thousand publications exist with the term 'drug discovery' in their titles. Most frequently, these reports share with readers how important the authors' particular area of

expertise is in this domain, covering almost everything from crystallography to screening and high-content screening, origin of the screened molecules, compound libraries, sample conservation, biochemical approaches, microarrays, proteomics, and aptamers. These publications are a useful source of inspiration for each new program. Clearly, the first step in drug discovery is inspired by the state of the art, and the literature will help drive research towards the 'bench-to-bed' goal of finding a new molecule that will eradicate some disease that affects our society.

We feel, however, that the complete picture of such processes is not very often characterized. Thus, here we exemplify how we built our drug discovery (DD) platform on basic science with the goal of following, in a unique location (a set of specialized laboratories), the logical successive steps of drug discovery from ideas to first hits. An oversimplified picture of the logical progression from start to end of these processes can be seen in **Figure 1**.

This configuration did not preclude failures but probably helped forward our successes. In the pharmaceutical industry, the attrition rate is around 9 of 10 projects, meaning that only 1 project in 10 will have a slight chance of ending up in clinical development, and a further 2–3 out of 5 of these will have a chance to reach patients. Therefore, in preclinical research, scientists will try to deliver on time and with maximal rigor the tools necessary for the pathological problem to be at least partially solved. In 9 cases out of 10, the efforts will be vain, as the programs will be terminated, either for lack of results or for a change in strategy. The reasons for attrition are many, and a key to decreasing number is certainly a better characterization of molecules entering the medicinal chemistry program that will drive candidates from hit to

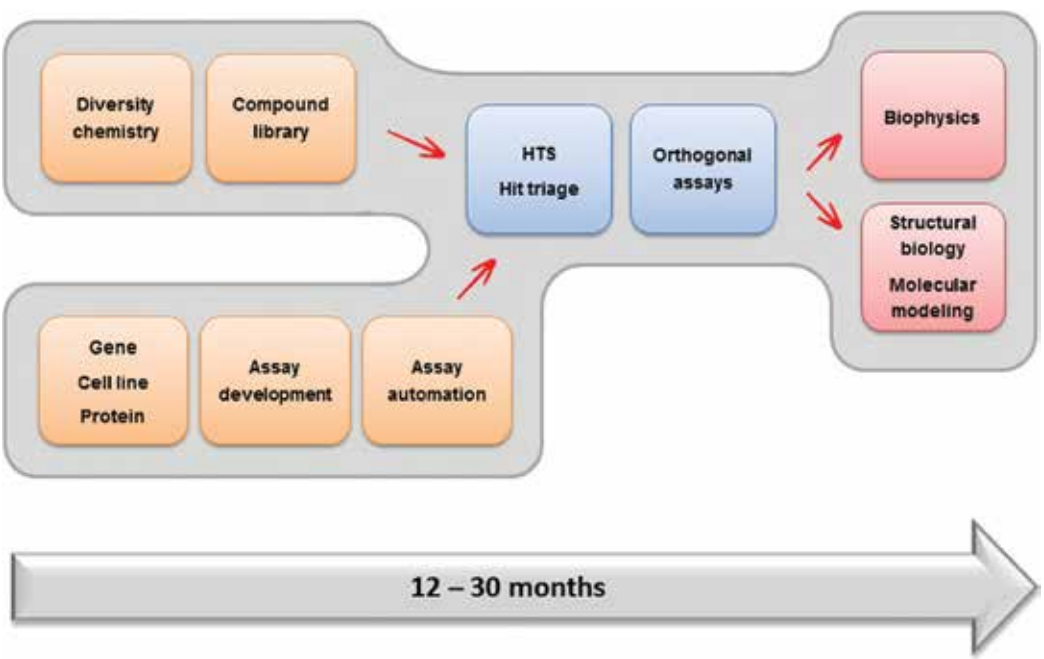

Figure 1. Schematic representation of a drug discovery process. Note that the exploratory/validation of the target process is not part of the schedule. Rather, these two steps might take several years to complete upstream.

lead. Among the characterizations that are essential is the specificity of the molecule within its class of targets (e.g., one given kinase among the 800 in the kinome) or its specificity towards the rest of the genome-encoded proteins. Another key is a better understanding of the molecule-target relationship at the atom level.

In addition to these scientific aspects, daily nonscientific aspects of business also require attention. Examples include practical questions, such as 'Can we hire new specialists to explore and assess a new field for a particular problem?'; economic questions, such as 'Can we spend the money necessary to acquire a particular technology'; and time-related questions, such as 'Can we spend the 12 to 20 months to deliver the results after the necessary assessment for a new technology/technique?'

Below, we offer our view through our experiences with all of these aspects, reflecting the work of a laboratory ultimately consisting of about 70 people, with some impressive achievements.

2. Basic sciences, organization?

Our group has a "simple" core job: the discovery of drug candidates. Programs are based on two types of knowledge: the pharmacological background linked to the various areas of therapeutics (e.g. cancer, cardiology, or neurosciences) and the techniques needed to explore the molecular side of the program (e.g. molecular biology, cellular biology, biochemistry, structural biology), in other words 'basic sciences for discovery'. That basis was a mixture of molecular and cellular pharmacology techniques that allowed starting from a protein target and finishing with a compound (or several) that interfered with this target in a reasonably specific manner. To this were added chemistry (for the compound collection) and molecular modeling (for dynamics and structural biology data interpretation). One of the major challenges was the identification of a given target in the pathological process, its role in the pathology, and its manipulation. These necessities meant that we also needed upstream access to experimental techniques such as siRNA, KO mice, alternative laboratory animals (such as yeast, flies, and zebrafish [1], and *Caenorhabditis elegans* [2]) often at a time when these techniques were barely validated. It also meant that we frequently had to make the choice of collaboration rather than pursuing these techniques internally.

2.1. Exploring pathology

"Exploring" means several things in this context, from exploring the disease to exploring the tools and the literature to validate a target. The literature is rather scarce in this context, but one resource is the essay by Scudamore, who tries to make us understand how difficult and diverse this area could be [3].

The exploration of pathology is the main objective of academia, but industry faces a challenging problem. Should it run brand-new programs using its internal research capacities or should it collaborate with academia to help them run such program? Considering that part of the work in industry is to choose relevant targets accessible to chemistry (even if chemistry has broadened its borders for the last decades) and that are truly and undoubtedly linked to

a pathological situation, it cannot start several million-euro projects without a validation that convinces everyone that the end discovery will help the patient.

Thus, industry must equilibrate its efforts among internal, confidential, and secured experiments that would identify and elucidate the role of a target in a pathological process while scouting externally in academia and biotech for new ideas, concepts, targets, and even sometimes candidates. In these last cases, however, internal reproduction of key data should not be underestimated in terms of time, money, and results (see Section 2.2).

We were involved intensively in several such programs. For example, N-myristoyltransferase is a protein-modifying enzyme with a key role in addressing substrate to the membrane [4], including oncogenes or viral proteins [5, 6]. Our discovery that one isoform was associated with rough endoplasmic reticulum led us to believe that this particular isoform potentially had a role in pro-virion maturation [7]. On another front, we discovered/characterized a new activity for the enzyme autotaxin, a lysophospholipase D, the activity of which explained why that protein was previously believed to be a migration factor [8, 9]. This enzyme could be deeply involved in fat accumulation processes [10] and in metastasis [11]. We pursued this program up to the discovery and characterization of a hit compound that failed for stability reasons [12]. Another example is N-arylalkylamine acetyltransferase (AANAT), an enzyme involved in the synthesis of melatonin, of which we characterized the properties of the human isoform [13], while its importance in regulating melatonin synthesis had been demonstrated [14]. Our approach to the uncoupling proteins was similar to this. We thought that UCP3 might be a good target for diabesity, if we could find an activator of this enzyme. The challenging idea was that by accelerating proton exchange, one could reasonably enhance the heat dispersed by muscles. We thus started to study this particular enzyme in various contexts [15, 16], up to an *in vivo* model for which elegant techniques were developed [17, 18].

Opportunities are numerous for scientists in the core of a network. As noted, the challenge is always to be original enough but not too much involved in fundamental research because the time frame is simply not suitable to the business of industry, at least for the most part.

2.2. Validating targets (as actor in a pathology)

The validation of the implied involvement of a given molecular target in a pathological process requires many different approaches and technical applications. A special issue of *Current Topics in Medicinal Chemistry* was published in 2017 on this subject (see the editorial by Henderson and Gibert in [19]). Furthermore, Kaelin published an interesting paper [20] on the pitfalls in preclinical cancer target validation. Indeed, one of the main aspects of the Kaelin essay is that many experiments reported even in major journals are hard to reproduce independently, if at all, and thus validation is a difficult task. Obviously, this has been an endless debate in the scientific community, and commenting on it further here will not add to the discussion. The *primum movens* is not the same between academia and industry. Basically, it is fair to say that the protocols are not regularly described in enough detail to be easily reproducible in a different laboratory context.

Without a strong, scientific knowledge about the pathology, cures will not be found except by chance. Thus, being able to present our program in a way that is reproducible is important.

One has to be able to ensure (or be reasonably sure) that the observations made are linked to the 'pathological' situation and not to experiment-generated artifacts. Of course, achieving this goal requires a mixture of pure luck, hard work, prepared minds, curiosity, and willingness to move forward.

Most of the time, however, only a portion of these experiments is feasible, either for timescale reasons or for cost. Therefore, the choice is often made to go to primary approaches such as knockout cells or knockout animals. We reported such an approach with cells [21], knocking out the product of a gene by measuring the catalytic activity of the corresponding enzyme, spontaneously expressed in a cell line, HT22, after siRNA and shRNA treatments. It took a double transfection with a shRNA encoding a siRNA specific of QR2 to knock down only 80% of the catalytic activity of the enzyme. This result suggests that knocking out a spontaneously expressed protein in a cell line—especially in a cancer cell line, which is often pluri-nuclear—is difficult, if not impossible beyond knocking it down somehow. It is interesting to compare this type of data, including those we obtained in similar conditions but that remained unpublished, with claims that cocktails of siRNA transfected directly into (cancer) cells would kill the activity of an enzyme within hours (if not minutes). A recent review on the subject [22] emphasized the fact that RNAi studies have been used and abused, suggesting that the key role of those RNAs should be kept to a tightly verified level to gain from this outstanding technology and fix once and for all the problem of *in cellulo* identification and engagement of a target in a given pathology. Technologies involving nucleases and CRISPR have changed the technological landscape of cellular biology because these techniques lead to complete knockout and are almost universal—until exceptions to their effectiveness or accuracy start to emerge.

Cellular validations are often not complete enough to give strong support to a pathophysiological hypothesis. It is thus necessary to move to another approach: the genetic deletion of the target in mice, or, more recently, in rats. The bias of this approach, besides its cost and often the length of the process, is linked to the potential capacity of animals to compensate for the absence of a particular gene (or the absence of a particular catalytic activity). This compensation might happen often, but proofs of such situations are rare. The two main difficulties we have encountered were either the lethality of the KO in mouse embryos [23] or the complete absence of an obvious phenotype due to the loss of this particular gene [24, 25], a surprisingly frequent feature. The former example offers a rare case. When we mutated a single amino acid in the catalytic site of lysophospholipase D, which renders the enzyme completely catalytically inactive, the mouse embryos died in the very early stages of their development [23]. This outcome was unexpected but proved at least that the enzyme activity was a key player in mouse embryogenesis [26, 27]. Knockout animals can also be fantastic tools to prove that the compound engages the target. For instance, our antagonist of the melanin-concentrating hormone (MCH) receptor, S38151, has no activity in MCHR1 KO animals, while it reduces feeding wild-type ones, demonstrating that the compound exerts its expected activity only through its binding to the receptor [28]. Other examples have been generated in our DD platform, such as with the deletion of trace amine 1 receptors, revealing the involvement of those receptors in the actions of the drug Ecstasy (MDMA) [29]. Unexpected properties of the modified animals are sometimes found long after the knockout has been performed, resulting in the resuscitation of the program. For example, the knockout of Ucp2, an uncoupling protein expressed in almost all mouse tissues, confers an unexpected resistance to *Toxoplasma gondii*

infection [30]. Finally, particularly difficult is the choice of the mouse strain usable for the KO line. Indeed, some examples of animals totally deprived of circulating melatonin led to slightly different observations about the impact of the knockout when compared to a strain presenting a "normal" level of circadian melatonin [31]; thus, not only are some strains partially blind but consequently their daily rhythm is profoundly affected.

Again, recent progress in cellular surgery, thanks to CRISPR techniques [32], might make the development of KO animals faster and even commonplace in the near future, but today the process remains a lengthy one.

Alternatively, in addition to these genetic manipulations *in vivo* or *in cellulo*, the pharmacological approach remains a possibility. Strategically, though, it is rare for a pharmacological set of tools for a "new" target to exist beforehand, and these molecules need to be specific and/or powerful enough to help validate the system. An upstream decision must be made about whether the high-throughput screening (HTS) approach that will probably deliver compounds can be used very early in the discovery process to bring tools rather than drug candidates to validate the target around which the program was/will be built. This theoretical situation can provide the community with very early tools to validate their activities (see reviews by [33, 34]).

Of interest, another approach has become possible in recent years, thanks to our collaborative work with the Shanghai Institute of Materia Medica (SIMM): a validation with molecules of poor specificity but engaging the target. Indeed, as soon as the first compounds are issued from this screening process, even if they are far from perfect, attempting high doses of such molecules on an animal model relevant to the pathology brings interesting and important information about the validation of the pathological hypothesis. Only a couple of examples have been published following these attempts, one with thioredoxin-interacting protein (i.e., TixNip) modulators [35] and the other with a gpr119 antagonist [36].

2.3. Cloning/expressing/purifying the targets

Obviously, targets come from independent experimental observations that have suggested their role in a given pathology in a given system through the measure of activity such as a catalytic activity or a potential binding site. Ultimately, purification should be performed and should lead to the unequivocal characterization of the protein the program targets. A recent trend is to consider that this given protein is a brick in the pathway(s) leading to the pathology. In this framework, the protein should retain its cellular context to allow for consideration of the potential role of its partners and cellular neighborhood. This situation is no doubt optimal. Nevertheless, finding compounds that will hit the target should begin with a simplification of the system. Without this simplification, one should consider the multiple interferences from the cell milieu (see also the HCS discussion below, Section 2.6.3).

This is an old debate between the pros and cons of protein purification. The pros consider that the candidate molecules should be aiming directly at the protein and that structural biology will deliver key information on the target-molecule relationship providing that the system is as simple as possible. The cons remind us that inside the cytosol, an enzyme interacts or is in

proximity to many other proteins, some of which can involve interference. Rather than being an assortment of simple linear pathways, the cytosol in reality is more like a bag of marbles—proteins—rolling around and bumping into each other.

As far as enzymes are concerned, is it acceptable to work on a partially purified extract or should we aim for the pure version? For screening purposes (the *nec plus ultra* of the work of such a DD platform), the need for membranes overexpressing a receptor is key to the success of the next processes, so are there alternatives?

Cloning remains a source of novelty. Obviously, using already published sequences for new targets involves a bias because these targets are already known in the literature, so that the results with them are less novel that would be desirable for the drug market. Thus, cloning efforts of new, not already described targets, are at best a challenge. Our first attempts were to control the expression of receptors or enzymes, together with an associated protein (whether naturally associated or needed for building an assay for this target). Particularly interesting was trying to find ways by which the expression of two proteins—or two peptides—could be stoichiometric. For such a goal, the best approach was to use and organize IRES promoters in different geometries [37]. This method led us to the successful expression of several multi-peptide proteins, such as the nicotinic receptors.

It is interesting to see now the progress in what was once called cellular surgery, first with nuclease approaches and now with CRISPR. In past years, we used nuclease-based techniques to produce cellular models (most of the time, unfortunately, based on cancer cells) that would express a single copy of the transgene, integrated into the cell genome at a single, neutral position [38]. This method was interesting because for comparisons of the molecular pharmacology of a receptor from different species, often from human and one or two rodent species, the comparison was more relevant because only the sequence of the receptors drove the differences between the pharmacological profiles. Indeed, it was integrated into the same cellular host—CHO or HEK cells—at an identical 'neutral' position, with a single copy of the gene, leading to a validity and accuracy in the data that was difficult to obtain whenever the transfection was less controlled.

When the enzymes have already been described/cloned, expression is easier in a host cell—often bacteria—but then the purification (of an active enzyme) often becomes the key. Typical examples of such purification strategies can be found for chymase [39], AANAT [13, 40], and indoleamine-2,3-dioxygenase [41].

Regarding other aspects of research the DD platform has conducted, we should mention work on already known targets that suddenly take on added interest because they turn out to be involved in an unexpected physiopathological situation. One example is the role of quinone reductase 2 beyond its presumed detoxifying role. The enzyme has been associated with cognitive processes on the one hand [42] and with melatoninergic systems on the other [43]. Aware of these observations, our attempt was to build a network around the notion that inhibitors of this enzyme could be interesting tools or drugs in understanding cognition and cognition deficits [24, 44], as well as better characterizing the MPTP Parkinson model in mice [45, 46]. We reported several examples in which we explored new pathways and

found evidence for new proteins or new activities. For example, the observation that lysoPLD activity was present and active in an adipocyte culture medium led us to hypothesize that this medium contained a protein capable of catalyzing the breakdown of lyso-phosphatidylcholine. We reported this discovery [8], after having purified, characterized, and discovered alternative splicing forms [9], built the KO mice [23], and started a search for active inhibitors [12]. It is interesting to look at it from a time-frame perspective. Indeed, the initial observations were made in 1999 while our initial report was published in 2003 [8] and the last report on the compound, S32826, in 2008 [12]. Thus, it took about 10 years before all tools were in place and the program had sufficient experimental evidence to allow for a process to lead to an actual drug discovery program *per se*. Only in 2011 evidence in the literature has shown the probable implication of this target in metastasis [47]. The work on autotaxin also concerned an already known motility factor [48] that turned out to be an enzyme generating this motility factor, with a potential role in metastasis [47, 49].

Broadening the observation that N-myristoyltransferase was key to the maturation and delivery of oncoproteins, such as src, to the membrane, we became interested in the other proteins that were myristoylated [6] and sought to differentiate their cytosolic activity from the poorly described membrane-associated activity. These studies led us to wonder if there was a target for inhibitors that would turn out to be antiviral compounds (myristoylation of the membrane viral protein gag is key in the virus life cycle). Very early on, we became interested also in kinases and some of their modifying enzymes (e.g., NMT, see above). Indeed, we started our program by choosing to explore the main tyrosine protein kinase expressed in the HL60 cancer cell line [50, 51] and purification was the only option at a time when cloning was rather rare.

We still believe that following the expression of the target (enzyme) in bacteria, for instance, activity must be purified to homogeneity. Indeed, the further process of testing the activity should lead to unambiguous attribution to the target protein and not to a similar activity catalyzed by a bacterial endogenous enzyme. Therefore, much effort was often put into the purification process, even though the current literature reflects a lack of enthusiasm about obtaining a pure enzyme. Furthermore, the need for biophysical as well as structural data necessitates a pure product, in any case. Therefore, what is done at that stage serves at least three purposes: obtaining uniformity of the enzyme in the preparations; gaining the possibility of measuring unambiguously the relationship between molecules and their targets; and finally, achieving crystallogenesis, which requires the purest possible preparation to start with.

Considering receptors, the situation might be slightly more favorable to a less simplified system. Indeed, the main property of receptor binding is certainly that the binder is specific to the receptor; thus, considering membranes as an acceptable receptacle for receptor studies seems to be an adequate compromise. For instance, in a program linked to our historical involvement in melatonin receptor pharmacology, we had to find a way to check for the activity of agonist candidates in an *in vivo* model of depression that was a non-rodent and diurnal animal (melatonin being heavily involved as a master switch of the circadian rhythm as well as the circannual one) [52, 53]. Such models were not that common, especially at a time when the exact nature of the chronobiology was not understood. We then turned to a sheep model with the difficulty that, at that time, it was believed that two animals were known to be natural

knockouts for one of the melatonin receptors (MT_2). Although this was clearly demonstrated for one strain of hamster [54], it was less clear for the sheep. Thus, we embarked on a challenge to finally clone, express, and characterize the MT_2 receptor from sheep, destroying this myth at the same time [55]. Such approaches became one of our interests in our multiple attempts to crystalize G-protein-coupled receptors (see also the structural biology section, below). In brief, a survey of the sequences of the receptors of a common family (e.g., melatonin) in various animals indicated variations of sequences with retention of specificity (towards melatonin). By measuring the thermal stability of the receptors, we can deduce the strongest structure that could be amenable to expression, solubilization, purification, and crystallogenesis attempts [56, 57], as anticipated a few years ago [58]. In other words, despite the apparent futility, cloning/expressing receptors from multiple sources might lead to findings of major importance. More recently, however, an attempt to clone the second melatonin receptor from the European hamster, previously reported as a natural knockout (as in the other hamster species), partially failed because of difficulty establishing the appropriate conditions to copy a particularly rich G-C region of the gene, as we had done for the sheep MT_2 receptor [55]. Finally, in our quest for stable versions of the melatonin receptors, we engaged in a series of cloning programs of these GPCRs from different animals, including bats, birds, snakes, and various mammals, most of which were not published (Guenin and Boutin, unpublished). Indeed, we ended up trying to characterize the melatonin receptors of the strangest mammal, the platypus. We did clone the ancestor of gpr50, a.k.a., Mel1c, and characterized its unique pharmacology; gpr50 has lost its melatonin-binding capacity in all mammals except for the platypus (Gautier et al., in press). Following the same line of work, we started a program aimed at validating the existence of a third melatonin-binding site [59], as reported earlier [60], to finally attempt and succeed in purifying it [43]. This effort led us to a series of studies demonstrating the key role of this enzyme in many different contexts [61, 62].

Of course, channels and receptors (as well as membrane-associated enzymes) require a system of expression in which the channel activity can be followed unambiguously because quite often, purification of these proteins is extremely difficult to achieve.

2.4. Testing the enzyme or the receptor (or else)

As noted, and as can be understood from the scheme in **Figure 1**, testing the enzyme or the receptor is the cornerstone of the whole process. Assaying a target involves several important requirements: the test should be robust, fast but reproducible, easy to handle and cheap and if possible should address only the activity that is targeted with minimal interference from the compounds in the assay ingredients. Several reviews have been published on this issue. For instance, the race for label-free solutions [63] or the use of instruments that measure directly the amount of product formed (or consumed) during the assay [64] are two perspectives on the never-ending moving/changing landscape that rises into view as soon as the screening process is involved. Books could be written on the way a screen must or can be done. Below are just a few examples of the choices we made.

Choosing the right test is a key decision that will influence the rest of the program. In the past, we spent some time trying to identify the best assay for our kinase program and evaluated the

whole literature for the best possible options [65] before finally attempting to run our own original version [66]. We frequently found that the assay described for our target class was not fully adaptable to our instruments or presented potential interference with the assay component(s). Several dozens of assays were adapted from the literature and/or from the material we had in hand, such as the systematic use of HPLC [9, 39, 41, 67–69] and more recently, mass spectrometry [70]. Worth noting here is the use of a global technique such as NMR. Indeed, a feature of this method is of great interest for us: it has poor sensitivity but a high robustness [71] and can be used not only for screening purpose but also for monitoring poor-affinity target/compound interactions, as in the first step of the search for receptor ligands. In addition to its obvious use as an analysis technique, we have adapted this method in various frames, including a way to better trace enzymatic activity [72], use a fluorine-labeled spy molecule as a ligand for an enzyme (that screened compounds can chase) [73], or verify changes in a component of a reaction [74]. These techniques are all automatable and thus can be used with less staff power as long as the automate is running by itself—day and night—and the validation of the assay guarantees stability of the components over time.

When turning to non-enzymatic assays, such as receptor binding or protein-protein interaction, the constructs necessary to reveal the activity of the candidate compounds might be complex. For the receptor assays, where binding is the known first step, the functionality is the key information: e.g., agonists as well as antagonists are ligands in a binding assay, and only the functional assay—often cellular [75–77], but with exceptions [78]—thus should be able to distinguish between both entity types.

The recent evolution in understanding of receptor bias ligands has considerably changed our view of receptology. Indeed, for GPCR studies, compounds can be specific antagonists of a given signaling pathway (see, for example, a recent survey on the melatonin receptors [79]) but partial agonists in another measured pathway. Even if the bottom line is that an agonist should lead to the internalization of the receptor—which the antagonists should not—there will be exceptions and changes in paradigm(s) ([80], for instance, and Legros and Boutin [unpublished]).

Of course, in some cases, we had to develop and assess a completely new assay, such as when we addressed the difficult question of PPARγ ligands and had to use a new method of binding onto this soluble protein [81].

Globally, it is fair to say that four molecular target types cover most of the druggable biochemistry of living organisms: the enzymes, receptors, channels, and protein-protein interactions. In fact, our view is that a full spectrum of possible techniques should be available to play with and adapt to the current project, while validating as much as possible the potential biases of such techniques. We decided very early to double-check the results of a screen (i.e., validate the hits) by using an orthogonal assay (see Section 2.9), that is, an assay that does not have the same technical approach as the initial one.

2.5. Automatizing the assay

Automation is the capacity for an industrial laboratory to run literally hundreds or thousands of assays a day and be able to extract from this formidable amount of data a promising

candidate that will fulfill at least some of the criteria for future development. It is thus important to have a decent number of automated stations without losing the capacity to understand and control the various parameters of such an assay.

We decided to go with small, independent stations that were not entirely automatized. One of the reasons for this choice was the fact that we could collaborate with the SIMM group that had all the capacities for running mammoth screen campaigns (over one million compounds). In addition, we believed—and still do—that some modularity is essential for the involvement of the technical personnel in these very repetitive tasks. Such modularity is more adaptive to numerous situations than a single, heavily integrated robot that will take weeks if not months to reconfigure for another assay, especially when the assays cover a very large spectrum of techniques. Indeed, we faced campaigns based on assays for widely different proteins and targets, including enzymes, receptors, protein-protein interactions, and channels, requiring being able to adapt the material at hand to the various approaches for screening these different target types.

Another aspect that is particularly important for us is repeatability of the screens. Indeed, we chose to run assays in independent duplicates at two different calendar dates and possibly with two different operators, leading to a higher level of robustness for the results, as long as both results were close to each other (see below).

2.6. Screening

Screening is the modern version of intelligently seeking the needle in the haystack. It concerns a test in which literally hundreds of thousands of compounds are evaluated, most of the time without any *a priori* information on their chemical structure or class (e.g., peptides, fragments, drug-like compounds, aptamers, toxins, natural extracts, natural compounds, etc.). Therefore, for cost constraint reasons, it may become important to screen in a smarter way [82]. This search has become a science in itself, a science that relies on chemical diversity. We view it as follows: the more diverse the compound library is, the more chemical space it covers, and the more successful we might be in finding one or several hits for a particular target.

In a given library of compounds, what are the chosen compounds that will lead to a new drug (i.e. active, specific, non-toxic, and patentable)? A perspective from some leading pharmaceutical companies was published in 2011 [83]. They gave their common view of the HTS programs, what they delivered, and what they failed to deliver. This particularly unusual publication (co-signed by the major pharmaceutical companies) exemplifies the reality of HTS expectations (too high) and of the quality/amounts of the results (the new drugs, too low). Nevertheless, as with the main progress in life science technologies in recent decades, the rise of HTS has led to plenty of new discoveries and approaches. We should not forget that all of these techniques accelerated discoveries, and even if those are not yet transformed into new drugs for the patient, they still add to the understanding of physiopathological processes. Mayr and Bojanic described the basic organization of an HTS laboratory—among other types—a few years back [84].

2.6.1. High-throughput screening

We embarked about 15 years ago on establishing a minimal HTS department in which a couple of screening robots together with an integrated analysis system permitted 'rapid' screening of several thousand compounds. The adaptability of the machine quickly became a problem: expanding our screening capacity—in terms of diversity of assays as well as number of compounds—would require several identical instruments running in parallel on which specialized personnel would apply their skills in an unvarying way. We chose something else for a time. We aimed at having small stations independent from one another to be able to treat or read between 40 and 50 384-well plates (~15,000 compounds). There are no general rules about how to arrange such a laboratory: it depends on the space, money, and personnel available. We felt that the whole process should be rigorously simple, with several key steps (go/no-go decisions) from the basic setup of the assay (and its validation with reference compounds) to the automation and test on a small scale of 10,000 compounds and finally both full-deck campaigns.

Figure 2 shows a series of typical examples of data obtained with this set of methods. In general, we screened about 10,000 compounds using the final conditions (set up previously) in two independent campaigns with the same compounds and different operators, if possible. The analysis of those data is what will permit moving forward to the full campaigns of 250,000 compounds, tested twice on the target. Without going too much into practical details, we found this methodology to be the most appropriate. Even if 2 or 3 out of 30 HTS campaigns turned out to be mute (no compound out of the HTS campaigns), the rest delivered several classes of compounds that could be pushed to the program downstream.

The figures show the repartition of the compounds according to their level of activity (left) while the graphs show the actual data where the results of the first test are plotted versus the second series of tests. The two tests are experimentally identical but run on a different day and if possible with a different operator. Because most of the compounds should be inactive, the Gaussian curves are centered to 0. The graphs should be aligned according to the diagonal of the rectangle because the data are theoretically identical, and should be massively located around 0. In **Figure 2A**, the first robotic setup led to a fat repartition of the compounds on the Gaussian curve, suggesting poor reproducibility, which is confirmed by the format of the correlation graph (or rather the lack thereof). In **Figure 2B**, with another setup, the data are more centered to the 0 and diagonal areas, respectively, in both figures, and in **Figure 2C**, the test was run first with a set of molecules, as in a feasibility attempt. We designated this process as a pre-HTS campaign. The data show a very good reproducibility of the tests, with compounds gathered along the diagonal of the graph.

2.6.2. HTS system: factory or small business?

It is the fashion to elaborate big systems that are very integrated and can run 'any' type of assays for any given number of compounds in a library. Unfortunately, despite beautiful examples of successful efforts with such machines, the issue of time frame in such situations is an elusive one. Indeed, moving on the same instrument from an enzymatic assay (e.g., with

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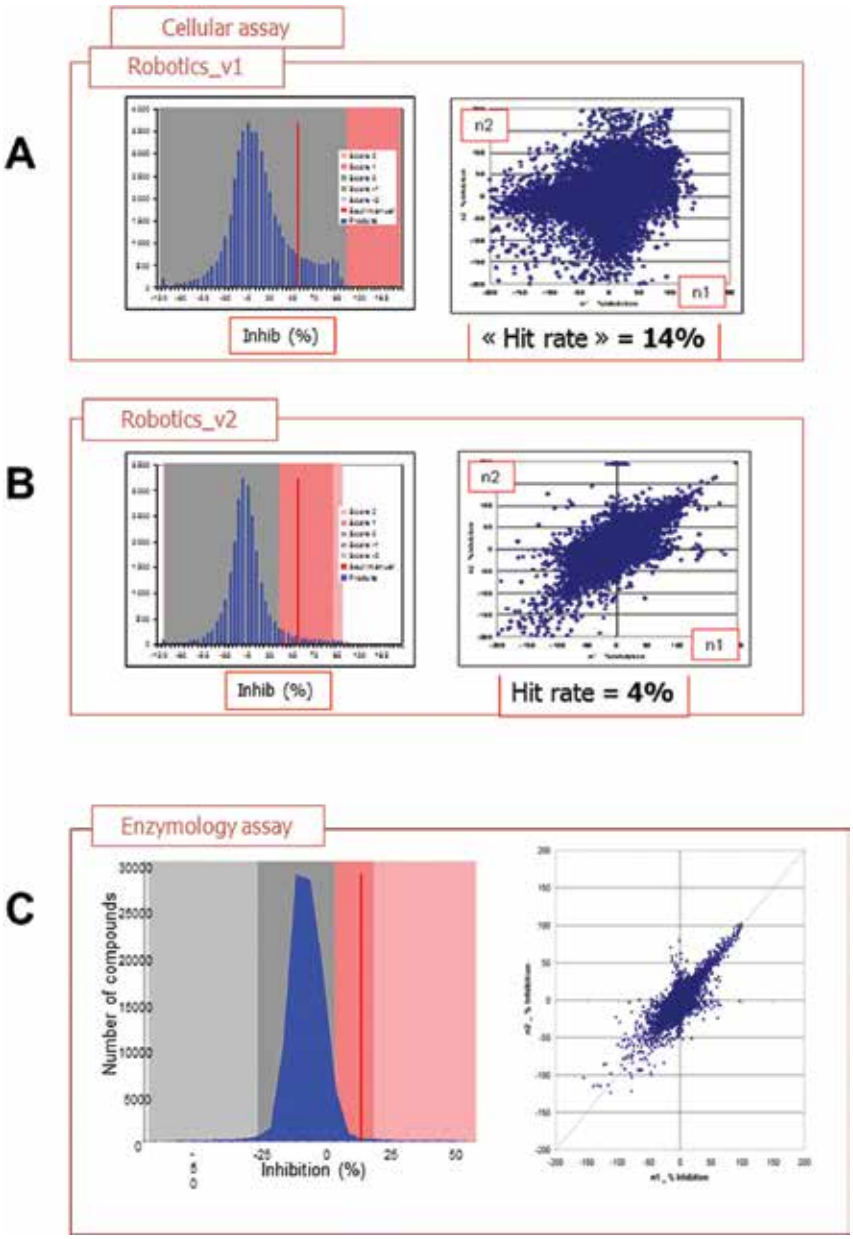

Figure 2. A typical and real example of the influence of the robotic setup on the quality of the screening results. The test was a cellular-based assay (*), comprising the detection of an intra-organelle color change potentially due to the tested compounds (see Jensen et al. for full description of the test [85]). A and B show cellular-based assay results; * note the use of differentiated primary cells; C shows an enzymatic assay. All results (whatever the biological sources) should resemble panel C. Poor results such as A and B, even if partially corrected by adapting robotics (between A and B), would lead to massive amounts of false positive hits.

fluorescent probes) to a cellular assay (e.g., with engineered cells) involves different instruments, setups, and prerequisites. One cannot honestly predict the length the preparation will require, and to a certain extent, the length of the global process (assessment + campaigns).

In the particular case of cellular assays, and as discussed elsewhere, the use of cancer cell lines brings difficulties, due to their genetic baggage, that is, to say the least, abnormal, leading to overexpression of many proteins. Alternatively, cells might be primary cells differentiated or not, the use of which leads to major variations from cells to cells, ultimately leading to massive false positive amounts (up to 14%, see **Figure 2**) and poor reproducibility from batches to batches. Finally one possible choice would be to work with differentiated stem cells, as their robustness is enhanced due to the current state of the art, with more and more publications describing works in this area. All these cells can be engineered with modern transfection techniques.

It is always possible for extreme cases to use different approaches. For instance, in a premodern time, we ran a HPLC assay for AANAT, ending with interesting hits despite the "low" number of compounds tested [67]. It is certainly an option whenever the available test involves extremely costly reagents or engineered fragile cells.

2.6.3. High-content screening (HCS) and its obligatory companion, functional genomic screening

High-content screening is a fast cellular-based approach that became possible in recent years, thanks to progress in imaging, computing, capturing, analyzing, and automating those processes. Moffatt et al. has summarized these approaches [86]. One also has to keep in mind that whatever the complexity of the engineered cell-based assay that is designed to identify compounds specific to given (cellular) pathways, it is probably less complex than the cellular metabolism. In other words, once the compound has been identified, one must check the *in situ* nature of its target. Treatment of siRNA libraries of the cellular systems and/or reverse pharmacology can be used to such an end. Saeidnia et al. [87] has reviewed the reverse pharmacology and possible solutions that are available.

The latest trend is to screen phenotypically engineered cells for the pathway in which the target is involved. If a compound is found, the validation process seems cumbersome to us. In fact, the deconvolution of the phenotype, to identify the actual target(s) of the hit compound(s), is a long process that is interesting, difficult, and expensive (in time and money). Cautions also have been published recently, warning scientists using such methodologies to be extremely careful in turning to these approaches because the number of biases is immense. We only very recently entered onto this path. In the meantime, we had serendipitously prepared the next steps by a very early attempt to industrialize our previous success in reverse pharmacology: the discovery of the third melatonin-binding site as quinone reductase 2 [43]. Nevertheless, this success has not been the rule. Several attempts done internally to find the target of given pharmacological agents have so far failed.

2.7. Screening what?

At the start of the year 2000, the question was, "How many compounds do you have in your library?" Without reference to the diversity of the compounds, some big players in the game had more than a million compounds available. Indeed, diversity is a complex problem that

has been discussed at length by various authors (see Gillet et al., in particular [88, 89]). In the chemist community, it is often joked that there are as many definitions of diversity as individual chemists. Briefly, we chose to stay on the following path. Based on a definition of diversity that we found clear and attractive [90] and that gather all the basic concepts of diversity (and their measurement), we chose to base our own measure of diversity on the pharmacophore concept [91]. We felt that the more we wanted to pinpoint this diversity concept, the more we became lost in endless descriptors of the molecules [92]. Ultimately, we used the approach of Lepp [93] to establish a representative sub-library of compounds that can serve as a simplification of our complete library for pre-test purposes (see above) or to limit the number of compounds whenever the screening campaign uses a test that is either too expensive or cellular (i.e., "too" complex). Another way to define diversity is to measure the similarity between compounds, another approach that has also been discussed at length [94].

The diversity of the product collection that will be tested is indeed important and allows for anticipation of the discovery of new series on which the medicinal programs will be developed. Achieving diversity, however, will require the inclusion in the library of compounds with unusual or poorly documented structures.

A company like ours has a 60-year history of chemical synthesis of drug candidates. These molecules are still available in the compound collection and constitute an original source of diversity. To complete this panel while maintaining the largest diversity possible, we chose to integrate into the compound library natural pure products, extracts, peptides, and more generally 'drug-like' chemicals either synthesized internally or commercially available. We targeted having a total of about 250,000 compounds screened, systematically, for any targets of interest.

2.7.1. Natural compounds

A fundamental in this domain is certainly the belief that ethnopharmacology can identify interesting natural molecule(s) from traditional medicine (see Heinrich et al. for background in ethnopharmacology [95]). Ethnopharmacology focuses on a compendium of mixtures that medical practitioners in native populations prepared and gave to their patients, sometimes with outstanding success. The complexity of these approaches is immense. Indeed, besides the type of the plants used—not always easy to trace and not always easy to find in historical locations—there is the nature of the preparation, including the mixture of several different plants and associated products (of animal [including insects] or mineral origin). We tried to access the two main available sources of such compounds: pure compounds or extracts that need to be deconvoluted, usually by bio-directed strategies. We have reported our experiences with the latter in three publications [96–98] but rapidly abandoned these approaches for the following reasons: paucity of results; tediousness of the process; difficulty in repeating the exact same experiment, including trouble finding plants at the same location with the same properties; and last but not least, often difficulties with the structure identified, which turned out to be common or of a complexity beyond the reasonable feasibility of industrial production. It remains interesting to view those compounds as a mold of the target binding site and/or to use them as an inspiration for a future drug. At the other end of the spectrum,

commercially available, pure compounds are hard to find in a decent quantitative supply. Often, compounds are available from vendors, but they are either common (flavonoids, coumarins, peptides, terpenoids, and alkaloids) or provided in minute quantities, with barely any reload possible. We therefore balanced our library with some of those pure compounds, as an internal control to assay, because often flavonoids—while poorly soluble—have turned out to be enzyme inhibitors (see, for example, [40, 99]).

In the interest of completeness, we add that one other path is to provide some biotech companies with a target. They screen the target with their proprietary sources of natural compounds (often as microorganisms, plants, and similar extracts to start with).

2.7.2. Peptide libraries

We became interested in peptide combinatorial libraries at the very beginning of this approach and studied several aspects of those libraries: numbers [100], quality control [101], and activity [102, 103]. For solid-phase synthesis approaches, the actual number of peptides in a given library is limited by the availability of amino acids on the resin, and that number is limited by the number of individual peptide repeats in the initial mixture, which is obviously by far the most complex one. The trick turned out to be the way syntheses were conducted, by having the last amino acids as a constant in each vessel (see Houghten et al. [104]). Regardless, these approaches are often interesting to follow, particularly when soluble or secreted targets are concerned.

2.7.3. Compound libraries

Despite the large diversity of compounds available for screening, whether through scientific collaboration, subcontracts, or vendors, the nature of the compounds is one of the most sensitive secrets of the pharmaceutical companies. As stated above, we attempted to complement our more than 60 years of internal chemical synthesis with compounds bought or synthesized with the greatest care to try to achieve the widest diversity of compounds and to be able, at the end of the screening process, to validate original molecules with great potential. This strategy is based on our capacity to analyze and understand chemical diversity and thus be able to predict what should be synthesized to fill in the gaps in this diversity picture.

2.8. Choosing the hits for further programs

The question here is, "Choosing among which hits and why?" In the industry, the answer is simple: to heal and to make a drug. For other scientists, the answer might be slightly different: understanding a pathway (such as those at the core of a pathology) or finding tools that permit the study of the pathology, the receptor, or the enzyme. In any case, the path from hits to lead and then from lead to candidate is particularly long and painful. Thus, the structure of the various candidates (often from two or three different families of compounds) must be chosen extremely carefully to avoid the various obstacles: chemistry difficulties, including that the drug will be synthesized at the ton level at the end; specificity towards the other proteins of the body (not less); pharmacokinetics (often the compound should be given orally);

and patentability. It is interesting to note that exceptional universities, such as the University of Michigan, have published their reflections on this subject [105]. On the other hand, one should realize that the hit rate of an *ab initio* screening is somewhere between 0.01 and 0.1%. For 250,000 molecules, that translates to 25 and 250 compounds. It looks like plenty to choose from, although the hope is that among those compounds, some are similar, suggesting a structure-activity relationship that enforces the results of the HTS.

Sometimes, as noted, researchers will choose compounds for their availability or their known lack of toxicity, despite other problems such as a lack of specificity. The purpose then is different: finding tools to validate a pathway or the engagement of a target in a given pathology (see Section 2.2). Examples like melatonin [106], curcumin [107], or resveratrol [108] are also interesting to note. Indeed, these compounds are fantastic tools to better understand a network of pathways involved in various pathologies, but none of them shows any type of specificity. To the contrary, they seem active in many pathological situations because they interfere with many targets.

A more recent approach has also involved the systematic use of chemoinformatics analyses with its formidable capacity of calculus and prediction [109, 110]. Starting from there, it is possible to rationalize the choice of the best hits: among others, the previous history of the compound in the earlier campaigns on other targets, similarity with known compounds from the literature, patentability, and accessibility to the chemistry (not too complicated to allow 'easy' generation of a large series of analogues).

2.9. Confirming the hit activity

Once hits have been identified, the process continues in validating the molecules in their recorded interaction with the targets. Genick and Wright [111] addressed this subject, among others, in their recent paper. Essentially, it comprises three aspects: biochemical validation, biophysical measurement, and structural data.

Our first step is towards an orthogonal confirmation of the activity of the compound. Obviously, whenever an enzyme is concerned, it is 'quite easy' to invent or copy an alternative assay that engages different physical parameters, going from the old, global, radioactive phosphocellulose paper-based detection assay [112] to a more specific radioactivity-based assay [113] for a kinase, for instance; trying to go from a protein-based phosphorylation assay [114] to a more specific peptide-based assay [66] or lastly, going from an antibody-based assay to a more specific ubiquitinated peptide-based assay [115]. There are multiple examples of such orthogonal assays, such as the use of HPLC, fluorescence polarization, mass spectrometry, and fluorescence resonance energy transfer (FRET) technology. We always felt that part of the reproducibility problem(s) was (were) due to the result of the poorly understood interferences between components of the assay, even sometimes the totally aspecific actions of the compound onto the target, for instance, by precipitating the protein rather than inhibiting it, with a similar effect in the assay (lack of activity in the presence of the compound). Compounds at the concentration used in the screening process—often 10 μM—tend to precipitate. The addition of dimethylsulfoxide (DMSO) as a compatible organic solvent limits this precipitation to a certain extent, but the presence of DMSO might affect the enzyme or the reactivity medium

(not to mention the receptor, membrane-bound assay). This is also why we argue for work on purified targets to simplify, at least in this context, the environment of the protein.

For the receptor assay, it can be more complicated to find an alternative, often because of the uniqueness of the radioactive ligand used in the binding assay. We tried to circumvent this problem by developing options in this context, for example, in synthesizing other ligands, to have more than a single binder in our melatoninergic [116, 117] or MCH toolboxes [118]. If this goal is achievable for receptors that have been studied for decades (serotonin, adenosine, histamine, and melatonin), for other more recently studied receptors, it has been completely impossible, including the obvious case of orphan receptors for which ligands are not known and assays are built around receptor functionality. Thus, the study of such receptors has largely been done through efforts to de-orphanize them [119, 120] and to fully characterize them with all the tools of molecular pharmacology, as for gpr103 [121–124] or gpr50 [125, 126]. An easier alternative is to work with known ligands as peptides, as in our extensive work on melanin-concentrating hormone for which alterations in the natural sequence led to alternative ligands [118], agonists [127], or antagonists [28, 128]. Beyond the thrill of executing a *tour de force* design and the joy of manipulating peptides of short sequences, with their infinite possible variations, the work brought a panel of new tools that might ultimately contribute to understanding such integrated systems.

2.10. Structuring: what is the hit/target relationship at the atom level?

As noted, part of the validation is linked to the visualization of the molecular structure of the complex between the hit and the target. The key role of this approach has been reviewed numerous times (e.g., Scapin [129], Hu et al. [130], and Zheng et al. [131, 132]). It is now clear that the progression of the compounds from a hit to an elaborate drug will benefit immensely from structural biology data. We contributed also to a certain vision of how alternative methods will complete (and possibly compete with?) crystallography, particularly cryo-electronic microscopy [133].

We made the choice of an active and ambitious structural biology approach about 10 years ago. Creating efficient collaborations with some groups, our first attempts were to characterize the interactions between some of our compounds and a given target in neurogenerative diseases [134–136]. We then embarked on several kinase-related projects that ended with the discovery of powerful compounds [137–140]. However, we needed more freedom to operate and establish proof of concept of the importance of these approaches to complement our drug discovery programs. For this reason, we chose to install a dedicated laboratory inside a synchrotron (Soleil, St. Aubin, France) to gain full access not only to the beamlines but also to the vast scientific community behind this instrument. This choice led to several collaborative academic-oriented lines of research [136, 141] and to more internal research programs. In preparing the next steps of crystallization—the step involving the receptor and more generally membrane proteins—we assessed a series of methodologies for the solubilization and purification of functionally competent receptors [56, 57]. Furthermore, we also studied the impact of microfluidics on crystallization of proteins for structural biology studies [142, 143].

The companion science of structural biology is molecular modeling. It is key for discovery programs, either at the early stage (explaining and rationalizing the molecule-target relationship

and even quantifying it) or at a later stage when new molecules, derived from a hit in the screening campaign, will be synthesized and need to be paused inside the structure of the target by calculation or co-crystallization. We have reported some examples of the use of those techniques for glucokinase [144], gpr103 [124], and rev-erbα [145].

2.11. "Biophysing": understanding and measuring the forces responsible for the relation between the molecule and its target

Many reviews have nicely summarized recent community perspectives on this aspect of compound characterization [146–148]. No doubt, the rise of all the physical biology methods affords more and more tools that are essential to a modern drug discovery program, particularly when trying to understand and measure the relationship between the compound—that is, the drug candidate or its ancestor—and its protein target.

From the seminal work—for us—of Shuman et al. on HIV proteases [149], we started to wonder if the complicated concepts of thermodynamics could be applied to the process of moving from hit to lead compounds. What would be the help of measuring the actual parameters of a target-compound association, even if the target were not a receptor but an enzyme? We became adopters of surface plasmon resonance (SPR) [150], calorimetric instruments, fast-flow machines to measure events of enzymatic activity that occur faster than a second [151], and native mass spectrometry to gain information on the minute conformational changes of proteins [152].

It is now clear that those tools have become part of ongoing studies for helping medicinal chemists better evaluate and thus better understand the impact of the minute modifications they make to their compounds. For instance, the use of calorimetry to determine the actual affinity of an inhibitor for its enzyme has proved to be accurate [134], while standard methodologies such as binding turned out to be cumbersome if not wrong [153]. Furthermore, the determination of the number of compounds and their nature associated with a given protein was also a key step in our understanding of quinone reductase 2 enzymatic behavior [70].

These "new" tools are increasingly reliable. "Structuring" and "biophysing" help give access to precise details on the structure of the co-crystal together with actual thermodynamics measures and thus the relationships at the atom level between the target and its ligand (at least, and for the moment, for enzymes); however, crystallization of the membrane-bound proteins (such as GPCRs) is still a challenge, despite recent major progress [154, 155]. These newer tools permit visualization and quantification of these interactions. The medicinal chemist can use this information as the basis for a synthesis strategy for the evolution of hits towards a series of compounds with *ad hoc* properties that are easier to predict and rationalize.

3. What next?

Research has always been a question of strategic and/or opportunistic decisions at a given time to explore areas that look promising for a better future. These decisions, at the industry

level, are always difficult to make. Money, time, and energy go into anticipating what will be important—technology-wise—for the next decade of discovery.

Among the published reports that we attempted to explore were many approaches involving new, non-invasive techniques to better understand the role of a compound and its target inside a cell, the future of stem cells and of chemical proteins, and the modifications of proteins inside the cells.

3.1. Stem cells as expressing systems

Stem cells have been the new frontier for some time and remain so. One debate has been whether to target therapy with stem cells, directly (in cell therapy) or indirectly (by using the secretosome of differentiated stem cells) or to use the cells as hosts for a target that would then be expressed in an optimal environment, as opposed to the classical use as hosts of cancer cell lines that are either very derived from the original cell lines or comprise numerous nuclei with profoundly abnormal karyotypes. We chose the isolation, differentiation, production, and characterization of such stem cell-derived cardiomyocytes [156]. Potentially, these techniques open new roads to the use of natural hosts that would be a better context for the expression of targets in a natural environment. Furthermore, as demonstrated in several instances, stem cells can be derived from patients, and some can recapitulate the disease at the cellular level, leading to a possibly better understanding of the molecular cause(s) of a given disease [157, 158].

It might be important in the coming years or decades to be able to cope with the therapeutic use of such stem cells, despite initial concerns [159] and the extraordinary characteristics [160] of these cell types.

3.2. Chemical proteins

A boom in the area of chemical proteins followed the discovery—among others—of chemical ligation [161]. It became possible to synthesize enzymes chemically and measure their catalytic activity. After the chemical synthesis (by solid-phase synthesis) and use of ubiquitin, described for several decades, we chose a small enzyme (120 aa), calstabin, that we completely synthesized with our partner. We crystallized this enzyme and measured its catalytic activity. In doing so, we learned two important things: (1) such an approach is feasible, even though it led to small quantities of material, at least to start with, and the material was indistinguishable from the recombinant version and (2) the apparent mono-peak analysis of the result of the synthesis revealed, after further analyses, that the protein could be separated in two distinct peaks, one a fully refolded protein with catalytic activity and the other a denatured protein with no catalytic activity and no refolding [141]. These findings led us to consider that large peptides/proteins that are chemically synthesized and often described as being used in the literature (e.g., EPO, growth factors) might be a mixture of active and inactive substances if not appropriately characterized.

Our aim was and still is the validation of a process encompassing both the recombinant expression of pieces of a large protein—such as an antibody—that contained only natural

amino acids and other pieces of the same protein, in which exotic amino acids were inserted. The fusion between those pieces would then be performed by chemical ligation(s). These approaches will open avenues to the incorporation of key functional groups in protein for derivatization with drugs (antibody-drug conjugates) or to incorporation into proteins of exotic chemical functions.

3.3. Ligases

Among the seminal studies of the *in cellulo* modifications of proteins are the numerous efforts of Alice Ting's group [162]. Using a modified ligase, it became possible to directly label target proteins in a cellular context. The label can be a fluorophore, radiolabeled compound, or NMR probe, for example, and the chemistry of the modification is the main limitation of this process. Coupling it with the power and resolution of magnifying instruments, including specialized beam lines in a synchrotron, can lead to the cartography of the protein inside a living cell. We began the process first by characterizing the enzymology beyond this ligase-catalyzed enzymatic reaction, using purified enzyme and reagents in an acellular context [163]. This technique will be adapted to intracellular processes with the goal of modifying a recombinant protein *in situ* and to allow labeling of a protein inside the cell to follow its fate.

4. Outside the box

Anticipating tomorrow's needs in modern research is a challenge that requires triangulating the many different areas necessary for drug discovery. It has thus been difficult to choose various avenues to make our efforts more successful, modern, and interesting and at the service of future trends and needs in drug discovery for therapies and cures. Choosing to enhance our capacities to incorporate new approaches is always a challenging but moderate risk, based on the way we perform these exploratory strategies. Indeed, one critical choice was to hire postdocs to scrutinize those ideas. In doing so, we not only identified and incorporated strong scientists but also could assign new technologies to categories such as 'dream', 'usable soon', 'needs more time', etc. Among the discoveries that we have published are the ligase use for *in cellulo* transformation of proteins [163], including antibodies; cellular imaging using large synchrotron instruments; synthetic proteins that can be as active as the recombinants [141]; and synthesis of proteins and peptides modified by ubiquitination the way it happens in the cell (as opposed to what was commercially available at that time) [115]. In addition, we started our first trials in thermodynamics, structural biology, new crystallization techniques, native mass spectrometry for structural biology approaches [152] and for target-molecule relationships, and the use of human stem cells as an alternative to rat cardiomyocytes in first culture, among other initiatives.

The engineering of cells and their use as therapeutic agents bring new challenges and new wonders almost daily. New challenges emerge because the necessary work to achieve therapies for tomorrow may not necessarily be what was done in the recent past. These challenges

often require new approaches, particularly in the use of treatments with stem cells from the patient, or with cells that have been engineered to fulfill a task that only science fiction writers dreamed about in the 1950s. Just one example is the seminal paper by Schukur et al. [164] on the possibility of engineering a cell to produce a factor (such as tumor necrosis factor) following a native regulatory pathway in the patient in whom these cells will be grafted. The number of new technologies required to reach that end is just immense. If we do not prepare for such wonders, the industry will have an extremely complicated time reorienting to tomorrow's health-related demands.

5. Conclusions

The body of techniques developed and applied in the DD platform throughout the years has contributed to the introduction of several molecules on the market. The contribution of this platform concerned the initial steps of compound discovery that proceeded towards final molecules. Or else, the platform was involved in providing data on the biophysics of the interaction, most of the time, all steps of drug discovery contributed to the final results. Some findings have been published and showed the completeness of the approach that laid the foundations for the discovery and early clinical development of drug candidates [138, 139, 165].

There is obviously confusion between the use of research that is the replication of data obtained elsewhere (the role of the industry) and the de novo research programs that are developed in the pharmaceutical industry. The confusion is that work consisting of new research should not be done in the industry, but in academic institutions, while repeating published data should be done in the industry. In that way, by limiting the novelty of the industrial work, the novelty of the cure(s) found in such context might be limited, thus leading to a situation where alternate paradigms must be found. For instance, a way to access to novelty might be to fund biotechnology companies to explore new research areas and new ideas.

To echo arguments already made above, most of the published research is difficult (if not impossible) to repeat. And as noted, there has been a long debate in the scientific literature on the repeatability of data [166, 167]. Therefore, part of any efforts at clarity and repeatability should be sorting the reality from the fantasy in what we are reading. Furthermore, the key to success is often to be the one company that delivers first on something for the patients' benefit. Anyone coming in after the first- or second-place entry will be less rewarded. As mentioned in the introduction, research attrition in the pharmaceutical industry is around 90%, meaning that 9 out of 10 projects fizzle before reaching the clinic. In the meantime, the (discovery) show must go on, and the company must sustain this endeavor with internal and investor money or turn to other sources of innovation.

Finally, research is a never-ending dynamic process. If development of new techniques and new concepts ceases, such a drug discovery platform can rapidly become obsolete.

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Chapter 3

Drug Treatment of Obesity: From Bench to Bedside

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Additional information is available at the end of the chapter

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Abstract

Obesity is a complex metabolic and behavioural disorder associated with increased health risk, including coronary artery disease, congestive heart failure and sudden cardiac death. Effective prevention and treatment strategies for obesity are needed. This unmet need for efficient and safe antiobesity medication resulted in many new therapies at various stages of development. Obesity has become one of the most intensively studied diseases because of the availability of suitable animal and cell culture models of adipocyte differentiation and appetite regulation.

Keywords: obesity, novel therapeutic targets, animal models of obesity, cell models of obesity

1. Introduction

1.1. Obesity: a global health issue

Obesity is a complex metabolic and behavioural disorder defined as an excess of body fat with a body mass index (BMI) greater than or equal to 30 kg/m^2. A combination of Western diet habits (increase in consumption of animal products, refined grains and added sugar) and sedentary lifestyle results in the excessive nutrient intake, leading to the hypertrophy and hyperplasia of the adipose tissue [1]. Obesity is associated with increased health risk, and the most perturbing danger is the increased predisposition for coronary artery disease [2], congestive heart failure and sudden cardiac death. Beside this, obesity is a major risk factor for a wide range of diseases, including type 2 diabetes, dyslipidaemia, obstructive sleep apnea, asthma, musculoskeletal disorders and certain types of cancer.

Since 1980, the worldwide prevalence of obesity has more than doubled [3]. The predictions for the course of obesity rates in the future are not optimistic because it has reached epidemic proportions in many countries, affecting more than one-third of adults regardless of socioeconomic status [4]. Also, the economic cost of obesity-associated diseases has been estimated at $147 billion annually [5]. The health and economic consequences carry a significant burden on the global population. Given the fact that cardiovascular disease (CVD) is still the leading cause of death globally [6], the harmful impact obesity has on cardiovascular health makes this disease one of the main global health problems.

1.2. Physiology and pathophysiology of adipose tissue

Adipose tissue is connective tissue which forms a layer under the skin with multiple functions: helps to regulate body temperature, attaches the skin to the underlying tissue and protects body parts. It is divided into the central and peripheral compartment, the former including the subcutaneous upper abdominal and visceral fat masses and the latter consisting of hip and gluteal-femoral fat. The forming of adipose tissue takes place when adipocytes accumulate in large numbers and become the predominant cell type. With the increase in their number and size, the adipose tissue expands. The most important enzymes involved in adipocyte metabolism are endothelial-derived and hormone-sensitive lipoprotein lipase, crucial to lipid storage and release, and acyl-coenzyme A synthetases essential for fatty acid synthesis. A cascade of enzymes is further included in beta-oxidation and fatty acid metabolism.

Adipocytes are complex and metabolically active cells, increasingly perceived as an endocrine gland that produces several metabolites and peptides relevant to the body weight control [7]. Some of the adipocytokines secreted by adipocytes play a role in inflammation (tumour necrosis factor, interleukin 6) or blood coagulation (prostaglandins) and others are engaged in appetite regulation (leptin) and insulin sensitivity (adiponectin). Adipogenesis is highly controlled process during which fibroblast-like preadipocytes differentiate into mature lipid-laden, insulin-responsive adipocytes [8]. This process of changes in morphology and gene expression of preadipocytes depends on the communication between the cells and their surrounded environment and between the cells themselves. Adipogenic stimulators are peroxisome proliferator-activated receptor γ (PPAR γ), single transducers and activators of transcription (STATs), enhancer binding protein α, β and δ (C/EBP α, C/EBP β and C/EBP δ), fatty acids, prostaglandins and glucocorticoids. Also, there are recently found activators such as Wingless and INT-1 proteins (Wnts), clock proteins (Bmal1 and Rev-erbα), interferon regulatory factors (IRFs; IRF3 and IRF4) and B-cell factor 1 (EBf1). Inhibitors of adipogenesis are growth hormone, transforming growth factor-β (TGF-β), glycoproteins and inflammatory cytokines.

The nucleus arcuatus of the hypothalamus is a key nucleus in the regulation of appetite which integrates many peripheral signals controlling food intake [9]. Two major neuronal populations in the ARC are involved in the feeding regulation. One of them increases food intake by coexpressing neuropeptide Y (NPY) and agouti-related protein (AgRP), and the other inhibits food intake through coexpression of cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC) [10]. Their neuronal projections communicate with other hypothalamic areas for appetite regulation [11], including areas involved in the reward system. Adipokines represent peripheral signals that influence the hypothalamic network [12].

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After crossing the blood-brain barrier, leptin binds to receptors in the hypothalamus [13]. Leptin receptors are a class I cytokine receptor which act through Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). Leptin activates anorexigenic POMC neurons and inhibits orexigenic AgRP/NPY neurons, leading to an overall reduction in food intake [14].

The GI tract releases a plethora of regulatory peptide hormones, which mediate short-term feelings of hunger and satiety by changes in their concentrations. Cholecystokinin is released postprandially and inhibits food intake [15]. The preproglucagon gene is expressed in the intestine, pancreas and brainstem. It is cleaved by prohormone convertases 1 and 2 to produce glucagon and glucagon-like peptide (GLP)-1 in the CNS and intestine. GLP-1 is released into the circulation after the meal in proportion to the calories consumed and inhibits food intake via the vagus nerve [16]. Ghrelin is produced by the stomach and binds to the growth hormone secretagogue (GHS) receptor. Ghrelin initiates hunger before a meal and increases food intake by increasing hypothalamic NPY mRNA expression [17]. Peptide YY (PYY) is released into the circulation following a meal and cleaved by dipeptidyl peptidase IV (DPP-IV) to create the truncated form PYY3-36, which excretes its anorexigenic properties via Y2 receptors.

The adipose tissue becomes dysfunctional in obesity, with an overproduction of proinflammatory and reduced production of anti-inflammatory adipokines [18]. During obesity, several cells of the innate and adaptive immunity are infiltrating the expanding white adipose tissue as a result of local microenvironment stimuli (fatty acids, adipocyte cell death products, increased hypoxia). The presence of such inflammatory conditions in the obese adipose tissue influences other organ systems and contributes to the development of cardiovascular disease and metabolic dysfunction [19]. When adipose tissue ceases to store energy efficiently, the lipid flux is directed towards non-adipose organs. This ectopic accumulation of lipids promotes lipotoxic insults in cells, leading to insulin resistance, apoptosis and inflammation [20].

2. Current approaches for drug treatment of obesity

Effective prevention and treatment strategies for obesity are needed. Diet, exercise and behaviour modification are the starting point. However, with the constant failure of lifestyle modifications to be successful, the use of antiobesity drugs is vital. Pharmacological treatment of obesity is available for patients with a BMI ≥ 30 kg/m^2 or a BMI ≥ 27 kg/m^2 with comorbidities. Pharmacotherapy is successful in improving obesity-related health risks and quality-of-life and preventing the development of comorbidities [21]. If a clinically significant effect (<5% weight loss in patients without diabetes and <3% in patients with diabetes) is not achieved after 3 months, the pharmacological treatment should be stopped [22]. Options for the medical management are still limited because of the paucity of drugs approved by the Food and Drug Administration (FDA).

2.1. Centrally acting medications

Phentermine is a sympathomimetic amine that reduces the appetite and increases the resting energy expenditure. As a norepinephrine-releasing agent [23], it augments the adrenergic signalling in the brain and peripheral tissues. It was primarily approved in 1959 for short-term

treatment of obesity. When taken in the morning, the patient can take advantage of the increase in energy during the day and avoid insomnia during the night. It is well tolerated with the most common side effect being dry mouth and constipation [24], although heart rate and blood pressure can potentially rise.

Topiramate was initially approved as an anticonvulsant in 1996, but during epilepsy trials, it has also shown effects on the weight loss [25]. It is a drug with multiple mechanisms of action although the mechanism responsible for weight loss is largely unknown. It is thought to be related to appetite suppression and enhanced satiety via blockade of sodium and L-type calcium channels, inhibition of AMPA/kainate receptors, facilitation of GABA-mediated chloride fluxes and inhibition of carbonic anhydrase [26].

The phentermine/topiramate (PHEN/TPM) combination product was approved in 2012 as a combination of a lower dose of phentermine and extended release formulation of topiramate [27]. So far, it has produced the highest weight loss effect. The exact mechanism of action may be related to a reduction in compulsive food craving via antagonism of AMPA/kainate receptors, decreased lipogenesis, increased energy expenditure due to GABA-receptors activation and modification of food taste by inhibiting carbonic anhydrase isoenzymes [25]. PHEN/TPM is taken once a day without regard to meals. The most common side effects are dysgeusia, constipation, dry mouth, paresthesia, headache, upper respiratory tract infection and nasopharyngitis [28].

Lorcaserin is a selective serotonin 5-HT2C agonist approved for long-term weight loss in June 2012. It has a high affinity for the 5-HT2c receptor in the POMC cell region of the hypothalamus. The activation of POMC neurons results in decreased food intake and increased satiety. Recommended dosing is 10 mg tablet twice a day with or without food. Lorcaserin is metabolised by multiple hepatic pathways to inactive metabolites, which are excreted in the urine and can accumulate in patients with severe renal impairment. It is usually well tolerated with a headache, nausea, dizziness, dry mouth, back pain and upper respiratory infections being the most frequent adverse effects [29]. Activation of 5-HT2A can cause hallucinations [30].

The combination of naltrexone and bupropion was approved in September 2014. The idea for this combination therapy emerged when bupropion was shown to promote weight loss in patients with obesity [31] who were treated for depression. Naltrexone, an opioid receptor antagonist, demonstrated a reduction in food intake in animals but failed to produce significant weight loss in obese humans [32]. However, animal experiments suggested that addition of an opioid antagonist to serotonergic, noradrenergic, or dopaminergic drugs could help activate POMC neurons in the CNS and thereby promote satiety. This combination is available as fixed-dose 8 mg naltrexone/90 mg bupropion tablet. The most common adverse effects are gastrointestinal (dry mouth, nausea, vomiting, constipation) and nervous system related (insomnia, anxiety, headache, dizziness). This therapy is contraindicated in patients with uncontrolled hypertension, seizure disorders and eating disorders.

Liraglutide is the representative of glucagon-like peptide one receptor (GLP-1R) agonists initially approved in 2010 for the treatment of type 2 diabetes. Weight loss and improved glycaemic control were often observed during clinical trials of liraglutide for the treatment of diabetes, which led to the approval of liraglutide for the treatment of obesity in December 2014. GLP-1 is a gut hormone secreted by the endocrine L-cells after food intake and presented

in the brain where it modulates CNS pathways involved in energy homeostasis. It suppresses glucagon production and stimulates pancreatic insulin secretion [16]. Liraglutide delays gastric emptying and increases satiety through stimulations of POMC neurons [33]. The recommended dose of liraglutide for weight management is 3 mg daily with the starting dose of 0.6 mg injected subcutaneously in the abdomen, thigh, or upper arm. It is well tolerated with gastrointestinal side effects, specifically nausea, which occurs in almost 40% of patients; vomiting and diarrhoea are also frequent. If weight loss is less than 4% after 4 months, the therapy should be discontinued.

2.2. Modulators of dietary absorption

One of the important factors contributing to the ongoing obesity epidemic is a Western diet rich in animal fat, where dietary fat intake provides >40% of the caloric content of daily food consumption. Although the energy is obtained through metabolism of protein and carbohydrates as well, the amount of energy gained from fat is almost twice that of other compounds, and subsequently, a fat-rich diet promotes obesity more rapidly [34]. Most of the dietary intake of fat is in the form of triacylglycerols, and their absorption is more than 95%. The triglycerides are composed of saturated or unsaturated long fatty acid chains. After consuming triacylglycerols, they undergo emulsification through the gastrointestinal tract and subsequently hydrolysis into diacylglycerol, monoacylglycerol and free fatty acids by pancreatic lipase. Incorporated into bile acid phospholipid micelles, they enter the circulation as chylomicrons. When they reach the membranes of hepatocytes, adipocytes, or muscle fibres, they can either be stored or oxidised for energy. An effective weight loss could be achieved by targeting the molecules participating in the absorption and digestion of fat. Pancreatic lipase is the key enzyme in this process, responsible for the catabolism of triacylglycerols to free fatty acids and monoacylglycerols. Inhibition of pancreatic lipase decreases the number of absorbed triglycerides by inhibition of the free fatty acids and monoglycerides production in the intestinal lumen [35, 36].

Orlistat is the tetrahydro derivative of lipstatin, a natural product of Streptomyces toxytricini [36]. It has been widely used for weight management in combination with reduced caloric diet since 1999. Orlistat works via the reduction in dietary fat hydrolysis and absorption in the gut by inhibition of gastric and pancreatic lipases. It prevents absorption of approximately 30% of fat, and it becomes effective when the fat content of patient's diet is at least 30% of their total dietary intake. Recommended dosing starts at 60 mg increasing to the full dose of 120 mg three times daily, administered during or up to 1 hour after the meal. The main side effect is steatorrhoea, followed by defecation urgency, diarrhoea, flatulence and abdominal pain. With the use of lipase inhibitors, there is a risk of inadequate absorption of the fat-soluble vitamins. For that reason, multivitamin tablets containing those vitamins should be taken 2 hours before or after the administration of orlistat.

2.3. Medications that increase energy expenditure

Inhibition of the sodium/glucose cotransporter (SGLT) 2 in renal tubules increases urinary glucose excretion [37]. SGLT2 inhibitors were initially designed to reduce hyperglycaemia in people with diabetes by competitive inhibition of SGLT2 transport system in the kidney. Since SGLT2 is essential for the glucose reabsorption, its inhibition led to reduced glucose reabsorption by the proximal tubule and increased glucose loss through the urine. However, glucose

reabsorption requires good glomerular filtration rate (GFR) and individuals with reduced GFR will probably experience little benefit from this class of drugs [38]. In obese patients with type 2 diabetes, 300 mg canagliflozin daily for 26 weeks resulted in weight loss of 3.3%, improved glycaemic control and lower blood pressure compared to placebo. However, there was a high incidence of genital and urinary tract infections [39]. In contrast, empagliflozin for type 2 diabetes showed impressive results concerning reduction of cardiovascular mortality along with kidney protective effects [40].

3. Translational research of obesity treatment

Translational research is needed to transfer basic science findings into novel therapeutical interventions. Obesity has become one of the most intensively studied diseases because of the increasing prevalence and the availability of suitable animal and cell culture models of adipocyte differentiation and appetite regulation that have permitted detailed studies impossible to conduct in other models.

3.1. Data derived from animal models of obesity

With the increased incidence of obesity, it is imperative that animal models sharing characteristics of human obesity serve in the quest for finding novel prevention and treatment approaches. Human obesity is considered to be polygenetic in addition to environmental influences. Animal models of obesity are therefore divided into different categories based on manipulations of individual genes, but genetically intact animals exposed to obesigenic environments were also used. Most animal models of obesity are small rodents (rats or mice), commonly the leptin-deficient ob/ob mouse, the leptin receptor-deficient db/db mouse and its rat counterparts.

Animals with a defect in the leptin-signalling pathway, including lack of leptin production and leptin resistance, develop a morbidly obese phenotype characterised by hyperphagia, reduced energy expenditure and hypothermia. A single-base spontaneous mutation of the ob gene terminates leptin synthesis prematurely, thus preventing the secretion of bioactive leptin. The transcription factor STAT3 is a key component of the signalling pathway that mediates leptin's effects on energy homeostasis. The amino acid tyrosine at position Ty 1138 plays a critical role in the activation of this pathway [41]. The specific replacement of the gene encoding the leptin receptor in homozygous s/s mice disrupts the transcription factor STAT3 [42]. The diabetic characteristics of db/db mice derive from a single autosomal recessive mutation, a Gly to Thr mutation in the leptin receptor gene on chromosome 4 (Leprdb). The abnormal mRNA splicing leads to the subsequent production of a nonfunctional Ob-Rb protein and defective leptin receptor with the result of overproduction of extracellular leptin [43]. Homozygous mice are hyperphagic and obese, but they are fertile and less hyperglycaemic compared to the db/db mouse.

POMC is the precursor of the α-melanocyte-stimulating hormone (αMSH), a potent anorexigenic neuropeptide that reduces eating and increases energy expenditure by activating MC3-R and MC4-R receptors. Transgenic mice lacking POMC ($POMC^{-/-}$) overeat and develop marked obesity that can be exaggerated by a high-fat diet [44], while heterozygous mutants develop

an intermediate phenotype. Treatment with αMSH or other agonists of the MC4 receptor can reduce obesity in POMC$^{-/-}$mice. The MC4 receptor subtype is involved in the control of food intake by mediating αMSH and AgRP influence on energy homeostasis. Specific inactivation of the MC4 receptor causes hyperphagia and morbid obesity [45]. MC4$^{-/-}$ mice also have high levels of insulin, glucose and leptin and do not respond to AgRP or αMSH. The lethal yellow (Ay) mutation of the agouti gene leads to ectopic agouti expression. The homozygous expression is lethal while heterozygous offsprings are viable, but develop obesity within the first few months of life [46]. The obesity results from the ectopic AgRP expression and α-MSH antagonism at MC3 and MC4 receptors. The mice are prone to developing type II diabetes and are infertile.

Cholecystokinin (CCK) plays an important role in satiation as a stimulator of fat digestion, an effect mediated by CCK-1 receptors. The Otsuka-Long-Evans-Tokushima Fatty (OLETF) rat is a spontaneous CCK-1 receptor knockout model for studying dysregulated control of eating and obesity [47]. The obesity phenotype is relatively mild, and as a result of obesity, they develop diabetes with hyperglycaemia, polyuria and polydipsia by the end of 5 months. OLETF rats respond less to CCK-induced stimulation of pancreatic secretions due to the lack of functional CCK1 receptors in the exocrine pancreas. However, these rats can prevent obesity if they have access to a running wheel.

Many Sprague-Dawley rats become obese (drug-induced obesity, DIO) when exposed to a high-energy diet, whereas others have a body weight similar to that of control rats on a low-energy diet (diet resistant, DR). The exposure of animals to high-fat (HF) diet often results in the development of obesity due to reducing the central actions of insulin and leptin by a post-receptor effect [48]. Additionally, HF diet directly affects intracellular signalling pathways in hypothalamic target neurons resulting in changes in neuropeptide expression. Offspring of DIO dams are heavier and more obese than offspring of DR dams [49] and that obesity extends into adult life.

The GLUT4 glucose transporter is vital for glucose transport in adipose tissue stimulated by insulin. Transgenic mice overexpressing GLUT4 develop early-onset obesity with a marked increase in the number but not the size of fat cells. Therefore, the mice have been used to study fat cell replication and differentiation during the development of obesity [44]. Mice deficient in β3-receptors are moderately obese [50] as a result of decreased activity of the sympathetic nervous system. Similarly, mice lacking functional serotonin 5-HT2C receptors develop hyperphagia [51], which results in marked body weight gain and adiposity. NPY1R-deficient mice are suitable for the study of obesity in the absence of overeating because they develop obesity independently of an increase in eating, which seems to be caused by decreased energy expenditure [52] due to a reduced expression of the uncoupling protein type 2 (UCP2) in white fat tissue.

Receptor-interacting protein-140 (RIP140) is a nuclear hormone co-repressor, which regulates fat accumulation by interacting with oestrogen, thyroid hormone and retinoic acid receptors through 2C-terminal receptor-interacting domains (RIDs). Mice with global RIP140 knockout are lean and resistant to HF diet-induced obesity. Silencing RIP 140 in animal models results in a long-lasting weight loss and enhanced metabolic rate [53]. SMRT is another nuclear hormone receptor co-repressor. In genetically engineered mice, the disruption of the molecular interaction between SMRT and nuclear hormone receptors causes increased adiposity and a decreased metabolic rate [54].

The synthesis of triacylglycerols occurs in two steps, both catalysed by enzymes of the endoplasmic reticulum. The synthesis ends with the conversion of diacylglycerol to triacylglycerol, which is catalysed by the enzyme diglyceride acyltransferase (DGAT) [55]. DGAT has two isoforms, DGAT1 and DGAT2, highly expressed in liver and white adipose tissue. DGAT1-deficient mice demonstrated significantly reduced adipose mass and increased insulin sensitivity [56], indicating that pharmacological inhibition of DGAT1 may be a useful strategy for treating human obesity and type 2 diabetes. The DGAT1 inhibitor XP620 reduced apolipoprotein B secretion, triacylglycerol synthesis and dietary fat absorption in mice. Another DGAT1 inhibitor, compound 4a, caused weight loss and a reduction in liver triglycerides [57]. Acyl-CoA carboxylase-2 (ACC-2) has a regulatory role in fatty acid oxidation. An increased fat oxidation by ACC-2 inhibitors could be a potential future approach to maintain weight loss [58]. On the other hand, stearoyl-CoA desaturase-1 (SCD-1) is an important enzyme in the synthesis of monounsaturated fatty acids. Spontaneous and targeted deletion of SCD-1 reduces triacylglycerol and cholesterol esters in the liver and increases insulin sensitivity and energy expenditure, as well as diet-induced obesity in animals [59].

3.2. Data derived from cell models of obesity

The process of adipogenesis has been extensively studied since the 1970s [60]. The ability to study the transformation of fibroblasts into preadipocytes in a tissue culture has enabled the exploration of general cellular mechanisms. Different cell culture models and protocols have become available to study adipocyte biology and to illustrate the transcriptional cascade that promotes fat cell differentiation [61]. Mature adipocytes, mesenchymal stem cells and preadipocytes can be easily isolated from adipose tissue homogenates and used for research purposes. The advantages when using this model is a homogenous population of cells that are all in the same stage of differentiation and the ability to passage the cells. On the other hand, the molecular events representing adipogenesis in a cell line are not necessarily transferrable in a human preadipocyte, and the ability of a preadipocyte cell line to differentiate often falls with increasing passage number [62].

3.2.1. Animal cell models

Studies in animal models of obesity offer valuable insights, but their applicability to humans is limited by the existing differences in the physiology and metabolism [63]. The most commonly used animal cell models are murine preadipocytes. The advantage of animal cell models is that they can be derived from various locations and from animals of different ages, which give valuable information about depot- or age-dependent adipogenic or secretory mechanism [64]. However, these cell lines have a considerable triacylglycerol store that interferes with biochemical and microscopy analyses, and they depend on the genetics and conditions of the animals from which they are isolated.

The 3T3-L1 cell line is a well-established preadipose cell line derived from disaggregated 17- to 19-day-old Swiss 3T3 mouse embryos, which display a fibroblast-like morphology. Under appropriate conditions, such as treatment with adipogenic agents insulin, dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (IBMX), they acquire an adipocyte-like phenotype.

These cells provide an equal response following treatments because they are homogeneous regarding the cell population [65]. Moreover, they are easier to culture, less costly and can tolerate an increased number of passages. The main goal of the research on 3T3-L1 cell lines is to establish the underlying molecular mechanisms of adipogenesis and to evaluate the effects of compounds or nutrients on adipogenesis to find the potential treatment of obesity [66]. It was found that compounds such as quercetin and resveratrol [67] inhibit adipogenesis in 3T3-L1 adipocytes. Additionally, these cells have been used to describe the effect of reactive oxygen species, antioxidants or melatonin on adipogenic differentiation [68]. Furthermore, different gene silencing techniques have been applied to study the function of various genes associated with adipogenesis in 3T3-L1 cells, particularly inflammatory pathways, adipokine synthesis and enzyme's function [69]. However, the adipogenic differentiation of 3T3-L1 cell line needs at least 2 weeks, and they require careful observation because when they become confluent, the differentiation into adipocytes cannot proceed.

The 3T3-F442A cell line is another important cell line derived from murine Swiss 3T3 cells and isolated from the third selection of clones that converts into bigger fat cell clusters capable of accumulating more fat than the 3T3-L1 cells and resistant to an early exposure to glucocorticoids in the matter of adipogenic differentiation [70]. Although significantly less than 3T3-L1, these cells have also been used to study the effects of different compounds and drugs on adipocyte differentiation. Additionally, gene silencing through siRNA has been carried out to study the role of alkaline phosphatase in lipid metabolism and gene expression and the secretion of adipokines.

OP9 cells are bone marrow-derived stromal cells that accumulate large triacylglycerol filled droplets only 3 days after adipogenic stimuli [71]. OP9 cells can be passaged for long periods of time and can differentiate into adipocytes even after reaching confluence. Furthermore, their rapid differentiation enables to detect protein expressed from transiently transfected DNA in fully differentiated adipocytes. This cell line has been used to evaluate the effects of different compounds on the adipogenesis process, especially the antiadipogenic activity of quercetin, and its effects on lipolysis [72]. Other studies investigated the inhibitory effects of Pericarpium zanthoxyli extract on the adipogenic differentiation of OP9 cells and the inhibition of adipogenesis in the OP9 cell line by ascorbic acid [73]. These cells are widely used to study the role of oxidative stress in the adipogenesis process, where it was shown that lipid uptake causes reactive oxygen species generation in OP9 preadipocytes.

The C3H10T1/2 cell line is extracted from 14- to 17-day-old C3H mouse embryonic stem cell precursors with the capacity to differentiate into mesodermal cell types such as adipocytes. The main research in this cell line is focused on investigating the molecular mechanisms related to adipogenic differentiation associated with obesity [74]. Additionally, a study of food contaminants was carried out on this cell line, finding that tributyltin, an endocrine disrupting compound, promotes adipogenic differentiation in vitro [75].

Primary mouse embryonic fibroblasts (MEFs) are derived from totipotent cells of early mouse mammalian embryos. What makes them different is their capability to differentiate into adipocytes with no need for pro-adipogenic transcription factors such as PPAR or C/EBP [76]. Their advantages are easy establishment and maintenance, rapid proliferation and producing

in large numbers. Nevertheless, because of the cellular heterogeneity of embryonic tissue, it is often difficult to culture them, and they reach biological ageing at passage 12. This cell line has been used to study mechanisms related to obesity such as genes or transcription factors, signalling pathways and obesity-associated (FTO) gene. In this sense, MEFs derived from FTO overexpressing mice had an increased potential for adipogenic differentiation, while MEFs derived from FTO knockout mice showed a reduced adipogenesis [77].

Porcine preadipocytes are highly similar to human cells and therefore a much better model for the study of adipogenesis and obesity-related diseases. The adipogenesis in porcine cell culture is shown in two steps: the recruitment of lipid-free preadipocytes and the stimulation of lipid growth in the recruited preadipocytes. Studies carried out on this cell line showed that phloretin enhances the lipid accumulation in a time-dependent manner [78], retinol-binding protein 4 (RBP-4) suppresses differentiation in porcine preadipocytes by decreasing the activation of insulin and that miR-125a promotes the differentiation of porcine preadipocytes upon inhibition [79]. In contrast, miR-199a promotes cell proliferation while attenuating the lipid deposition in porcine adipocytes. Furthermore, miR-181a overexpression represses the tumour necrosis factor in porcine preadipocytes [80].

3.2.2. Human cell models

Human cells have been rapidly developed and are gaining more importance as candidates for in vitro studies. They are derived from the human stromal vascular fraction, a mixture of stem cells, endothelial cells, preadipocytes and immunological cells. That is the reason why the obtained results are more reliable than those from animal models and find better applicability towards human diseases such as obesity.

Stromal vascular fraction (SVF) of adipose tissue contains adipose-derived stem cells (ADSCs), the multipotent cell population firstly discovered in the last century and remained a focus of many studies to characterise their nature, including their potential to differentiate into numerous cell types [81]. There are many reasons why ADSCs are suitable for conducting studies—multipotency, a high number of passages and high expansion capacity, the reflection of donor- and depot-specific characteristics and the possibility of being cryopreserved for long periods of time. Once differentiated into adipocytes, ADSCs display phenotypic characteristics of genuine adipocytes, the most important being responsive to hormones including insulin and adrenergic agonists. During last 5 years, ADSCs have been used to study the effect of different compounds on adipogenesis for the characterisation of molecules and cellular processes involved in adipogenesis [19] and to investigate the role of different genes associated with adipocyte metabolism. Finally, because of their capability to convert from white to brown adipocytes, this cell line has been used in the study of the differential effect of specific molecules, such as p53, in white and brown adipogenesis.

Preadipocytes emerged as an excellent model for the study of adipogenesis and fat cell biology. They can be easily obtained from adipose tissue and differentiate into mature adipocytes under appropriate conditions. Unlike ADSCs, which retain multilineage differentiation capacity, preadipocytes from the SVF are already committed to adipogenic differentiation. Human primary preadipocytes are an excellent model for the study of obesity-related alterations because they

reflect a situation close to that of adipose tissue due to the presence of depot-specific properties. Their reflection of donor characteristics makes them useful in studies assessing differences between individuals [82]. Moreover, human preadipocytes do not require extensive proliferation in vitro to differentiate and can successfully differentiate in serum-free conditions, which rules out possible effects of serum components on inhibition of adipogenesis. Preadipocyte adipogenic differentiation protocols are divided into an induction period characterised by the presence of insulin, IBMX, a PPAR-agonist or indomethacin, and a maintenance period. The induction period can be prolonged from three to 7 days, resulting in a significantly higher proportion of cells with adipocyte morphology and higher adipogenic marker expression. In the last years, human preadipocytes have been widely used for the characterisation of regulatory molecules during the adipogenic differentiation process [83]. Finally, ADSCs have been used to validate findings from animal adipocyte models.

4. Antiobesity drugs in the pipeline

Current therapeutic approaches often fail to achieve clinical efficacy or have adverse effect profiles that limit their use. This unmet need for efficient and safe antiobesity medication resulted in the investigation of some new monotherapies or combination therapies (**Table 1**) [84]. Targeting of endogenous endocrine circuits regulating energy homeostasis is a mechanism shared by many antiobesity drugs under development. Most of these endocrine circuits are anorexigenic, starting with the postprandial release of peripheral peptide hormones. The receptors for peptide hormones are expressed in the dorsal vagal complex in the medulla and nucleus arcuatus of the hypothalamus. Some of the medications act through direct binding in CNS, other affect peripheral tissues, and some therapies act both centrally and peripherally.

The history of antiobesity drugs has seen the fall of many pharmaceutical agents that were highly effective yet ultimately dangerous. This is why the research has given up to find a magic pill and turned to the goal of providing a safe and effective drug regimen that will achieve a sustainable reduction in body weight in combination with exercise and improved diet habits.

4.1. Central targets

Melanocortins regulate energy balance through the MCR3 and MCR4 receptors and mediate the effects of leptin in the central nervous system. That is why some MCR4 agonists have been under development. The first generation failed because of increased blood pressure or lack of efficacy [85]. In contrast, the injectable MCR4 reached phase 1/2 trials in obese humans (US), demonstrating increased resting energy expenditure and a weight loss ranging from 2.5 to 4.8% after 12 weeks without any adverse cardiovascular effects.

Neuropeptide Y participates in weight determination by stimulating the creation of new fat cells. It stimulates food intake and reduces energy expenditure by activating NPY receptors Y1 and Y5 present in the hypothalamus. The idea for some of the new therapies was to target the NPY pathway in the hypothalamus, but it has shown less promising results. One of those

Name	Type of agent	Current status
Velneperit	NPY antagonist	Phase II, abandoned
Tesofensine	5-HT/DA/NA reuptake blocker	Phase III
Bupropion/zonisamide	Antidepressant/anticonvulsant	Phase II
Cetilistat	Lipase inhibitor	Phase III
PAZ-320	Carbohydrate hydrolysing inhibitor	Phase III
Beloranib	Methionine aminopeptidase-2	Phase II, abandoned
ALSL1023	matrix metalloproteinase inhibitors	Phase II
IONIS-FGFR4Rx	Fibroblast growth factor receptor 4 inhibitors	Phase II
MB-11055	AMP-activated protein kinase activators	Phase II
JNJ-16269110	MTP inhibitor	Phase II
Resveratrol	SIRT1 activator	Phase II
Langlenatide	GLP1 agonist	Phase II
Semaglutide		Phase III

Table 1. Current status of antiobesity drugs under development.

medications was Velneperit that prevented the binding of NPY to the receptors, decreasing hunger and controlling energy balance. In one study on 656 patients, 800 mg Velneperit showed an average loss of 3.8 kg compared to the placebo group and higher than 5% loss of body weight in 35% of all patients. Despite preliminary reports, Velneperit was discontinued in 2013 after phase 2 data demonstrated no clinically significant benefit over placebo [86].

Modulators of monoamine neurotransmitters (dopamine, serotonin and norepinephrine) can suppress appetite efficiently by enhancing POMC neuronal activity [87]. However, a major concern for the use of this therapy is the risk of adverse cardiovascular events and psychiatric morbidity. Tesofensine is the noradrenalin/serotonin/dopamine reuptake inhibitor initially developed for Alzheimer's and Parkinson's disease and currently under development for therapy of obesity. It primarily not only acts by suppressing appetite, but possibly also works by increasing thermogenesis. Tesofensine has completed phase 1 and showed promising levels of weight loss after 24 weeks compared with placebo, in addition to improved glucose and lipid metabolism with reduced waist circumference. It is currently in advanced phase 3 testing and is yet to enter confirmatory phase. It was well tolerated with side effects such as dry mouth, dizziness, insomnia and gastrointestinal disorders, but has recently been under inspection for serious side effects such as elevated heart rate and blood pressure [88].

Bupropion/zonisamide SR is a combination of the antidepressant bupropion and the anticonvulsant zonisamide. Bupropion reduces weight by the dopamine/norepinephrine reuptake inhibition. Increase in the levels of dopamine decreases appetite. Zonisamide is the anticonvulsant GABA-receptor agonist, and it is believed that the mechanism for inducing weight loss includes modulation of the sodium channel, carbonic anhydrase inhibition and

enhancement of dopamine and serotonin transmission. This medication completed phase 2 in approximately 1500 participants with the result of weight loss up to 15% after 48 weeks, along with the improved cardiometabolic risk markers such as triglycerides levels, blood pressure, waist circumference and insulin sensitivity [57]. The most common side effects were nausea, headache and insomnia. The drug is now in the process of initiating phase 3 clinical trial with the fixed-dose combination.

4.2. Peripheral targets

Pancreatic lipase is a key enzyme that causes breakdown of triglycerides into free fatty acids. Inhibition of pancreatic lipase reduces this conversion and the absorption of free fatty acids in the intestine, resulting in increased excretion of triglycerides in the urine. Cetilistat is a pancreatic lipase inhibitor with the similar mechanism of action to orlistat. During phase 2, it was as effective as orlistat but showed superior safety profile with less gastrointestinal side effects [89]. A significant reduction in body weight, LDL and haemoglobin A1c (HbA1c) levels was also noted. Cetilistat has completed phase 1 in Europe and in the USA and has now undergone phase 3 clinical trial.

Carbohydrate hydrolysing inhibitor PAZ-320 works by blocking the enzymatic breakdown of complex carbohydrates into simple sugars, resulting in the reduction in the intestinal absorption of glucose, fructose and other monosaccharides. It is now undergoing phase 3 clinical trial for type 2 diabetes and obesity.

Expansion of adipose tissue requires continuous remodelling of capillary networks. Since obesity involves the pathological formation of angiogenetic vessels, inhibiting angiogenesis could be a novel treatment target [90]. Methionine aminopeptidase-2 (MetAP2) inhibitors hold antiangiogenetic properties and were primarily used as a therapy for solid tumours. During these trials, they have been demonstrated to effectively lower bodyweight, not just by inhibiting angiogenesis, but via converting stored fats into useful energy and reducing the production of new fatty acid molecules. Beloranib caused dramatic weight loss compared with placebo, along with the improved cardiometabolic risk markers, including circulating lipids, waist circumference and glycaemic control. The development of this drug was terminated during phase 2 due to increased incidence of thromboembolic events.

Matrix metalloproteinases (MMP) are endoproteinases that break down extracellular matrix, thus participating in tissue remodelling and angiogenesis. High levels of MMP are associated with obesity and cardiovascular diseases. The target of the oral angiogenesis and MMP inhibitor ALSL1023 is visceral fat, which is closely linked to the metabolic risks of obesity [91]. In a phase 2 study on 126 patients, ALSL1023 demonstrated 15% reduced visceral fat after 12 weeks of daily oral administration of 1200 mg, and the effect was superior to placebo with no significant adverse events.

Fibroblast growth factor receptor 4 (FGFR4) is involved in the regulation of fat storage, energy expenditure and overall body weight. It is highly expressed in the liver and other peripheral tissues. The antisense oligonucleotide IONIS-FGFR4Rx lowers the production of FGFR4 [92]. It significantly suppressed liver FGFR4 expression in obese mice after 10 weeks which

was accompanied by a 20% placebo-subtracted weight loss in addition to improved glucose metabolism and lipid levels. The drug distributes to peripheral tissues but poorly enters heart muscle and brain, hopefully avoiding cardiac and CNS side effects. The phase 2 trial for IONIS-FGFR4Rx has been initiated along with the development of four other antisense drugs.

AMP-activated protein kinase (AMPK) is activated by cellular stress such as exercise, when the depletion of cellular ATP levels leads to a concomitant rise in AMP. AMPK activation in liver, muscle and fat tissues increases oxidation of glucose, fatty acids and triglycerides and decreases their storage by switching to catalytic processes [93]. Activation of AMPK by pharmacological means is, therefore, a potential way of reversing the metabolic abnormalities of obesity. The plant-derived AMPK activator resveratrol mimic the beneficial metabolic effects of energy restriction. The AMPK activator MB-11055 is currently in phase 2 trial for obesity.

A long-acting ß2-adrenergic receptor agonist, salmeterol xinafoate, is administered via series of multiple subcutaneous injections where it reduces the fat by increased lipolysis. In 54 healthy adults after 4 weeks, it reduced abdominal circumference and skin-fold thickness. It is currently in phase 2 trial in 160 obese subjects.

Microsomal triglyceride transfer protein (MTP) is an essential chaperone highly expressed in the intestine and liver that transfers several lipids including triacylglycerols and phospholipids. The triacylglycerol transfer activity of MTP was used to identify several potent antagonists as a potential therapeutic approach for obesity and hyperlipidaemia. However, MTP inhibition results in triacylglycerol build-up and gastrointestinal disturbances, but more important leads to steatosis and increases levels of transaminases [94]. SLx-4090 is a compound currently in phase 2 clinical trial. In 24 patients with dyslipidaemia, this drug demonstrated reductions in postprandial triacylglycerols and clinically significant weight reduction with no effect on liver. Another selective intestinal MTP inhibitor, JNJ-16269110, showed dose-related weight loss reduction after 12 weeks in 321 nondiabetic obese subjects.

Sirtuins (silent information regulator 1 proteins, SIRT1) participate in critical pathways such as lipid metabolism and insulin secretion. SIRT1 suppresses the expression of the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) that is responsible for fat storage. Resveratrol is an allosteric activator of SIRT1 that increases the mitochondrial activity in brown adipose tissue and skeletal muscle. Besides resveratrol, several other sirtuin activators are in early clinical development. Phase 2 study for SRT2104 gave optimistic data on the metabolic profile of elderly patients [95].

4.3. Agents acting through central and peripheral mechanisms

Since the approval of liraglutide, several long-acting GLP-1 receptor analogues are being evaluated with the goal to provide a more stable blood profile of the peptide and avoid the peak levels more likely to cause side effects, in particular, nausea [96]. Langlenatide is intended for weekly/monthly subcutaneous administration, which achieved placebo-subtracted body weight loss of 7.3 kg in a phase 2 trial in 297 obese patients. Semaglutide has completed a phase 3 trial in 632 obese patients with type 2 diabetes, where a daily intake of 40 mg semaglutide for 26 weeks resulted in improved glycaemic control and placebo-subtracted bodyweight reductions of 5.7 kg.

Glucagon increases resting energy expenditure by inducing thermogenesis [97]. This effect in combination with GLP-1-mediated reduced appetite has demonstrated interesting results in preclinical trials. The natural gut hormone oxyntomodulin (OXM) acts at both the GLP-1 and glucagon receptor. The crucial step in developing OXM analogues is to identify the ratio of GLP-1/glucagon receptor co-agonism with maximal weight loss and without impairing glucose tolerance. Six agonist agents are in phase 1 trial, and at least one agent is in phase 2 for diabetes and obesity.

Although leptin replacement successfully treats obesity caused by congenital leptin deficiency, results are still disappointing in obese patients because of leptin resistance in obesity. Some molecules may re-establish responsiveness to leptin. Different combinations of leptin analogues and other weight loss agents are now in preclinical development [98].

5. Challenges in obesity drug development

Due to the unmet need for the right medication, growing of the antiobesity market created the clinical development of several promising weight loss therapies. When choosing a weight loss drugs, several things, such as safety, cost and effectiveness, must be equally considered. Several challenges need to be overcome to achieve substantial weight loss. Improved medications are needed to help reduce costs of obesity to society. These new pharmacological agents will either replace the existing ones or be used in combination with them. The advantage of combinational therapy is the need for lower doses of the individual drug, which lowers the rate of side effects and suppression of counter-regulation and increases the weight loss effect. Like any other chronic condition, polytherapeutic and long-term treatment strategies are required to achieve the sustained efficacy of weight loss therapies, although the probability of maintaining weight loss is low with an available drugs [77].

A significant challenge for the development of new antiobesity drugs is the different clinical efficacy criteria by FDA and EMA. For the FDA, the placebo-subtracted weight loss should be 5%, or that amount of weight loss from the baseline of body weight must be achieved in 35% of patients. Both those differences between drug and placebo effects must be statistically significant. EMA puts the accent on weight loss from baseline as more clinically relevant than the weight loss compared to placebo, so the primary criterion is 10% weight reduction regarding to baseline. Another factor that discourages pharmaceutical industry in obesity research is the reluctance of medical insurers to pay for antiobesity drugs, which directly affect the sales of new drugs. An additional reason for drug sales to be disappointing is the expectations of prescribers and patients [99]. Current antiobesity drugs can produce modest weight loss of 2–9 kg, which is not satisfactory to the majority of obese patients [100]. They end up discontinuing medication to avoid the cost and side effects connected to the potential lifelong treatment. The major problem with stopping the medication is weight loss maintenance because patients can weight more than before the intervention [101]. From this fact, it is evident that weight reduction of 5–10% will not be enough for many patients and physicians—any new entrant to the obesity market will be considered clinically effective only if the reduction in body weight will be 10–15%.

Now that most drugs target endogenous pathways regulating energy homeostasis, the off-target effects are hard to avoid. The withdrawal of several previously marketed antiobesity medications encouraged the regulatory authorities to require pre-specified safety data for all antiobesity drugs. Monitoring of adverse cardiovascular and psychiatric effects is of particular importance for drugs modulating the monoamine system. Additionally, some of the peripherally acting drugs work in multiple tissues by poorly understood mechanisms, which can cause the appearance of unexpected adverse effects. Besides acceptable safety profile, an antiobesity drug must meet minimum efficiency criteria to be considered relevant. Those criteria apply not only to weight loss but also to beneficial effects on the comorbidities such as diabetes type 2, cardiovascular disease and NASH [102]. In the end, treatment must be effective in the long term to sustain weight loss, which can be limited by receptor desensitisation and tachyphylaxis [103]. The progression of active weight loss to its maintenance will cause retention of patients to antiobesity drug especially when the patient has to contribute to the cost of the drug. All these unmet needs will have to be covered by any new medication entering the market to have a chance of being the commercial success.

6. Conclusion

Given the increasing prevalence of obesity worldwide, researchers are putting a lot of effort to find new therapeutical strategies for treatment of the disease and its prevention. Rising understanding of the body weight control and the molecules participating in this process lead to the breakthrough of potential novel therapeutic opportunities. Also, genome-wide studies are developing to give insights and answer to a lot of remaining questions. Furthermore, recently established clinical guidelines and increased number of monotherapy and combination medications enabled an individual approach to different types of obesity. However, there is still a long way to win a battle against obesity by pharmaceutical means. As for any other chronic condition, there is a small chance that super drug will ever appear to resolve obesity. Thus, we must focus on individually tailored approach with the accent on the metabolic, genetic and molecular background.

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Chapter 4

Pharmacologic Interventions for Preventing Chondrocyte Apoptosis in Rheumatoid Arthritis and Osteoarthritis

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Additional information is available at the end of the chapter

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Abstract

Chronic inflammation drives the progression of rheumatoid arthritis (RA) and osteoarthritis (OA) to synovial joint failure. The inflammatory state in both musculoskeletal diseases is associated with significantly elevated levels of pro-inflammatory cytokines in joint synovial fluid, which is best exemplified by increases in interleukin-1β (IL-1β), IL-6, IL-17, tumor necrosis factor-α, among others, as well as increased activity of soluble mediators such as nitric oxide and certain growth factors including vascular endothelial growth factor and fibroblast growth factor. The multitude of these factors activate chondrocyte signal transduction pathways resulting in programmed cell death, otherwise known as apoptosis as well as compromising chondrocyte autophagy. Importantly, chondrocyte apoptosis causes a loss of articular cartilage vitality which dampens cartilage repair mechanisms because at present, the possibility that chondrocyte progenitor cells could replace those differentiated chondrocytes lost via apoptosis remains debatable. Certain pharmacologic interventions which have been proven to induce apoptosis in various cancer cell studies *in vitro* suggest the possibility that drugs could be developed to specifically suppress or completely inhibit chondrocyte apoptosis in RA and OA cartilage. This review supports that contention and indicates that apoptosis can be inhibited by identifying novel cellular targets which promote apoptosis and autophagy.

Keywords: apoptosis, chondrocyte, osteoarthritis, rheumatoid arthritis, signal transduction

1. Introduction

Controlled cell death otherwise known as programmed cell death or apoptosis constitutes a critical event which is germane to the normal development of the immune system, the appropriate

integration of cells within tissues and organs and organ homeostasis [1–7]. However, the aberrant frequency of apoptotic cells can compromise normal tissue architecture and, in doing so, contribute to the loss of cell vitality [8–10]. This is especially the case in explaining the loss of chondrocyte viability in arthritic conditions of synovial joints, such as rheumatoid arthritis (RA) and osteoarthritis (OA).

Significant progress has been achieved over the previous decade or so in furthering our understanding of the cellular and molecular events that trigger the increased frequency of chondrocyte apoptosis in RA and OA [11–16]. These advances include (1) an appreciation that the significant increase in the levels of pro-inflammatory cytokines in synovial fluid from RA and OA patients also can induce chondrocyte apoptosis *in vitro* [17–21]; (2) that the elevated frequency of chondrocyte apoptosis by these cytokines is deregulated by altered signal transduction which can involve continuous activation of stress-activated/mitogen-activated protein kinases (SAPK/MAPK) [22–25], the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway [26–31], the phosphatidylinositide-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway [32–36], and other protein kinase pathways [37–40]; (3) that specific co-factors are capable of regulating the activation of these signaling pathways [41–44]; and (4) that micro-RNAs (mi-RNAs) can regulate the activity of these co-factors and, in this manner, control the induction of apoptosis via these signaling pathways [45–48].

Furthermore, the increased frequency of chondrocyte apoptosis now confirmed in guinea pig OA cartilage [49] as well as human RA and OA articular cartilage [50–53] presents a particularly onerous scenario for the survival of synovial joints under these conditions. Although a population of chondroprogenitor cells was identified in several studies of adult normal and diseased articular cartilage [54–56], significant repair of damaged articular cartilage in RA and OA by these cells is effete. This effect on cartilage repair may result from elevated levels of pro-inflammatory cytokines such as IL-17, which was recently shown to inhibit the chondrocyte maturation lineage emanating from progenitor cells in RA [57]. Therefore, even if chondrocyte precursor cells exist in adult articular cartilage which could potentially become authentic chondrocytes lost from articular cartilage via apoptosis or chondrocytes lost via diffusion of cartilage extracellular matrix fragments into synovial fluid, the reduction in chondrocyte vitality via apoptosis would be a challenging event to overcome, especially in a synovial joint microenvironment replete with pro-inflammatory cytokines.

In this chapter, we have systematically examined the mechanistic underpinning for identifying novel targets in order to suppress or even inhibit chondrocyte apoptosis in RA and OA. However, the scenario in RA, in particular, is even more complex than in OA because in the context of devising therapeutic strategies designed to inhibit chondrocyte apoptosis in RA, one must also take into account the fact that in the hyperplastic RA synovial tissue, comprised of activated synoviocytes, immune cells, macrophages and other inflammatory cells are generally considered to be relatively "apoptosis-resistant" [58, 59]. Thus, this characteristic of the RA joint ensures a plentiful source of immune-mediated cells and non-immune inflammatory cells which drive the progression of RA. It is also likely that at

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some time during the course of the progression of OA, immune-mediated inflammation may also cause a similar chronic inflammatory microenvironment, as found in RA, to arise and persist in OA synovial tissue [55] resulting, in part, in an increased frequency of apoptotic chondrocytes [60].

2. Compelling evidence that many factors relevant to RA and OA promote or induce chondrocyte apoptosis *in vitro*

Analyses of synovial fluids and sera from RA and OA patients with active disease showed that these samples contained significantly elevated levels of various pro-inflammatory cytokines and growth factors when compared to a control group [52–68]. Of note, incubation of rat [69], non-arthritic or human chondrocytes from OA cartilage [70–76] or immortalized lines of human chondrocytes [77] with physiological levels of these cytokines, growth factors (e.g., VEGF and FGF) or additional soluble mediators (e.g., nitric oxide) were shown to induce apoptosis, which was accompanied by activation of SAPK/MAPK, JAK/STAT or PI3K/Akt/mTor signaling in these cells [78–81]. In addition, mediators of inflammation, including prostaglandin E_2 and neuropeptides (e.g., Substance P), are also implicated in perpetuating chronic inflammation [38]. The induction of apoptosis was also shown to be related to altered levels of various down-regulators of apoptosis. These included BCL-2-like protein-11 (Bim) [18], B-cell lymphoma-2 (Bcl-2) [75], cell-derived inhibitors of apoptosis proteins (IAPs) [81–84] and Suppressor of Cytokine Synthesis (SOCS) [85, 86]. Furthermore, alterations in the functions of mitochondria [87] and endoplasmic reticulum (ER) [88–91] related to cell stress, the generation of reactive oxygen species [92] and the recently described advanced oxidation protein products [93] with respect to their capacity to induce apoptosis were reported as well. Taken together, these results provided compelling evidence that pro-inflammatory cytokines, growth factors and soluble mediators germane to the progression of RA and OA are responsible for inducing chondrocyte apoptosis in these conditions.

3. The relationship between apoptosis and autophagy

A recent review of RA pathogenesis by Angelotti et al. [94] emphasized the view that numerous cells comprising the innate immune system, including macrophages, dendritic cells, mast cells, natural killer cells and neutrophils as well as T-cells and B-cells, regulators of adaptive immunity, are primarily responsible for perpetuating the state of chronic inflammation through their capacity to alter the survival of resident synovial fibroblasts. It is also likely that such cell combinations are also involved in the progression of OA as well, as evidenced by the skewing of cartilage homeostasis toward catabolism, and the loss of chondrocyte vitality via the induction of apoptosis [95–97]. However, it was previously shown that the classical apoptosis cascade can be activated by various pro-inflammatory cytokines, and certain growth factors, chemokines, chemokine receptors (**Table 1**) and other interleukins, including

IL-8 (CXCL8) and adhesion molecules [64]. Thus, these factors are likely to be the most influential in inducing apoptosis pathway RA. However, a non-classical form of apoptosis, termed, "chondroptosis" is just as likely to be activated in OA. In that regard, "chondroptosis" involves an increase in the number of ER and Golgi apparatus reflecting an increase in protein synthesis that accompanies the loss of viable chondrocytes [98].

Furthermore, changes in the number of viable articular chondrocytes in experimentally induced arthritis [99], human RA [100], and OA are almost certainly associated with the autophagic-mediated cell death of chondrocytes [101–105], which occurs in concert with the activation of the extrinsic apoptosis pathway, the latter mediated by Tumor necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL), Death Receptor-5 (DR5) and caspase-3 [106, 107]. In that regard, it was noteworthy that Huang et al. [108] reported that exogenous leptin promoted chondrocyte apoptosis while inhibiting chondrocyte autophagy via the up-regulation of lysyl oxidase-like 3 (LOXL3). Thus, in their study [108], overexpression of LOXL3 inhibited chondrocyte autophagy by activating mechanistic target of rapamycin complex-1 (mTORC1) [36]. In contrast, cartilage-specific

Cytokine, chemokine, chemokine receptors and growth factors	**Reference**
IL-17, IL-18BP	[62]
IL-17, IL-20, IL-21	[63]
IL-7	[64]
IL-12/IL-23	[64]
IL-15/IL-16	[64]
IL-17/IL-18	[64]
IL-19/-20/-21	[64]
IL-32	[64]
VEGF[1]; TGF-β_1[2]; Leptin; FGF[3]	[64–66]
CXCR3; CXCR4,-5; CXCR-1,-5,-6; IL-8; MIP-1α[4]; GRO-α[5](CXCL1); GRO-$\beta\gamma$[6]; MCP-1[7]; RANTES[8]; Eotaxin-1	[65, 68]
IL-6	[64, 67, 68]
TNF-α	[64, 67, 69]
IL-1β	[64]

[1]Vascular endothelial growth factor.

[2]Transforming growth factor-β_1.

[3]Fibroblast growth factor.

[4]Macrophage inflammatory protein-1 (CCL3).

[5]Growth-related oncogene-α.

[6]GRO-$\beta\gamma$.

[7]Monocyte chemotactic protein-1.

[8]Regulated on activation, normal T-cell expressed and secreted.

Table 1. Cytokines, chemokines, chemokine receptors and growth factors in RA and OA.

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deletion of mTOR resulted in the up-regulation of autophagy [102]. Autophagy protected mouse cartilage from degeneration [109]. Autophagy was also shown to protect chondrocytes from glucocorticoid-induced apoptosis via reactive oxygen species, Akt and FOXO3 signaling [110] as well as from advanced glycation end-product-induced apoptosis which was accompanied by lower levels of MMP-3 and MMP-13 in rat chondrocyte cultures [111].

4. Do micro-RNAs play a critical role in chondrocyte apoptosis?

Micro-RNAs (miRs) are critical mediators of mRNA degradation as well acting as repressors of translation. MiRs have been implicated in the development of skeletal long bones via their multiple effects on osteogenesis [112]. However, recent evidence has also improved the recognized role of miRs in RA pathology as a result of evidence that many miRs including miR-16, miR-146a/b, miR-150, miR-155 and miR-223 are over-expressed both in the peripheral circulation of RA patients and in the RA synovial joint [113], although other miRs relevant to RA, such as miR-21, miR-125a, miR-223, and miR-451 are principally found to be at elevated levels in the plasma and sera of RA patients.

Additional evidence has been presented to show that several of these miRs also regulate apoptosis. For example, the level of the pro-apoptotic protein, Bim, was increased when activated T-cells were incubated with a repressor of miR-148a resulting in an increase in the Th1 apoptotic response [114]. Thus, accumulating evidence showed that both the immune cells and chondrocyte apoptotic and autophagic response can be manipulated by either experimental overexpression or repression of various miRs which are relevant to OA [115] and RA [116] pathophysiology.

The results of numerous studies have confirmed the role of specific miRs as directly affecting apoptosis or indirectly affecting apoptosis through their activity on other molecules that regulate chondrocyte apoptosis. For example, mIR-146a was reported to be over-expressed in OA [117]. In that regard, mIR-146a was shown to promote human OA chondrocyte proliferation and to inhibit apoptosis by targeting tumor necrosis factor receptor-associated factor 6 (TRAF6) via NF-κB [118, 119]. By contrast, mIR-146a was also shown to target IL-1β, to induce VEGF production, and to promote rat chondrocyte apoptosis via Smad4 [120]. On the other hand, silencing of miR-34a inhibited rat chondrocyte apoptosis [121], whereas overexpression of mIR-34a promoted apoptosis in normal human chondrocytes by targeting SIRT1/p53 signaling [122], although other evidences indicated that miR-34a was increased in intervertebral disc degeneration and was associated with an elevated frequency of apoptotic cartilage end plate chondrocytes [123]. In another study, glycerol-3-phosphate dehydrogenase 1-like protein was shown to be a target for mIR-181a, which is deregulated in OA wherein human chondrocyte apoptosis was increased [124]. In yet another study, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [36] was identified as the target for mIR-181 whereby mIR-181 up-regulated the expression of proteins associated with apoptosis, including caspase-3 and PARP. However, mIR-181 also up-regulated MMP-2 (gelatinase A; 72 kDa gelatinase) and MMP-9 (gelatinase B; 92 kDa gelatinase) [125] which are two MMPs directly relevant to cartilage matrix protein degradation in arthritis [126].

Of note, several miRs were identified as potential targets for inducing chondrocyte survival and therefore could be considered anti-apoptosis factors; these included mIR-98 [127–129], mIR-9 [130], mIR-15a-5p [131], mIR-142-3p [132] and mIR-502-5p [133]. Additional mIRs, exemplified by miR-195 [134], mIR-139 [135], mIR-29b-3p [136], mIR-488-3p [137] and mIR-203 [138] could very well be included in this group. For example, by employing the C28/I2 line of immortalized human chondrocytes, Zhao et al. [138] showed that knockdown of mIR-203 targeting the myeloid cell leukemia-1 (MCL-1) protein activated Wnt/β-catenin and JAK/STAT signaling promoted chondrocyte survival.

To summarize this section, gaining a further understanding of how to manipulate specific mIRs to achieve increased or decreased synthesis of specific targets known to influence chondrocyte apoptosis may signal the next major advance in targeted OA and RA therapy designed to promote chondrocyte survival.

5. Pharmacologic interventions designed to specifically inhibit chondrocyte apoptosis

5.1. Signal transduction pathways

Lewis and Malemud [82] previously reviewed several potential pharmacologic strategies designed to limit the loss of chondrocyte vitality via apoptosis. These included, targeting x-linked inhibitor of apoptosis (XIAP), tumor necrosis factor-like weak inducer of apoptosis (TWEAK), TRAIL, decoy-receptor-3 (DcR3), tumor necrosis factor receptor protein-like molecules, p53 up-regulated modulator of apoptosis (PUMA), and apoptosis-signal-regulating kinases. In that regard, we proposed several interventional strategies which we acknowledged involved developing a deeper understanding of which signal transduction pathways were altered in RA and OA chondrocytes [19, 28, 36, 77, 82, 106]. For example, XIAP, an inhibitor of activated caspase-9, and caspases-3 and -7 [82] was also shown to interact with mitogen-activated protein kinase kinase 2 (MEKK2) [139]. The interaction between XIAP and MEKK2 resulted in a biphasic activation of NF-κB, a known downstream effector of TNF-α-mediated apoptosis. This finding is relevant to the regulation of chondrocyte apoptosis in both RA and OA primarily because XIAP is a well-known inhibitor of apoptosis protein-3 (IAP3) [140]. In fact, we had previously shown that recombinant human TNF-α (rhTNF-α) induced human chondrocyte apoptosis via activation of p38, JNK1/2 and STAT3 [72], whereas apoptosis of the immortalized human chondrocyte line, C-28/I2 induced by rhTNF-α, but not by rhIL-6, was dependent on upstream MEK1/2 [77]. Therefore, we posit that in order to consider using potential drug interventions that alter the activation of various signaling pathways it will be useful to consider what we know about how alterations in receptor-mediated signaling pathways (reviewed in [19]) may influence apoptosis.

Activation of SAPK/MAPK signaling is most often associated with induction of chondrocyte apoptosis. Thus, induction of rabbit articular chondrocyte apoptosis by the nitric oxide (NO) donor sodium nitroprusside (SNP) was linked to inhibition of c-Jun-amino-terminal kinase (JNK) by virtue of the finding that the JNK small molecule inhibitor (SMI) SP600125, reduced

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the frequency of apoptotic chondrocytes along with NO-induced NF-κB, p53 and caspase-3 [141]. IL-1β, another potent inducer of chondrocyte apoptosis, was also shown to be JNK-dependent as both chemical inhibitors of JNK as well as RNA interference with Bim, the latter up-regulated by IL-1β, were shown to be phosphorylated-JNK-dependent [142]. In other studies, chondrocyte apoptosis was again linked to IL-1β-induced activation of p38 kinase [143–145], along with JNK [145], and MMP-3 gene expression with IL-1β negatively regulating chondrocyte autophagy [145]. Of note, AG490, a pan-JAK SMI significantly reduced leptin-induced chondrocyte apoptosis *in vitro* as well as reducing STAT3 phosphorylation, reactive oxygen species, MMP-13 and B-cell lymphoma 2-associated X protein [146]. Interestingly, Li et al. [147] showed that the PI3K/NF-κB pathway was activated by TNF-α in human chondrocytes. However, the effect of leptin did not involve mTOR, suggesting that newly developed small molecule mTOR inhibitors (reviewed in [36]) might not be useful for neutralizing activated PI3K/NF-κB in response to leptin-induced apoptosis.

Several attempts to employ various compounds and/or natural products to inhibit chondrocyte apoptosis have taken advantage of various findings related to the role of these compounds and natural products in many of the aforementioned signal transduction pathways. For example, IL-1β-induced chondrocyte apoptosis was inhibited by oligomeric proanthocyanidin, a water-soluble plant polyphenolic compound [148]. Thus, the over-expression of peroxiredoxin 4 (PRDX4), a member of the PRDX family (a molecule essential for scavenging free radicals and reducing reactive oxygen species) reduced IL-1β-induced rat chondrocyte apoptosis [149]. Importantly, AZD5363, an inhibitor of Akt activation also reduced the apoptosis protective effect of PRDX4. In another aspect, chondrocyte apoptosis induced by IL-1β not only involved reduced Bcl-2 levels, activated (i.e., phosphorylated) Akt, and activated PRAS40, a proline-rich 40 kDa Akt substrate and an inhibitor of mTORC1 kinase activity, but was also linked to increasing the levels of Bax, and activated caspase-3/-9 [149].

Shikonin, a compound with anti-tumor, anti-inflammatory, anti-viral and pharmacological efficacy significantly inhibited apoptosis by decreasing IL-1β, TNF-α and inducible NO synthase (iNOS) in rats with experimentally induced OA [150]. The effect of shikonin in this animal model of OA was accompanied not only by reduced caspase-3 and cyclooxygenase-2 activity but also by increased activation of Akt, indicating a prominent role for PI3K/Akt signaling in this rat model of OA. Finally, a few novel targets, including protein kinase R-like endoplasmic reticulum kinase and activating transcription factor 6, were identified as potent regulators of chondrocyte apoptosis *in vitro* and *in vivo* [151], although the precise signaling mechanisms attributed to these molecules have yet to be completely established.

5.2. Additional potential targets related to apoptosis

Several experimental studies, of note, have focused on several cellular components which may eventually become suitable pharmacologic targets for altering chondrocyte apoptosis and/or autophagy in osteoarthritis (**Table 2**). However, the results of these mainly *in vitro* studies point out why it will be necessary to determine the underlying mechanisms regarding how these factors work to inhibit apoptosis. Thus, only after successful evaluation in animal models of RA and OA, can we envision that these targets could eventually be employed in a clinical setting.

Factor	Target	Reference
HIF-1α/HIF-2α[1]	HIF-1α—SOX9; HIF-2α—Fas	[152]
Integrin-β1	G1T1[2]	[153]
IGFBP-3[3]	Nur77[4]	[154]
SGBT[5]	Caspase-3/Hsp70[6]	[155]
UCP4[7]	ROS[8]	[156]
DEL1[9]	Caspase-3/Caspase-7	[157]
Rela[10]	Pik3r1[11]	[158]
Beclin[12]	Bcl-2; Bcl-2 associated X	[159]
AST[13]	LC3-II/I[14]; P62/SQSTM1[15]	[160]
AQP-1[16]	Caspase-3	[161]
Mt1/Mt2[17]	ROS[8]	[162]
Sirt1[18]	Bcl-2; Bax	[163]

[1]Hypoxia-inducible factor-1α/-2α.
[2]G-protein-coupled receptor kinase interacting protein-1.
[3]Insulin-like growth factor-1 binding protein-3.
[4]Nerve growth factor 1B.
[5]Small glutamine-rich tetratricopeptide repeat-containing β.
[6]Heat shock protein-70.
[7]Uncoupling protein-4.
[8]Reactive oxygen species.
[9]Developmental endothelial locus-1.
[10]RelA/p65 of NF-κB complex.
[11]Pik3r1 encodes a p85α regulatory protein that is a subunit of phosphatidylinositol 3-kinase (PI3K).
[12]Beclin-1, a product of the *BECN1* gene is a mammalian ortholog of the yeast autophagy-related gene 6 (Atg6) and BEC-1 in the *C. elegans* nematode.
[13]Astragaloside IV.
[14]Microtubule-associated protein 1A/1B-light chain-I/II.
[15]P62/sequestosome-1.
[16]Aquaporin-1.
[17]Metallothionein-1/metallothionein-2.
[18]Silent information regulation of transcription 1.

Table 2. Cellular factors that regulate chondrocyte apoptosis and/or autophagy *in vitro*.

6. Conclusions and future perspectives

It is now generally agreed upon by many investigators that a chronic state of inflammation is, in part, responsible for driving and perpetuating the progression of RA (reviewed in [94]) and OA (reviewed in [58, 164]). Moreover, additional recent evidence has indicated that both apoptosis and an altered state of autophagy are critical events in chronic musculoskeletal disorders, such as RA and OA [60, 102–105, 164–167]. Although the loss of chondrocyte vitality

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is a common pathological finding in RA and OA, cellular mechanisms that result in synovial fibroblast and immune-cell-mediated "apoptosis-resistance," such as those that were found to underlie B-cell activity in RA [168–171], aids in distinguishing between the fundamental underpinning responsible for bony abnormalities in the two conditions. However, equally important is that the increased frequency of apoptotic chondrocytes in both RA and OA constitutes a major contributor to inefficient cartilage repair and synovial joint failure. We contend that any pharmacologic strategies designed to simultaneously target the "apoptosis-resistance" in the RA hyperplastic synovial tissue and the elevated frequency of chondrocyte apoptosis would be an onerous undertaking. So for the present, concentrating on developing agents that suppress or inhibit chondrocyte apoptosis might be the initial way to proceed. For example, the current literature on this subject has already indicated that certain drugs used in the medical therapy of RA suppress chondrocyte apoptosis *in vitro*. In that regard, the drug sulphasalazine, commonly employed in combination with methotrexate, for treating RA [172] was found to inhibit rabbit chondrocyte apoptosis induced by SNP [173]. In this study, the reduced frequency of chondrocyte apoptosis was accompanied by an increase in phosphorylated p38 kinase and ERK1/2 expression compared to treatment of chondrocytes with SNP alone.

Another area worthy of consideration is to employ *in vitro* studies to, in effect, rule out for further considerations for developing drugs for treating potential targets of chondrocyte apoptosis. A suitable example of this strategy was a recent finding by Nasi et al. [174] that the NALP3 inflammasome (reviewed in [175]) was not involved in chondrocyte apoptosis characteristic of several alterations in cartilage characteristically found in a murine menisectomy model of OA. Although alterations in the structure of articular cartilage such as cartilage destruction, synovial inflammation, cell death and calcification were seen in this OA animal model, a deficiency in IL-1α had no impact on these features. Importantly, deficiencies in IL-1β and NALP3 actually resulted in an enhancement of cartilage damage in this OA animal model.

The suppression or more favorably the complete inhibition of chondrocyte apoptosis in RA and OA using pharmacologic interventional strategies would be a laudable achievement in the continuing search for novel disease-modifying-anti-rheumatic drugs. A variety of potential novel targets have now been identified during the previous 3 years that at least employing cancer cells induces the frequency of apoptosis. Thus, targets have been identified to induce apoptosis in these cancers and therefore, may be eventually exploited for blocking chondrocyte apoptosis. For example, the activity of 2,5-dihydroxy-3-undecyl-1,4 benzoquinone, 6 g, also known as embelin was reviewed [82] as an inducer of apoptosis in inflammatory breast cancer cells and pancreatic cancer cells. Embelin was also shown to block the transcription of several gene products relevant to tumor cell survival, proliferation, invasiveness and metastatic cancer cell proliferation. Of note, treatment with non-toxic concentrations of embelin could also sensitize cultured malignant glioma to TRAIL-induced apoptosis [176]. Lewis and Malemud [82] reviewed the findings showing that embelin blocked the activation of NF-κB, RANKL and, STAT3, the latter finding demonstrating "proof-of-principle" that STAT3-activated transcription could also be employed to probe the extent to which this signaling pathway was required for maintaining chondrocyte survival *in vitro* [177]. In that regard, these pre-clinical results may provide a suitable platform for exploiting the overall objective of preventing synovial joint failure in RA and OA through the maintenance of normal articular chondrocyte viability and cartilage integrity.

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Chapter 5

Medicinal Plants of West Godavari

Sudhakar Pola and Venkata Narasimha Kadali

Additional information is available at the end of the chapter

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Abstract

Medicinal plants are one of the nature's greatest gifts to the mankind. Each plant will have an exquisite deal of character, which can act as an antidote to various of diseases. Traditional medicine has become a vital alternative source of medicine all over the world today with some approximation of about 80% of the primary health care system in some developing countries. Medicinal plants are known to comprise of hundreds of active constituents that may be potentially useful for the development of therapeutic agents. The development of therapeutic agents involves the isolation and identification of bioactive compounds from plant materials which is crucial for drug discovery. Researchers from all around the globe have focused on drug discovery from the natures' wonder medicinal plants, forming an important group of complementary and alternative medicine (CAM) therapy. West Godavari is a part of Andhra Pradesh, India, which hosts several plants that have high therapeutic significance. Each of the plants has a unique feature which can be employed for healing of various lethal diseases. The present examination intends to review the therapeutic plant assets of West Godavari area Andhra Pradesh. This evaluation also offers the critical elements which include medicinal properties of various medicinal plants found in West Godavari district of Andhra Pradesh, India.

Keywords: drug discovery, traditional medicine, West Godavari district, nature, antidote

1. Introduction

The great thing that nature has given us is the medicinal plants. Each plant will have a great deal of character, which can act as an antidote against different types of diseases.

Traditional medicine has become a vital alternative source of medicine all over the world today with some approximation of about 80% of the primary health care system in some developing countries (e.g., Nigeria, Ghana, China, and India [1, 2]).

India has a recognized traditional system of medicine, that is, Ayurveda, Siddha, Unani, Homeopathy, Yoga, and Naturopathy which placed India in a unique position in the world [3].

As the growth of knowledge has increased, the number of new plant-derived drugs grow at an accelerated pace. India has an enormous wealth of medicinal plants, and often it has been referred to as the Medicinal Garden of the world [4].

Medicinal plants are known to comprise hundreds of active constituents that may be potentially useful for the development of therapeutic agents. The development of therapeutic agents involves Identification and segregation of bioactive compounds from plant materials that are crucial for drug discovery [5].

Researchers from all around the globe have focused on drug discovery from the nature's wonder medicinal plants, an important group of complementary and alternative medicine (CAM) therapy [5].

The man has acclimated himself with plants and utilized them in an assortment of courses all through the ages. Primitive man looking for nourishment and to adapt efficiently to human sufferings started to recognize those plants appropriate for restorative reason from others with complete pharmacological activity. This connection between plants and man has developed, and many plants came to be utilized as medicines. The development of information to cure infections proceeded at a quick pace and various new plant-derived drugs expanded in this manner.

Herbs employed in ancient medicines represent a tiny, low portion of present plants solely. With the advances in informative innovation and science, many bioactive concoction substances are distinguished in plants or foodstuffs through phytochemical and pharmacological studies.

The clinical utilization of plants portrayed in Indian Vedas for curing distinctive maladies. In the present setting, the conventional arrangement of pharmaceutical is broadly acknowledged and drilled by individuals around the world.

The natural solution is drilled around the world. For a considerable length of time, individuals have swung to natural solutions for basic cure illnesses, for example, colds, sensitivity, annoy stomachs, and toothaches, and the pattern is continually expanding. In this way, there has been a move in general pattern from manufactured to homegrown solutions.

The advancement of plant-derived drugs began when improvement of science. The homegrown drug is compelling, minor symptom, and moderate than the medications purchased from an allopathic pharmaceutical. Homegrown medications incorporate herbs, homegrown materials, natural arrangements, and homegrown items that contain diverse parts of plants or other plant materials as dynamic fixings.

As the worldwide utilization of homegrown therapeutic items keeps on developing and numerous, the newest items are brought into the market, general medical problems and concerns encompassing their well-being are likewise progressively perceived. Although some homegrown herbs have promising potential and are utilized, a considerable lot of them stay untested and their utilization likewise not observed.

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It has turned out to be fundamental, subsequently, to outfit the overall population incorporating human services experts with adequate data to encourage better comprehension of the dangers related to the utilization of these items and to guarantee that all solutions are protected and of reasonable quality. Discussion in this review is restricted to lethality-related issues, and significant well-being concerns are emerging from the utilization of homegrown drugs and additional factors advancing them. Some essential difficulties related to the successful checking of security of these homegrown cures are additionally featured to refocusing appropriate administrative offices on the requirement for viability and guaranteeing sufficient assurance of general well-being and advancing well-being.

The worldwide acknowledgment and utilization of natural medicines and related items keep on assuming exponential increment. Issues identifying with unfavorable responses as of late are additionally ending up more distinctive, expanding in common and no longer accessible to refute as a result of prior misguided judgment with regards to or classifying homegrown restorative items as "protected" because they are getting from "characteristic" source. In this manner, regulatory approaches on homegrown pharmaceuticals should be institutionalized and reinforced on a worldwide scale. Pertinent administrative experts in various nations of the world should be proactive and keep on putting proper set up measures to secure the general well-being by guaranteeing that every single natural prescription endorsed available to be purchased sheltered and of reasonable quality. Suppliers of pharmaceuticals, for example, doctors, attendants, and drug specialists, regularly have little preparing in and comprehension of how homegrown solutions influence the well-being of their patients. Satisfactory preparation is present extremely basic since most patients are regularly on different sorts of the solution or non-professionally prescribed drugs. Additionally, it is vital that all suppliers of natural solutions are adequately enabled to assume a part in observing well-being of homegrown prescriptions. This, be that as it may, ought to be in a joint effort with the traditional healers. For this to be successful, it is basic to make a climate of trust to encourage satisfactory sharing of information about the utilization and well-being of homegrown solutions. Truth be told to suppliers of homegrown solutions, and patients/shoppers are imperative for the version of possibly genuine dangers from misuse of natural medications. The supplier must demonstrate adequate responsibility toward understanding the utilization of homegrown drugs.

West Godavari is a part of Andhra Pradesh, India, which hosts several plants that have high medicinal importance. Each of the plants has a unique feature which can heal lethal diseases. Some researchers have drawn some plants into attention from lots of surveys. It also hosts traditional healers who can efficiently cure some of the deadly diseases.

The traditional plant healing has brought a significant breakthrough in curing diseases very efficiently. The nutraceutical companies have isolated plant compounds based on the knowledge provided by the traditional healers. An adequate amount of knowledge is still to be known from the traditional healers. There is a substantial increase in the use of Ayurvedic medicines and medicines derived from plant sources. The reason being its efficacy and lack of side effects, and there is an economic point of view as well. Based on the information given by the traditional healing system, several modern scientific studies are being conducted on the various medicinal plants.

Some of the medicinal plants have been perished because of urbanization. The ancient knowledge about medicinal plants had not been documented, and many of the valuable plants are

at the edge of extinction. The government and NGO should take necessary conservatory steps to avoid extinction of valuable medicinal plants.

Several bioactive compounds isolated from medicinal plants are in excess demand in nutraceutical companies.

The present study aims to review the medicinal plant resources of West Godavari district, Andhra Pradesh. This review also deals with the critical aspects such as medicinal properties of various medicinal plants present in West Godavari district of Andhra Pradesh, India. This article deals with the medicinal properties of plants which are implicated in various diseases such as jaundice, cardiac diseases, asthma, cancer, skin diseases, diarrhea, conjunctivitis, ulcers, diabetes, leprosy, syphilis, and neural diseases.

This article also deals with the scientific studies conducted by the researchers on numerous medicinal plants present in West Godavari.

2. Medicinal plants of West Godavari

2.1. *Annona reticulata* L.

Annona genus (Annonaceae) has about 119 species [6]. *Annona reticulata* belongs to the plant family Annonaceae and is a semi-evergreen and small deciduous tree [7]. *Annona* species are having a place in custard apple family and is cultivated everywhere in India for its fruit. All components of genus *Annona* are employed in natural medication within the tropics. It is thought to be a smart supply of natural antioxidants for various diseases.

It is being cultivated in Peru and Brazil and is grown mostly in the Bahamas and occasionally in southern Florida, Bermuda, the east coast of Malaysia, and throughout Southeast Asia and the Philippines [8, 9].

2.1.1. Scientific evidences

This plant is known to possess antioxidant activity [7], anticancer activity [10–13], anti-helminthic activity [14], anti-inflammatory activity [6], analgesic, and CNS depressant activity [15].

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2.2. *Abutilon indicum*

Abutilon indicum (Linn.) belong to family Malvaceae and it is scattered throughout Andhra Pradesh, India, and it is being used for treating various diseases like diabetes, leprosy, ulcer, and jaundice [16].

In Siddha System of Medicine, it has been using as a remedy for jaundice, piles, ulcers, and leprosy [17].

2.2.1. Scientific studies on Abutilon indicum

This plant is proved to have diuretic activity [18], antimycotic activity [19], anti-arthritic activity [20], anti-inflammatory and anti-asthmatic activity [21], hypoglycemic activity [22], anticonvulsant activity [23], wound healing activity [24], antidiarrhoeal activity [25], antimalarial [26], and hepatoprotective activity [27].

2.3. *Abrus precatorius*

Abrus precatorius belongs to family Fabaceae. *Abras precatorius* is a plant originating from Southeast Asia. The name *Abrus*, means beautiful or graceful, is used to describe the appearance of the seed [28]. The seeds of *Abrus precatorius* have a history in a variety of roles because they have uniform size and weight. They were once called as Rati, and utilized as weights for measuring gold and silver [28].

2.3.1. Scientific studies on Abrus precatorius

This plant is demonstrated to have antibacterial activity [29], diuretic activity [30], nephroprotective activity [31], neuroprotective activity [32], bronchodilator activity [33], effect on neuromuscular antioxidant activity [34], anticonvulsant activity [35], antispasmodic activity [36].

2.4. *Acacia Arabica*

Acacia is the most remarkable variety of family: Leguminosae, as a matter of first importance, portrayed by Linnaeus in 1773. It is assessed that there are approximately 1380 types of Acacia around the world, and two-thirds of them local in Australia and rest of spread around tropical and subtropical districts of the world [37–39].

2.4.1. Scientific studies on Acacia Arabica

This plant is proved to have antidiabetic activity [40], antimutagenic activity [41], antimicrobial activity [42], antifungal activity [43], antidiarrhoeal activity [44], antiviral activity [45], nematicidal activity [46], antioxidant activity [47], and abortifacient activity [48].

2.5. *Bambusa arundinacea*

Bambusa arundinacea, belong to Gramineae family, is a highly reputed Ayurvedic tree commonly known as the Bamboo [49]. Bamboos contrast from alternate individuals from the grass family

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because of the nearness of branches at every node. A bamboo culm comprises of an internode (which is empty for most bamboo) and a node, which is robust and gives basic structural integrity to the plant. The buds on the node later develop into side branches [50].

2.5.1. Scientific evidences

This plant is demonstrated to have antifertility activity [51], anti-bacterial activity [52], anti-inflammatory [53], and anti-ulcer activity [54].

2.6. *Boerhavia diffusa* L.

Boerhaavia diffusa L. (Nyctaginaceae), generally known as "Punarnava" in the Indian arrangement of medicine, is a perennial creeping herb found all through the wastelands of India [55]. The roots are reputed to be diuretic and laxative and are given for the treatment of anasarca, ascites, and jaundice [56].

2.6.1. Scientific evidences

This plant is verified to have antidiabetic activity [57], antibacterial activity [58], hepatoprotective activity [56], analgesic/anti-inflammatory activity [59], antitumor activity [60], anticonvulsant activity [61], antiproliferative and antiestrogenic activity [62], cytological activity [63], bronchial asthma [64], and anti-fibrinolytic activity [65].

2.7. *Calotropis procera*

Calotropis procera belong to family Asclepiadaceae is a tropical plant growing wild in warm climate up to an altitude of about 1050 m. It is a native plant of North Africa, and it is well distributed throughout India, particularly it is abundantly found in Rajasthan. It also found in Pakistan, Africa, Mexico, Australia, Egypt, Central and South America, and Caribbean islands [66, 67].

2.7.1. Scientific evidences

This plant is verified to have hepatoprotective activity [68], antioxidant activity [69], antipyretic activity [70], anthelmintic activity [71], anti-inflammatory activity [72], antidiarrhoeal

activity [73], spasmolytic activity [74], antidiabetic activity [75], antiulcer activity [76], and wound healing activity [77].

2.8. *Momordica charantia*

Momordica charantia is a climber that have its place in family Cucurbitaceae, is commonly known as bitter gourd or bitter melon. This plant typically grows in tropical areas of Asia, Amazon, East Africa, and the Caribbean and it is being cultivated throughout the world for its use as a vegetable as well as medicine [78].

2.8.1. Scientific evidences

This plant is demonstrated to have antioxidant activity [79], antidiabetic activity [80], anticancer and antitumoral activity [81], antiviral activity [82], antifertility activity [83], and antineoplastic activity [78].

2.9. *Punica granatum*

Punica granutam is widely known as pomegranate. It belongs to Punicaceae family, which is a large deciduous shrub or small tree native to Asia. *Punica granutam* have been used in

folk medicine for centuries in the Middle East, India, and China, and it has been used to treat disorders ranging from inflammation and rheumatism to the pain of a simple sore throat. The most famous usage worldwide has been as a vermifugal or taenicidal agent [84–87].

2.9.1. Scientific evidences

This plant is verified to have healing activity [88], anti-inflammatory activity [89], antidiabetic activity [90], and anticancer activity [91].

2.10. *Pongamia pinnata*

It is a medicinal plant native to the Western Ghats and is chiefly found in tidal forests of India. *Pongamia pinnata* also was known as *Derris indica*, is a monotypic genus and grows profusely along the coasts and riverbanks in Myanmar and it has multi-purpose benefits and as a potential source of biodiesel [92, 93].

2.10.1. Scientific evidences

This plant is proved to have antihyperglycemic and antilipidperoxidative effects [94], antihyperammonemic effect [95], anti-inflammatory activity [96], antiviral activity [97], antifilarial potential [98], ulceroprotective activity [99], nootropic activity [100], and antinociceptive activity [101].

2.11. *Piper longum*

Piper longum Linn. has been named under the family Piperaceae is a flowering plant in the *Piper* family. *Piper longum* commonly known as long Indian pepper, it is widely being used as a spice and flavoring agent in various foods and herbal formulations. It is widely cultivated in India, Nepal, Indonesia, Malaysia, Sri Lanka, Timor, and the Philippines. In India, it is extensively grown in the central Himalayas to Assam, Khasi and Mikir hills, lower hills of West Bengal and evergreen forests of the Western Ghats from Konkan to Kerala and also from Car Nicobar Islands because of its therapeutic potential [102–105].

2.11.1. Scientific evidences

This plant is shown to have anti-apoptosis and antioxidant [106], analgesic activity [107], immunomodulatory activity [108], anticancer and antitumor activity [109], antidiabetic activity [110], antifertility activity [111], anti-snake venom activity [112], melanin-inhibiting activity [113], and antiulcer activity [114].

2.12. *Ricinus communis*

Ricinus communis, belong to a family Euphorbiaceae and it is most commonly known as castor oil plant. *Ricinus communis* as a tropical plant, known as castor bean, distributed widely across the world, and it is a local of India and developed all through the nation in greenery enclosures and fields and furthermore develops wild in squandering places [115, 116].

2.12.1. Scientific evidences

This plant is shown to have antimicrobial and antifungal [117], antioxidant activity [118], anti-implantation activity [119], anti-inflammatory and free radical scavenging activity [120], central analgesic activity [121], anti-tumor activity [122], larvicidal and adult emergence inhibition activity [123], antiulcer activity [124], molluscicidal, insecticidal and larvicidal activity [125], antidiabetic activity [126], cytotoxic activity [127], and antihistaminic activity [128].

2.13. *Syzygium cumini* (L.)

Syzygium cumini Linn. is a huge evergreen tropical tree belongs to the family Myrtaceae, and this plant is also mentioned in literature as Jamun, synonym as black plum or jambolana, since ancient age this plant is very well-known for their pharmacological properties [129, 130].

2.13.1. Scientific evidences

This plant is revealed to have antiallergic activity [131], gastroprotective activity [132], antioxidant activity [133], CNS activity [134], anti-inflammatory activity [135], antihyperlipidemic activity [136], antidiarrhoeal activity [137], antipyretic activity [138], antispasmodic activity [139], and antiviral activity [140].

2.14. *Sida cordifolia*

Sida cordifolia L. belongs to family Malvaceae and commonly called as Country Mallow and Bala (Sanskrit). This herb is extensively spread throughout the tropical and subtropical regions of India [141].

In Ayurvedic practices, *Sida cordifolia* has three basic applications: Mashabaladi Kvatha, where the plant seeds are blended with different fixings to soothe relieve muscular pain; Balataila, a procedure for the treatment of sensory system grievances and stomach issues and as a heart tonic; and the squashed leaves of the plant as an astringent for the treatment and dressing of wounds or skin wounds [142].

2.14.1. Scientific evidences

This plant is shown for its antioxidant activity [143], anti-inflammatory activity [144], antiulcer activity [145], antidiabetic activity [146], nephroprotective activity [147], cytotoxicity [148], anti-hypercholestrolemic activity [149], hepatoprotectivity [150], cardiovascular activity [151], and anticancer activity [152].

2.15. *Sapindus mukorossi*

Sapindus mukorossi belongs to family Sapindaceae and has some common names such as soapnut, soapberry, washnut, reetha, aritha, dodan, and doadni. It is an attractive medium-sized deciduous tree found in diverse geographical provinces like Gangetic Plains, Western Ghats, and Deccan Plateau in India [153].

2.15.1. Scientific evidences

This plant is proved to have an anti-mosquito activity or larvicidal activity [154], cytotoxic activity [155], tyrosinase inhibition and free radical scavenging [156], antigonorrhoeal activity [157], antifungal activity [158], and molluscicidal activity [159].

2.16. *Tribulus terrestris* L.

Tribulus terrestris L. is a well-known plant that belongs to genus Tribulus. The genus *Tribulus*, having a place with family Zygophyllaceae, involves around 20 species on the world. Among them, *T. terrestris* is a well-practiced medicinal herb by Ayurvedic practitioners as well as by modern herbalists [160].

2.16.1. Scientific evidences

This plant is demonstrated to have diuretic activity [161], aphrodisiac activity [162], anti-urolithic activity [163], immunomodulatory activity [164], antidiabetic activity [165], hypolipidemic [166], central nervous system (CNS) activity [167], anticancer activity [168], and larvicidal activity [169].

2.17. *Terminalia chebula*

Terminalia chebula is a moderate plant which is being utilized as a part of conventional solutions. It has a place in the Combretaceae family. It is typically called as Black Myrobalan, Ink tree (or) Chebulic myrobalan and furthermore known as "Ruler of the drug." It is widely utilized as a part of Unani, Ayurveda, and homeopathic prescription. *Terminalia chebula* is a well-known conventional plant utilised in the pharmaceutical industry in India as well as in different countries of Asia and Africa [170]. It stimulates the liver and ensures it is promote by removing the excretory waste items from the digestion tracts. It is shown in protracted loose bowels with hematochezia and prolapse of rectum. It is a decent nervine, utilized as a part of anxious shortcoming, apprehensive crabbiness.

2.17.1. Scientific evidences

This plant is demonstrated to have antibacterial activity [171], antiamoebic and immunomodulatory activities [172], antianaphylactic and adaptogenic activities [173], antiviral activity [174], antimutagenic and anticarcinogenic activities [175], anti-arthritic activity [176], antidiabetic and retinoprotective activities [177], and hepatoprotective activity [178].

2.18. *Tephrosia purpurea*

Tephrosia purpurea or Sharpunkha has its place in the family Leguminosae (subfamily-Papilionaceae). The genus *Tephrosia* encompasses between 300 and 400 species of annual and perennial woody herb, scattered in the tropical and subtropical local of the world. This plant has excessive economic cost due to the presence of phytochemicals like flavonoids, sugars, gums tannins and phenols, alkaloids and glue, settled oils and fats, and saponins and lipids [179, 180]. As per Ayurveda writing, this plant has additionally given the name of "Sarwa Wranvishapaka" which implies that it has the property of mending a wide range of wounds. It is an imperative part of a few arrangements, for example, Tephroli and Yakrifitused for the liver issue. In the Ayurvedic arrangement of different pharmaceutical parts of this plant, they are utilized as a solution for impotency, asthma, diarrhea, gonorrhea, ailment, ulcer, and urinary issue.

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2.18.1. Scientific evidences

This plant is verified to have anticarcinogenic and anti-lipid peroxidative [181], anti-inflammatory and analgesic [182], in vitro antioxidant [183], anticancer activity [184], and in vitro anthelmintic activity [185].

2.19. *Tectona grandis*

T. grandis Linn. belongs to family Verbenaceae is one of the most well-known timbers in the world and is famous for its dimensional stability, extreme durability, and hardness which also resists decay even when unprotected by paints and preservatives. This plant is commonly called as teak. It is one of the most famous heartwood of the world. Timber value of teak has been well-known for decades [186, 187].

2.19.1. Scientific evidences

This plant is verified to have hair growth activity [188], cytotoxic activity [189], anti-hemolytic anemia activity [190], hypoglycemic activity [191], anti-inflammatory activity [192], diuretic activity [193], and gastroprotective activity [194].

2.20. *Tamarindus indica* L.

Tamarindus indica or tamarind regarded as a tropical fruit tree native to the African savannahs and it is found in numerous tropical nations. It is arranged as a monospecific class in the group of Leguminosae. The sweet and harsh taste of its natural product mash is utilized to add flavor to neighborhood cooking styles. Other than culinary, tamarind is likewise utilized as a part of the conventional drug as purgative, diuretic, antibacterial operators and also in the treatment of fever and malarial contaminations [195, 196].

2.20.1. Scientific evidences

This plant is verified to have antipyretic activity [197], laxative activity [195], anticancer activity [198], antiemetic activity [199], antimicrobial [200], hepatoprotective [201], and analgesic activity [202].

2.21. *Withania somnifera*

Withania somnifera (WS) belongs to the Solanaceae family, commonly known as Ashwagandha. Traditionally this plant was named for its potential to calm the mind, the capacity to improve learning ability, memory power, and to improve poor eyesight. It is also named for anti-inflammatory potential in the treatment of joint diseases and an appropriate remedy for asthma and bronchitis [203]. Ashwagandha is one among the vast assorted variety of the restorative plant, which is exploited well for its phytopharmacological impact. The restorative properties of *Withania somnifera* are accessible both in the composed and non-composed arrangement as conventional information since time immemorial. In the conventional framework, the plant

has been utilized as a calming, antitumor, antistress, cell reinforcement, immunomodulatory, and adaptogenic medicate. It likewise applies a positive effect on the endocrine, cardiopulmonary, and focal sensory systems with next to zero-related poisonous quality. It has the ability to battle growths by lessening tumor size and ended up being a decent regular wellspring of a sturdy and moderately safe radiosensitizer/chemo-remedial specialist.

2.21.1. Scientific evidences

This plant is proved to have anti-inflammatory activity [204], antioxidant property [205], anticancer properties [206], immunomodulatory potential [207], neuroprotective effects [208], cardioprotective and hypocholesteremic [209], and antimalarial potential [210].

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Chapter 6

Biomedical and Pharmaceutical Applications of Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

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Additional information is available at the end of the chapter

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Abstract

Medical science is a field of study that is relevant to all people, but the development of pharmaceutical, biomedical and life science is of particular importance. In these fields, further studies are being established to determine with incredible accuracy the quantities and concentration of inorganic elements and organic compounds, such as nucleotides, sulphur and phosphorous containing peptides and proteins, to be used in all kinds of drugs. Since 1980, inductively coupled plasma-mass spectrometry (ICP-MS) has emerged as a new and powerful technique for elemental and isotopic analysis. It provides a means for the analysis of an extremely wide range of elements and the co-analysis of most elements in the periodic table. It can also be used for qualitative, quantitative and semi-quantitative analysis and for the measurement of isotopic ratios through mass-to-charge ratios. In recent years, ICP-MS has emerged as the best technique for the quantification of inorganic impurities in pharmaceutical and biomedical applications. This chapter focuses on introducing the applications of ICP-MS in the pharmaceutical and biomedical fields. Some problems facing ICP-MS are also presented at the end of this chapter.

Keywords: pharmaceutical analysis, biomedical analysis, inductively coupled plasma-mass spectrometry (ICP-MS)

1. Introduction

Within the field of pharmaceuticals, two fundamental issues are necessary for drug therapy: safety and efficiency. The impact of elemental impurities in drugs must be investigated using

pharmacological and toxicological profiles so that the safety requirements and dosage forms of bulk drug scan be determined. This is vital because unwanted pharmacological and toxicological effects may result from elemental impurities and improper drug dosage forms. As such, all products intended for human consumption must be characterised as completely as possible to ensure quality and safety and to ensure that elemental impurities are being monitored and controlled. Thus, pharmaceutical analysis is one of the most important activities related to ensuring the safety of drugs [1].

1.1. Heavy metals in drugs

During the synthesis of pharmaceuticals, inorganic impurities can result from several different sources and phases, including solvents, raw materials, reagents, catalysts, electrodes, reaction vessels, plumbing and other equipment used. These impurities are often a result of the manufacturing process. Routinely monitored as impurities in several drugs are cadmium, copper, chromium, mercury, iridium, molybdenum, nickel, osmium, lead, tungsten, palladium, platinum, rhodium, and ruthenium and vanadium and their derivatives [2]. For the synthesis of many pharmaceuticals, tungsten-containing catalysts are used in the intermediate reaction process. In the pharmaceutical industry, monitoring heavy metals is an important activity for both reaction intermediates and final drug substances, not only because of their ability to catalyse decomposition but also because of their potential for toxicity. Even at very low doses, heavy metals such as lead, cadmium, mercury and chromium pose a serious health risk when used for pharmaceutical purposes [3]. Long exposures can cause physiological and behavioural issues. For instance, daily exposure to 0.06 mg of lead for a period of 1 month is sufficient to cause long-term problems such as kidney impairment, demineralization and obstructive lung disease. In pharmaceutical and biomedical substances, the presence of heavy metals is monitored and limited by regulatory authorities. Tests are performed to ensure that no inorganic-based reagents or contaminates are introduced into the drugs at any step during the manufacturing process. The determination of residues of metal catalysts and reagents has moved increasingly into the focus of regulatory guidance within the last 5 years [4]. The European medicines agency has published guidelines on the specification requirements for inorganic metal catalysts and reagents [5]. The United States Pharmacopoeia (USP), British Pharmacopoeia (BP), European Pharmacopoeia (EP) and Japanese Pharmacopoeia (JP) have proposed the collective monitoring of the total metal content in pharmaceutical products. For example, the EP has proposed a limit of 20 μ.g/g of platinum in calcium folinate. In aqueous solutions, metal sulphide precipitation results in a clear visual difference of colour, but this are not the case for similarly treated standard metal solutions. In both cases, determining the exact presence of heavy metals is a process that is time consuming and not selective, specific or accurate. Thus, there is a great need to develop a highly sensitive and more selective technique to determine the presence of heavy metals in pharmaceutical substances and, accordingly, to ensure the safety and efficacy of drugs intended for human consumption.

1.2. Atomic spectrometric techniques used in pharmaceutical and biomedical analysis

Spectrophotometric techniques are widely used for trace elemental analysis, including atomic absorption spectrophotometer (AAS), inductively coupled plasma-atomic emission

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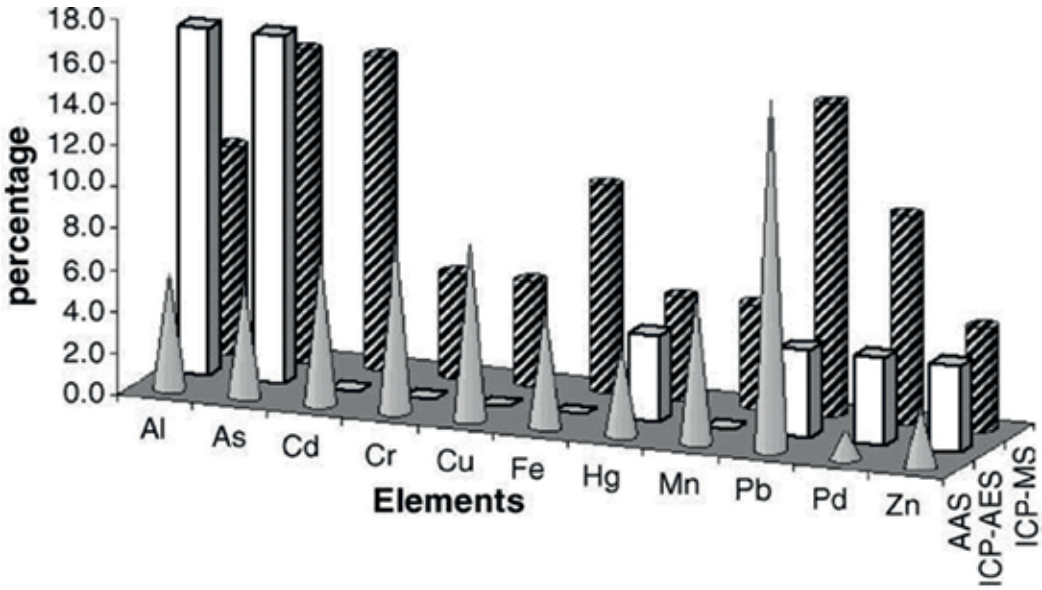

Figure 1. Usage pattern of different spectrophotometric techniques in detection of trace metals in drugs and pharmaceutical industry.

spectrophotometer (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) [3, 6]. AAS is used extensively, often for analysing different samples. Flame-AAS (F-AAS) or low graphite furnace (GF-AAS) is used for samples with a high concentration of metals. Because of its high detection limit, the application of this technique to the analysis of impurities is limited. However, each metal present can be individually analysed to a high level of specificity because of the hollow-cathode lamp used.

ICP-AES is an important technique for the trace elemental analysis of pharmaceutical samples [7, 8]. While it is a powerful technique for multielemental analysis, it does suffer from spectral interferences and low levels of accuracy. At present, ICP-MS has emerged as the most powerful, sensitive and selective technique for the determination of elemental impurities in trace and ultra-trace concentrations in pharmaceutical and bulk drug substances. Hence, ICP-MS provides a major service to the pharmaceutical and bulk drug industries in allowing them to identify and quantify elemental impurities. However, there are limitations to ICP-MS in that it is very expensive and lacks certified reference standards for most drugs and pharmaceutical products. The pattern of the various spectrometric techniques used recently is shown in **Figure 1**.

2. Principle and instrumentation of ICP-MS

The hyphenated ICP-MS principle and construction are essentially identical. Plasma employs the ionisation source for the ICP, and positively charged ions detect the mass analyser in the mass spectrometer (MS). Generally, argon gas is used to generate plasma at a high frequency (30 MHz), with energy in the range of 1000–2000 W, since in the periodic table most elements

excite and ionise at plasma temperatures in the range of 6000–10,000 K. The torch is made with quartz and consists of three concentric tubes through which the argon flows. The periodic table shown in the **Figure 2** makes multielemental analysis possible. A schematic diagram of the ICP-MS instrumentation is shown in **Figure 3**.

When a sample is introduced into the plasma, it goes through desolvation, vaporisation, atomisation and ionisation before entering the mass analyser. There are many methods for introducing the samples, which can be solid, liquid or gas, into the plasma. Most commonly, liquid samples are introduced using pneumatic nebulization.

From ICP, the MS extracts the ions at a low pressure, via sampling and skimmer cones. Later, ions travel towards the quadrupole mass analyser through a series of ion lenses. Positively charged ions are then separated according to the mass-to-charge ratio. Ions can then be detected by an electron multiplier and amplified. The quadrupole mass analyser is widely used ICP-MS, and the double-focusing field mass analyser is used to achieve more separation and to decrease isobaric interference (such as ^{40}Ar, $^{35}Cl^{+}$ interference with $^{75}As^{+}$). A speciation analysis can also be performed using time-of-flight mass spectrometry (TOFMS). Another possible separation technique, the introduction of samples into the source of ICP at atmospheric pressure makes its possible of coupling separation techniques to ICP-MS. This decreases the isobaric overlap of the high-resolution mass analyser and interests ions with different mass-to-charge ratios differently via chemical reaction. The percentages of the different elements analysed by ICP-MS in pharmaceutical substances and bulk drugs are shown in **Figure 4**.

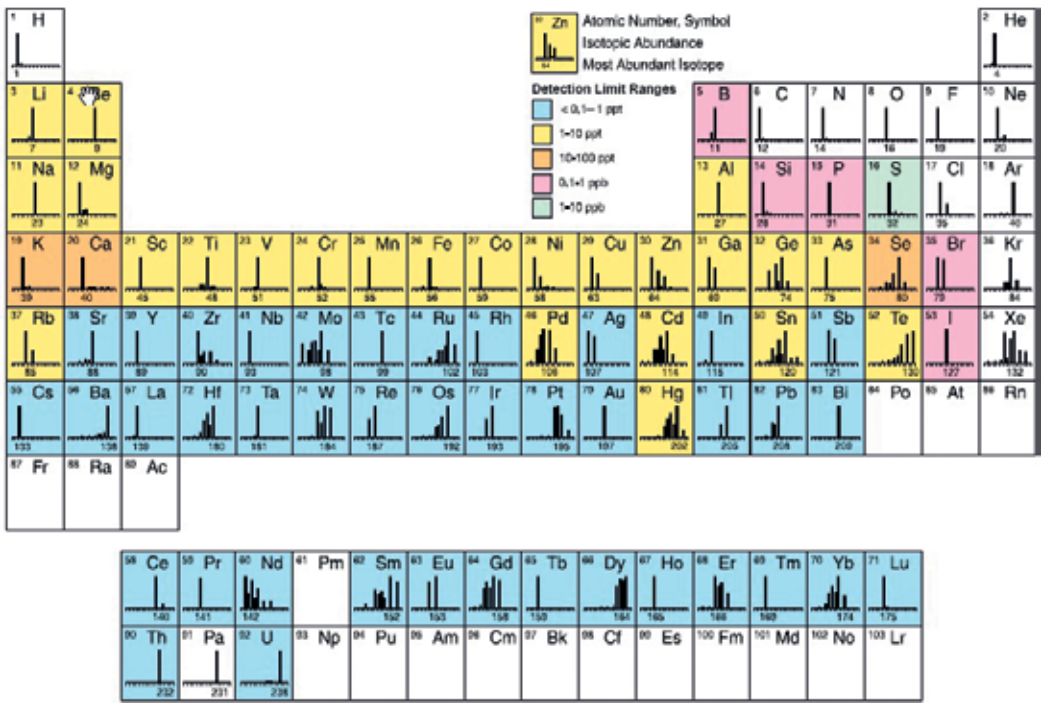

Figure 2. Elements detectable by ICP-MS analysis.

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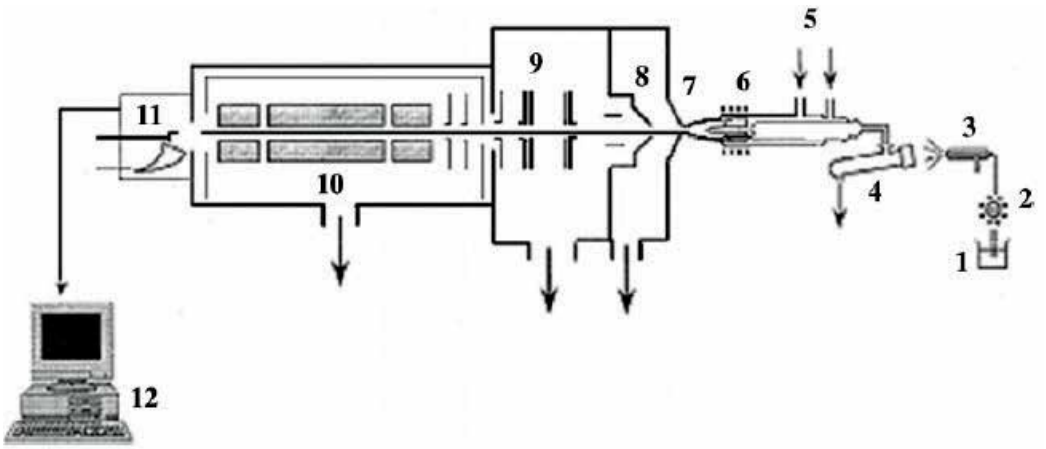

Figure 3. Schematic diagram of inductively coupled plasma-mass spectrometer: 1. Liquid sample, 2. Pump, 3. Nebuliser, 4. Spray chamber, 5. Argon gas torch inlets, 6. Torch, 7. Sampler cone, 8. Skimmer cone, 9. Ion lenses, 10. Quadrupole mass analyser, 11. Electron multiplier detector, and 12. Data collection.

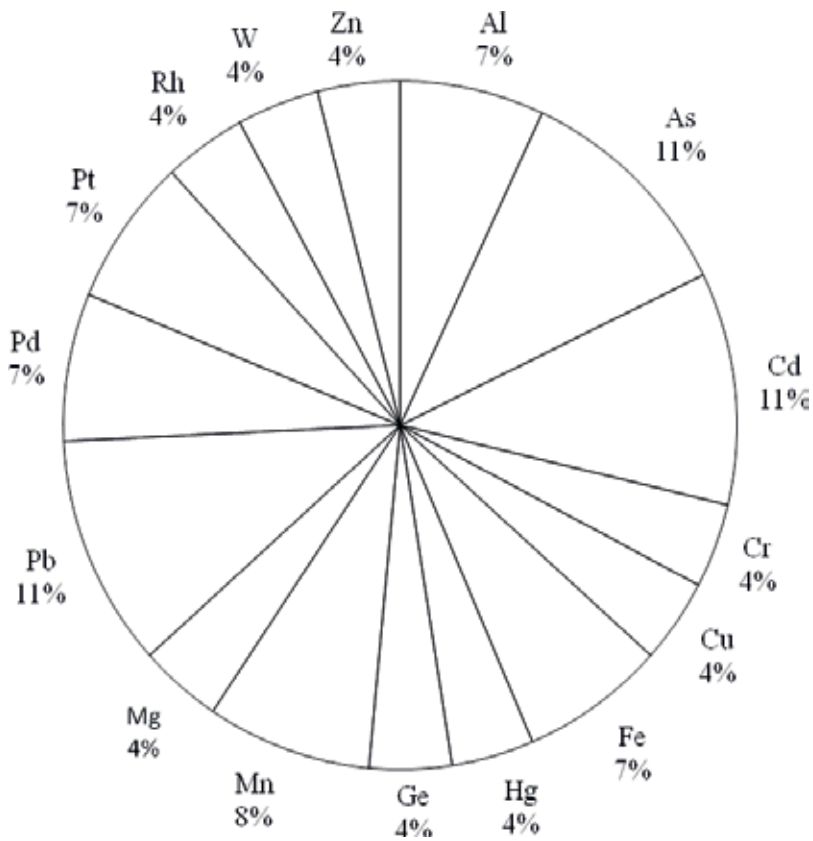

Figure 4. Individual elements analysed by ICP-MS in pharmaceutical substances and bulk drugs.

3. Sample preparations

Samples are normally prepared using dissolution or digestion with a low concentration of mineral acids [9, 10]. For the reproducibility of volatile, low-concentration and low-volume elements, microwave digestion is better than acid digestion. However, microwave digestion and acid digestion are both appropriate for pharmaceutical samples due to the volatility of some metals and trace detection limits. The laser ablation technique [11] can be used for solid samples. Some of the sample preparation methods are summarised in **Table 1**.

Element	Bulk drug/ formulation	Procedure	Medium	Calibration[a]	Reference
W	Bulk drugs	Dissolution	80:20 (v/v) HNO_3	EC with ^{209}Bi for IS	[12]
Fe	Methotrexate	Microwave-assisted	H_2SO_4 + HNO_3	EC with ^{56}Fe	[13]
Pd, Pt, Rh	Enalapril maleate	Dissolution	1:1 HNO_3, 0.3 M,	EC with In for IS	[14]
	Calcium folinate	Dissolution	0.2 M HNO_3, 0.2 M HNO_3		
	Levodopa	Dissolution			
Pd	Fosinopril sodium	Dissolution	25% (v/v) 2-butoxy ethanol and water	EC with In for IS	[15]
69 elements	Drug substances, intermediate and raw materials	Dissolution, sonication	80% (v/v) HNO_3	EC	[16]
Cr, Ni, Sn, Pb	Vitamin E	Microwave digestion	HNO_3 + H_2O_2, Triton X + Tetralin	EC, Y, In and Tl for IS	[17]
		Emulsion preparation			
Na, Br, Pd, Ba, I	Methamphetamine HCl	Dissolution	H_2O	EC	[18]
As, Se, Mo, Ru, Pd, Cd, In, Sn, Sb, Pt, Hg, Bi, Ag.	API with various functionalities	Dissolution	2-butoxy ethanol/ water (25:75 v/v)	EC with Co, Au and Rh for IS	[19]
Cr, Cu, Mg, Mn, Mo, P, Se, Zn.	Multimineral and multivitamin	Microwave digestion	HNO_3	EC with Co for IS	[20]
Li, B, V, Cr, Mn, Ni, Zn, Cu, Br, Sr, Sn, Ba, Pt, and Pb	Ecstasy tablets	Dissolution	1% (v/v) HNO_3	EC with Rh for IS	[21]
Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, Hg, Pb	Dicyclomine HCl,	Dissolution and digestion	5% (v/v) HNO_3	EC	[22]
	Ethambutol,		HNO_3 + H_2O_2 + H_2O		
	Pyrazinamide,				
	Furazolidone				
As, Cd, Hg, Pb	Dietary supplements	Microwave digestion	HNO_3	Ec with Rh, IN Lu, Bi for IS	[23]

[a]EC: external calibration; IS: internal standardisation.

Table 1. Sample preparations procedures for analysis of trace elements in pharmaceutical substances and bulk drugs using ICP-MS.

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4. Applications

4.1. Applications of ICP-MS in pharmaceutical samples

4.1.1. Determination of multielements in drugs and pharmaceutical substances

Supplements in the form of gluconate contained the lowest concentration of cadmium, which was detected in many zinc supplements, according to ICP-MS [26]. In calcium supplements, cadmium and lead were detected using ICP-MS, F-AAS and electrothermal-atomic absorption spectroscopy (ETAAS) [27]. Using GF-AAS and inductively coupled plasma-optical emission spectroscopy (ICP-OES), iron and palladium were detected in the bulk drug methotrexate; and the results were compared which obtained from ICP-MS [13]. To avoid polyatomic interferences before quadrupole mass analysis (such as ^{40}Ar, ^{16}O with ^{56}Fe), hexapole collision cell was used. Using ICP-MS, lead and its isotope ratio was investigated in antacids and calcium drug supplements using rhenium as an internal standard [28–30].

Tungsten has been identified in bulk drug substances and their intermediates, with bismuth used as an internal standard [31]. With the help of different isotopes, spectral interferences have been monitored in bulk drug substances. In bulk drug samples, ICP-MS was also used to detect up to 2500 ppm of tungsten [31]. The ICP-MS results are in accordance with those of microwave induced plasma-mass spectrometry (MIP-MS). Hence, ICP-MS has been used for the expeditious screening for inorganic impurities such as rhodium, palladium, platinum, beryllium, vanadium, manganese, cobalt, nickel, copper, zinc, molybdenum, cadmium, tin, thorium and lead, using Rh^{103}, Pd^{105}, Pd^{106}, Pt^{195} and In isotopes as an internal standard.

4.1.2. Multielements in synthetic drugs

Sodium, bromine, palladium, barium and indium have been investigated in methamphetamine hydrochloride, which is prepared using catalyst and reagent methods [18]. Barium, palladium, bromine, iodine and sodium were detected by Emeds and Nagais using ICP-MS. The levels of these elements in N-methylamphetamine were measured, and the results were compared with neutron activation analysis (NAA). ICP-MS has been used to detect multielements in synthetic drugs; these elements include arsenic, selenium, cadmium, indium, tin, antimony, lead, bismuth, silver, palladium, platinum, mercury, molybdenum, and ruthenium. Several ICP-MS procedures have been reported for performing single and multielemental analysis in drugs and pharmaceutical substances, as shown in **Table 2**.

4.1.3. Multielements in herbal drugs

In herbal drugs such as dietary supplements, inorganic impurities are a major concern due to their major toxicity and impurities (e.g., As, Cd, Hg and Pb) [23]. Normally, dietary supplements are analysed using high-resolution ICP-MS. Microwave digestion method is also an accepted method according to National Institute of Standards and Technology (NIST) reference materials.

Drug/formulations	Elements	MDL	Detector	Reference
Dicyclomine HCl, Ethambutol, Pyrazinamide, Furazolidone	Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd, Hg, Pb	Not reported	Varian ultramass 700	[22]
Neusilin	Al, Mg	40 and 6 μg/g	Laser ablation system: CETACLSX-100, Perkin-Elmer SCIEX, ELAN 6000ICP-MS	[23]
Drug substances, intermediate and raw materials	69 Elements	0.004–19 ppm	Elan 6000 ICP-MS, 1300 Wrf, cross flow nebuliser, Scott spray chamber	[16]
Vitamin E	Cr, Ni, Sn and Pb	3.02, 0.22, 2.92, 0.07 ppb (emulsion) 0.13, 0.05, 0.70, 0.08 ppb (15% HNO_3)	Aglient 7500 ICP-MS, 1100 Wrf Meinhard nebuliser	[17]
Methamphetamine HCl	Na, Br, Pd, Ba, I	Not reported	Seiko ICP-MS SPQ-6100, 1.35 kW quadrupole	[18]
Fosinopril sodium (monopril)	Pd	0.1 μg/g	Plasma Quad PQ11 Turbo plus ICP-MS jacketed Scott type spray chamber, cooled to 5°C, platinum sample cone	[15]
Enalapril maleate, calcium folinate, levodopa	Pd, Pt, Rh	15, 2.8, 2.5 ng/g	Plasma Quad II STE peristaltic pump: spray chamber: double pass, water cooled (10°C)	[14]
API	As, Se, Mo, Ru, Pd, Cd, In, Sn, Sb, Pt, Hg, Bi, Ag.	0.37, 0.42, 0.08, 0.2, 0.18, 0.03, 0.17, 0.35, 0.16, 0.03, 1.82, 1.51, 0.15 μg/g	VG Plasma Quad PQII TURBO plus, ICP-MS and Micro mass platform ICP-MS	[19]
Multimineral and multivitamin	Cr, Cu, Mg, Mn, Mo, P, Se, Zn	48.3, 47.1, 24.3, 9.0, 43.2, 750, 24, 109 ng/l	VG Plasma Quad 3 ICP-MS, 1380 W	[20]
Methotrexate	Fe	0.2 μg/g	Thermo elemental ICP-MS with collision cell technology	[13]
Bulk drug substances	W	0.04 ppm	Perkin-Elmer Elan 6000 ICP-MS, AS-91 auto sampler 1300rf	[12]
Ecstasy tablets	Li, B, V, Cr, Mn, Ni, Zn, Cu, Br, Sr., Sn, Ba, Pt, and Pb	Not reported	Perkin-Elmer SCIEX Elan 6000 ICP-MS, Gibson peristaltic pump, rf1000W, nebuliser ryton cross flow with gem tips	[21]
Dietary supplements	As, Cd, Hg, Pb	8.2, 18, 140, 25 ng/l	Micro mass Plasma Trace-2 HR ICP-MS, rf1350W	[23]
Chinese medicinal material	As, Hg	0.19, 0.32 μg/l	ELAN 6000 ICP-MS rf 1000 W, HP 4500 ICP-MS rf 1200 W, Babington type cross flow nebuliser, peristaltic pump, double pass spray chamber	[24]
Dietary supplements	Pb, Hg, Cd, As, U, Cr, V, Cu, Zn, Mo, Pd, Sn, Sb, Tl, W	Not recorded	Micro mass platform ICP-MS Meinhard concentric nebuliser	[25]

Table 2. Determination of single and multielements in drugs and pharmaceuticals using ICP-MS.

An analysis of arsenic and mercury in Chinese Medicinal Materials (CMM) was completed using ICP-MS, with trace amounts of both arsenic and mercury found [24]. This is notable because of the leaching characteristics of these elements. Mercury and arsenic are often used in traditional Chinese medicine (TCM) and have insoluble sulphides due to the low leaching efficiency of the cinnabar or realgar minerals in the drug formulations [24]. ICP-MS has been used to determine elemental impurities in plant and dietary materials, [25] and indium has been used as an internal standard.

In herbal materials, quality and safety is of major importance. Lead, arsenic and cadmium, in particular, are of considerable importance in herbal materials such as mineralised mint, nettle and black-wrack leaves [32]. In herbal materials, arsenic, cadmium and lead have been quantitatively determined to be in the range 0.2–200 ppb, 0.1–20 ppb and 0.1 ppb–10 ppm, respectively. Also, arsenic was investigated in uncoated TCM using ICP-MS with high-pressure microwave digestion [33].

4.2. Application of ICP-MS in biomedical analysis

The continuous improvement of techniques and their applications in the life sciences sphere, including the combining of organic and inorganic MS, has helped to further advance the field. For inorganic MS and life sciences, ICP-MS has emerged as a key technique in biomedical research. In this section, some of the applications of ICP-MS in the biomedical field will be explored.

4.2.1. Analysis of DNA using ICP-MS

The development of cancer cells and modifications in DNA are commonly caused by the chemical modification of nucleobases, such as styrene oxide. The detection of cancer and DNA adducts is essential, and there are various methods based on standard reference materials, such as mass spectrometry, ^{31}P labelling and immunoassays (however, these are not readily available as they have not been made for three decades). The problem is that identification and unknown modification is not possible. Such problems as selectivity, sensitivity, and qualitative and quantitative technique soften arise when using internal standards, regardless of their structure.

For example, ICP-MS quantitatively engages phosphorus signals in modified nucleotides using bis(4-nitropenyl)phosphate (BNNP) as an internal standard [34, 35]. Hence, ICP-MS has independent elemental selectivity, sensitivity and detection capability.

4.2.2. Analysis of proteins using ICP-MS

In recent years, ICP-MS was used extensively in the field of proteomics [36] for the determination of metal concentrations in proteins and biomedical analysis. In these studies, similarly, bromine was detected quantitatively in rats and human plasma metabolism using ICP-MS [36]. In biological systems, and especially in proteins, metals play an important role as cofactors. Without proteins, there will be an absence of essential metals such as manganese, iron, copper, cobalt, molybdenum and zinc and of essential non-metals such as selenium and iodine.

A deficiency in these metals and non-metals cause diseases as well as catalytic cytotoxic reactions. The field of proteomics determines the metals contained in proteins and, therefore, faces the challenging task of identifying metals and their concentrations, which requires a more selective, sensitive and powerful analytical technique. ICP-MS has been used to identify phosphorus, iron, zinc and copper quantitatively within brain proteins and has been used in the study of neurodegenerative diseases [37, 38]. ICP-MS also represents a powerful technique for the detection of copper, iron and zinc in proteins in the study of Alzheimer's disease and has also analysed copper, zinc and iron with respect to $^{54}Fe/^{55}Fe$, $^{65}Cu/^{63}Cu$ and $^{67}Zn/^{64}Zn$.

4.2.3. Analysis of trace elements in human health

Everybody knows that in human health, some of the elements are essential and others are toxic. However, the benefits and risks of each element depend on the quantity of the intake, the accumulation, the mobility of the element, the storage and interaction of the element in metabolites, the oxidation number, the metal-ligand ratio and the complexity of its interaction with different elements. ICP-MS has become one of the most powerful techniques for elemental speciation studies. In HepG2 cell and liver cell lines, copper and its isotope ratio $^{63}Cu/^{65}Cu$ has been measured accurately using ICP-MS [39], and in human blood and livers, iron and its isotopic ratio has been measured. In the liver, iron and its isotopic composition has been found to be higher than that of the blood and muscles [40].

In vivo, many essential and non-essential elements can interact with each other. Because of these interactions, it is important to anticipate deficiency and excess and to know elemental toxicity levels. To use elemental analysis to determine concentrations, ICP-MS has proved to be an advantageous technique. ICP-MS has been used to identify trace elements that are toxic and those that are essential [41–44]. In human blood and serum, many trace elements can be found, including cobalt, copper, zinc, selenium, rubidium, rhodium, palladium, cadmium, tungsten, platinum, mercury, thallium and lead. Both blood and serum selenium correlated with blood can contain the trace elements lead, mercury, copper and zinc.

5. Brief solution to problems in the application of ICP-MS

ICP-MS is a good analytical method when compared to the other analytical methods available. However, it is not without its drawbacks. These include signal fluctuation and the matrix effect, which occurs when elements that are very close in terms of mass number experience an indistinguishable influence [45]. The matrix effect might be a result of the plasma, the deposition of salts in the orifices, ion extraction or the internal standard [46]. The matrix effect can cause severe interference, but it can be eliminated by selecting a matching standard for the matrix of the isotope being diluted or by ensuring a greater dilution of the specimen. It can be of particular concern for double electric charge ions, such as barium and cerium, as these elements have low second ionisation energy levels. Oxides can be controlled by elevating the temperature of the plasma or slowing the rate of flow. Polyatomic ions and isobars can impede the detecting the mass-to-charge ratio via quantitative analysis, making them very important to analysts.

6. Conclusion

ICP-MS has the power required to sensitively analyse the presence of trace elements; the method has low detection limits, allows for more selectivity of elemental mass, provides insight into the isotope ratio and performs ultra-trace elemental detection. It can be used for the elemental analysis of inorganic and organic elements in all kinds of drugs, including nucleotides, sulphur, and phosphorus containing proteins, providing both a qualitative and quantitative determination with accuracy, making it a vital method for trace element analysis in the pharmaceutical and biomedical fields.

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Chapter 7

Cell-Based Screening to Identify Cytoprotective Compounds

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Additional information is available at the end of the chapter

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Abstract

Prevention of cellular injury and consequent cell death is expected to provide therapeutic benefit in various diseases, but with the complexity of cell damaging pathways involved, identification and validation of novel potential drug targets is not a trivial task. New drug targets are expected to take part in complex responses with wide-ranging effects on gene expression and cellular function and drug candidates rather modify these effects than act as simple agonists or antagonists to ultimately protect the cells from an injury. Phenotypic screening may help identify cytoprotective compounds in diseases, in which the lack of drug targets makes target-based approaches unfeasible. This chapter gives an overview of the strategy of cell-based assay development, primary screening, hit selection and confirmation. Considerations about the choice of small molecule compound libraries utilized in cell-based models are discussed as well as the use of clinical drugs for drug repurposing or repositioning. The choice of cell types and issues associated with cell culture techniques are overviewed and the most common assays and readouts are briefly described. Finally, the potential pitfalls of data analysis and hit selection are discussed.

Keywords: cell-based screening, high-throughput screening, cytoprotection, drug discovery, chemical genomics, drug target

1. Introduction

The development of small molecule therapeutics for the treatment of diseases has gone through various phases over the last few decades. While the number of approved drugs continuously increased from the 1970s till 1996, when a record number of drugs (53 drugs) was approved by the Center for Drug Evaluation and Research of the U.S. Food and Drug Administration (FDA), a decline has been observed since then with no more than 20–30 new drugs approved each year [1]. In the second half of the twentieth century, drug discovery saw a rise of synthetic organic

chemistry that made it economically feasible to produce large combinatorial libraries and biology progressed to provide many novel drug targets with structural details that promoted both high-throughput screening of small molecule libraries and computer-aided drug design as potential methodologies to identify novel therapeutic agents. In 2000, the number of known human drug targets were less than 500 but with the overestimated number of human genes and an expected number of 5–10 drug targets per disease genes, the number of potential drug targets were estimated to lie between 5000 and 10,000 [2–4]. The number of human genes shrank to 19,000 and the identified small molecule drug targets rose to mere 557 (549 for FDA-approved drugs and 8 further targets for drugs approved in the rest of the world) in 2017, while 146 additional human protein targets were curated for biological drugs [5, 6]. In fact, the number of biological drugs and respective targets increased considerably over the last 20 years: in 2000 only 59 biological drugs (recombinant proteins and monoclonal antibodies) were introduced, while 250 unique biological agents were available in 2016 [3, 5]. In 2002, Hopkins and Groom argued that only those disease-modifying genes can be used as drug targets that are druggable (contain domains that small molecules can bind to) and the overlap between the druggable genome and genes linked to diseases may be between 20 and 50% [7]. They predicted that no more than 600–1500 small molecule drug targets existed in humans if we had 30,000 genes. With fewer genes than anticipated, the number of potential drug targets also decreased, which partly explains the lower number of new molecular entities over the last decades. Examining the new first-in-class drugs (compounds that modulate a novel target or biological pathway that was not targeted before their introduction) approved by the FDA during the first decade after 1999, Swinney and Anthony found that 28 of the small molecule drugs were discovered using a phenotypic screening approach compared to 17 drugs discovered by a target-based approach suggesting that phenotypic screening may be more successful because of the unbiased identification of drugs with new molecular mechanisms of action [8–10]. However, the discovery of a first-in-class drug may not be simply attributed to a phenotypic screen or a target-based approach, the two approaches do not mutually exclude each other [11]. The launch of a new chemical entity often roots in a discovery that occurred one or two decades earlier and the development of a drug that reaches the market often involves both approaches [11]. For example, a phenotypic assay may be used for the discovery of a new drug target, while subsequent optimization of drug candidates may occur via a target-based approach, or the two strategies may be combined in multiple cycles during the process. The term of 'phenotypic screening' may be broadly used for all non-target-based approaches or more selectively for testing of compounds in a system-based approach using a target-agnostic assay that monitors phenotypic changes, thus the contribution of phenotypic assays may be differently interpreted in various contexts. Nonetheless, phenotypic screening is making a comeback in drug discovery with large pharmaceutical companies like Novartis AG and GlaxoSmithKline plc admittedly promoting it, but its overall share remains unclear in the efforts of pharmaceutical companies and academics [12].

2. Planning a phenotypic screen

A clear plan is needed for any early drug discovery projects and a phenotypic screen is not an exception [13, 14]. While the steps of the drug discovery project occur in a different order

than in a target-based project, it is necessary to establish a clear go/no-go plan for all the steps (**Figure 1**). Since the target is unknown in the beginning of the project and there might be rather wide expectations about the activity of a potential cytoprotective compound, it may not be necessary to define cut-off values in the beginning. However, it is necessary to make

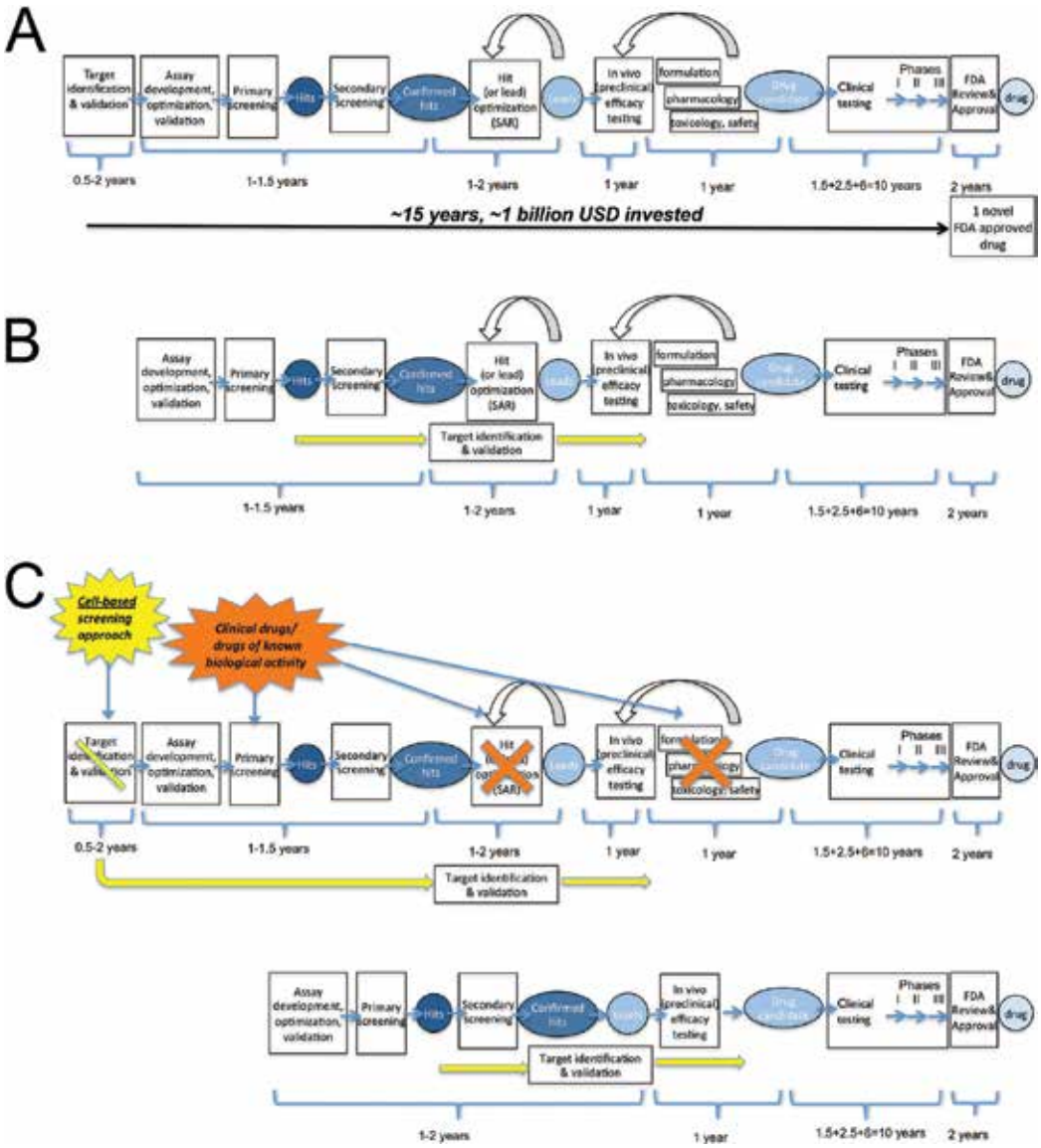

Figure 1. Basic plans of target-based and cell-based drug discovery approaches. (A) Target-based drug discovery represents the standard procedure in drug discovery. (B) Cell-based phenotypic screening skips the target identification step in the beginning of the project. This may lead to considerable time saving, since this step is usually performed simultaneously with the hit optimization. (C) Drug repurposing is possible in cell-based screening projects and it may accelerate the drug discovery process by skipping the hit optimization and toxicology steps. Please note the changes in the order of the respective steps and the expectable savings.

estimations about the market potential of a prospective drug candidate for the disease, so that we can decide on the necessary steps when the costs and potential investments arise. Fortunately, the earliest steps (optimization of the cell-based model and the primary screening) represent relatively low cost, foreseeable expenditures. Actually, the costs of the cell-based screening may not exceed the expenses associated with the identification of a novel target to start up a target-based project. Decisions can be made based on the results of the primary screen whether it is worth proceeding with a hit compound and what kind of secondary tests or models to be used based on the chemical nature of the compound. In general, higher level of flexibility is necessary during a cell-based drug discovery project than in a target-based project due to the unforeseeable nature of the drug target. Still, apart from the cell culture model of the disease it is worth having secondary *in vitro* models and a well-established animal model of the disease planned in the beginning of the project.

Phenotypic assays may be better suited for different disease areas than target-based approaches but there is no clear rule about its applicability. Santos et al. analyzed the therapeutic areas in which new drugs appeared and found that the majority of recent innovation occurred in the areas of cancer and immunology, while very little progress was seen in cardiovascular drugs [5]. On the other hand, the annual direct costs of cardiovascular disease and stroke were double the amounts of cancer-associated costs ($193.1 and $88.7 billion, respectively, in 2011) suggesting that higher progress is expected in this area [15]. When new molecular entities are considered, the lag behind other areas becomes even more apparent over recent years: cancer, infectious diseases and nervous system disorders are the leading areas of drug discovery and they all precede cardiovascular diseases [16–18]. One potential explanation for the disproportional representation of this area is the lack of new druggable targets in cardiovascular diseases, whereas the new cancer drugs and anti-infective agents are new molecular entities, they bind to novel protein targets. The other problem may be related to the nature of the injury: both anti-infective agents and cancer drugs are expected to kill the cells, while in cardiovascular diseases the expectation is to protect the cells from a harmful injury. In most instances, the mechanism of cell killing involves an inhibitory effect on the target, which is easier to attain than a stimulatory effect, just like it is simpler to produce an antagonist than an agonist for a given target since various compounds may block a binding site even if they do not fit perfectly in the active center but only a perfect molecular match can activate the target [19]. Phenotypic screening may provide a solution for the difficulties of cardiovascular drug discovery, since it is possible to find compounds that reduce the cellular injury even in the absence of a known drug target [20].

Orphan diseases represent another potential area of drug discovery using cell-based assays. Orphan diseases are rare disorders that affect small percentage of the population and thus possess a limited market potential, which led to the loss of interest by pharmaceutical companies and the lack of drug treatment in the majority of cases. The definition of orphan diseases are somewhat questionable, since there is no exact prevalence value associated with the term but in most cases if a disease affects less than 1 in 1000 or 2000 people, we call it a low prevalence or orphan disease [21–24]. While the incidence of rare diseases is low, the European Organization for Rare Diseases (EURORDIS) estimates that there are 5000–7000 distinct rare diseases and they affect approximately 6–8% of the population of the European Union [25].

The majority of these disorders are inherited diseases and drug therapy may be necessary throughout the lifetime that increases their market potential. Most governments have recognized the disproportionality of the potential profits and the necessary investments in case of rare diseases and have issued legislations to promote the development of new drug therapies for orphan diseases [26, 27]. As most of these diseases have a disease-linked gene and the mutations are easily reproduced in cellular models, they may represent the most important target diseases for cell-based phenotypic screening. Furthermore, if we accept the prediction about the number of druggable targets by Hopkins and Groom [7], the logical consequence is that many of these diseases will not be cured by an independent molecular entity but will share therapeutic drugs either with other orphan diseases or with more common illnesses. Thus, testing clinically used compounds with a drug repurposing approach may prove successful in many of these disorders, which reduces the overall costs of subsequent steps.

Cellular injury and cell death are the major challenges in today's drug discovery portfolio. While cancer drugs are on the rise, with cell killing as the principal mechanism of action of drugs, cellular injury remains the major theme of scientific research. In most diseases, researchers focus on cellular damage and cell death and investigate the underlying mechanisms that may help us understand how to interfere with the process. Unfortunately, this approach is not that successful in the discovery of new drug targets, but provides us with a multitude of cellular and animal models of various diseases [28]. These models usually allow only low throughput assays to be performed but may represent a good starting point for phenotypic screens.

In many diseases, the question arises whether reduction of the damage will be possible if currently there is no therapeutic drug for the disease in clinical practice. If, under experimental conditions, protection of the injured cells is accomplished by gene silencing or by gene therapy, there will be greater chance for establishing a pharmacological intervention in the future since it indicates the existence of disease-linked genes. It may not be necessary to induce orders-of-magnitude changes or to fully suppress the expression of a disease-linked gene to attain cell survival benefit, because the cell fate in an injury may be modified by small changes in the level of interacting proteins. Also, the existence of other experimental methods (as it was the case with ischemic pre- and post-conditioning) that induce cell protection may indicate the existence of potential targets prior to the identification of a disease-linked gene. Since phenotypic screening is a target-agnostic methodology, the outcome is of primary importance and not the underlying mechanism.

The mechanism of cytoprotection may not be identical with the blockage of known cell death pathways. Apoptosis and necrosis represent the two major cell death processes and they were long regarded as examples of "programmed" and "unprogrammed" cell death. Distinguishing a clear pathway or program in the mechanism of cell death allows us to interfere with specific components of the process and to block the execution of the program. In this respect, caspase inhibitors are the prototypical inhibitors of apoptosis [29]. However, necrosis can also be blocked by poly(ADP-ribose) polymerase (PARP) inhibitors and thus its unprogrammed classification is no longer valid, even if it took us longer to fully understand the process because of its rapid execution [30–32]. Many other regulated forms of cell death (autophagy, pyroptosis,

necroptosis, parthanathos and mitoptosis) have been identified and various drugs that block these cell-based processes played a key role in their discovery [33]. Since cell death may occur simultaneously via multiple pathways, our classification of the dominant cell death form may change over time in various diseases as we understand more details about an injury [34]. Understanding the key features of a disease or an injury and reproduction of these mechanistic details in a cellular model may be of higher value than close mimicking the cell death process, as the latter often represents the final steps in the damage that we have to prevent and not to interfere with. Interestingly, neither caspase inhibitors nor PARP inhibitors went through clinical trials in their originally conceived application, but paradoxically PARP inhibitors reached clinical practice in diseases, in which they had seemed to play a lesser role. Thus while PARP was discovered as the main contributor to necrotic cell death and the first PARP inhibitor started a trial in ST-Elevation Myocardial Infarction (STEMI) [35], PARP inhibitors reached the market later as cancer drugs: currently olaparib, rucaparib and niraparib are approved for ovarian cancer [36, 37]. Similarly, caspase inhibitors were initially suggested to play a role as potential drugs in a wide array of diseases including acquired immune deficiency syndrome (AIDS), ischemic diseases (myocardial infarction, stroke), neurodegenerative diseases, myelodysplatic syndrome and toxic liver injury [38], but clinical trials were only started in epilepsy, hepatitis C virus (HCV) infection and non-alcoholic steatohepatosis (NASH) and none of the caspase inhibitors have reached FDA approval [39–41].

3. Compounds libraries

The number of compounds used in a cell-based assay is often lower than what can be screened in a simple enzymatic reaction. The cell-based models are usually more complex and the assays typically require longer time to perform. Furthermore, the maintenance of a cell culture lab for high number of assays is more costly than what is needed for simple biochemical assays, thus it is worth considering whether the associated costs can be limited by testing fewer compounds. Also, the measurement results may show higher variance in cell-based assays than in biochemical assays and may require higher number of repeats that will considerably increase the expenses. Since there is limited information about the number of compounds that may protect the cells against an injury, we can start the screening with a validation set of compounds that may contain compounds that are known to reduce the cellular injury in that model and also include various other compounds to see the data variability. This can give us information about the expectable number of hits in larger sets of compounds and help us plan the screening strategy. A reference compound that protects against the injury may not be available when we study a new disease model, thus we may need to consider the use of controls in which the injury has not been induced and introduce the use of positive controls in the assay once we identified drugs that protect against the injury.

A two-step procedure may be preferred in the majority of phenotypic screens: starting with a smaller set of compounds with higher expectable hit ratio followed by a second screen of larger sets of compounds that may achieve a lower hit ratio. The number of hits is higher in sets of compounds that mostly contain biologically active compounds than in sets of drug-like

molecules. It is easily understandable that drugs that interfere with biological processes definitely possess binding sites, whereas those compounds that only show resemblance to other compounds do not necessarily have any targets in a cell. However, many of the biologically active compounds may show higher level of toxicity in the cells, since their known activity may not be related to cytoprotection and it may increase the data variability.

Hit selection may also include two steps in cell-based screens: (1) identification of hits in the primary screening and (2) a secondary confirmation assay of the hit molecules. Because of the higher variability of the assays and measurement values, the active compounds may show less cytoprotection in a single measurement than their average effect, thus it is better to use cut-off values that allow us to select a broader set of initial hits. These compounds will include many false positives, which will fail to show protection in repeated tests. If we expect a hit ratio around 1 in 100 molecules in the primary screen, and run confirmatory tests in repeats (e.g. in 3–6 repeats), it gives an the assay burden of ~50% for the hit confirmation step, which is substantially less than running the primary screen in duplicates. In many cases, the number of confirmed hits will be around or below 1 in 1000 compounds tested, so the set of test compounds should include a few thousand compounds in the first primary screen to produce a meaningful set of data. Fewer compounds may not contain protective molecules at all and the lack of confirmed hits often results in discontinuation of the project.

Clinical compounds can be used for initial screening efforts and a repurposing approach can speed up the drug discovery process. The number of drugs approved for human use is around 2400 in the USA and there are no more than 4000 molecular entities approved worldwide (including the US market) [24]. There are various sets available from a few vendors that contain a selection of clinically used drugs and may also include other compounds that went through toxicology studies but failed in the clinical phases (**Table 1**). If multiple of these sets are obtained from different companies, there is usually substantial overlap in the provided drugs but the vendors mostly use independent sources for the drugs and various salts of the same compound might be included in the different sets. Another option is to use a compound library of biologically active drugs. In this case, the majority of the drugs will have an annotated target in the cells but not all compounds will possess a binding site: for example, the drug target may not be expressed in the cell type in use or an anti-infective compound may not have a mammalian homolog. In general, these libraries mostly contain a similar number of potentially cytoprotective drugs as clinical libraries but there is a huge difference between them in the subsequent steps. Those compounds that have gone through formal toxicology studies, may be directly reused for other diseases, but those compounds whose toxicity have never been investigated will require more follow-up work.

Larger chemical libraries comprising 10,000–100,000 drugs may be screened as a second step following the screen of small libraries. The number of possible drug-like molecules is not indefinite, but it is certainly large enough to forget about testing all possible compounds. Virshup et al. estimated that the set of all synthetically feasible organic molecules of 500 Da molecular weight or less contained over 10^{60} structures ("the small molecule universe") [42]. Depending on the disease and target tissue, it might be possible to exclude certain chemistries and by using chemoinformatics filtering methods, the composition of the library might be limited to a set that is easier to handle [43]. A key concept is druglikeness, prediction of

Compound library	Number of drugs	Short description	Link
NIH Clinical Collection	450	Compounds that have already been in clinical use or in clinical trials	http://www.nihclinicalcollection.com/index.php?cPath=21
LOPAC (Library of pharmacologically active compounds)/ TocriScreen collection	1280/1120	Known receptor agonists, antagonists, modulators of cellular responses and signal transduction	http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=LO1280|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC http://www.tocris.com/dispprod.php?ItemId=5381
US Drug Collection	1140	Compounds that have reached the stage of clinical trials in the USA	http://www.msdiscovery.com/usdrugs.html
International Drug collection	240	Compounds that have reached the stage of clinical trials in other countries	http://www.msdiscovery.com/
Killer Plates	160	Compounds with known effects on cellular viability	http://www.msdiscovery.com/killer.html
New Prestwick Chemical Library	1200	Drugs that are in clinical use	http://www.prestwickchemical.com
FDA Approved Drug Library	640	Compounds selected from another library of clinically used compounds	http://www.enzolifesciences.com/BML-2841/fda-approved-drug-library/
Apexscreen Library	5000	Diverse compound library, representative set of various chemotypes for screening	http://www.timtec.com/apexscreen.html
Chembridge Diversity Library	10,000	Drug-like diverse compound library for screening library	http://www.chembridge.com/screening_libraries/diversity_libraries/#DIVERSet
Myriascreen Library	10,000	Library of drug-like diverse compounds	http://www.timtec.com/myriascreen-diversity-collection.html
Actitarg-K library	6600	Library of kinase modulators and similar structures	http://www.timtec.com/kinase-modulators-actitarg-k-library.html
Natural Product Libtrary	640	Purified natural compounds, products	http://www.timtec.com/natural-compound-library.html
AMRI Diverse sample library	10,000	Diverse selection of 'lead-like' compounds that covers Albany Molecular Research Institutes' small molecular compound collections	http://www.amriglobal.com/products_and_services/products_detail_sub.cfm?prodID=1&subServID=4&subServID2=5

Table 1. Examples of compound libraries for cell-based screening projects.

the properties of a molecule based on the physicochemical properties of approved drugs and filtering the compound library according to these parameters [44]. The absorption, distribution, metabolism and excretion (ADME) of drug-like compounds may be predicted and subsets of drugs can be chosen for a specific organ or disease based on these data [45]. Lipinski introduced his "rule of five" concept, the filtering of molecules by solubility and permeability

prediction using the following parameters: the molecular weight is less than 500 daltons, CLog P is less than 5, the number of H-bond donors (the sum of OHs and NHs) is less than 5 and the number of H-bond acceptors (all nitrogen and oxygen atoms) is less than 10, that greatly reduces the number of potential drug-like molecules [46, 47]. This approach suggests that the number of drug-like molecules that we potentially use is closer to 10,000 than to a million drugs, since these compounds are sparsely distributed through "the small molecule universe" [47]. The use of a targeted library, which consists of drugs that are known to bind to certain types of targets and also contains highly similar molecules, is commonly used in chemical genomics and may prove useful in phenotypic screening, as well [43, 48].

The concentration of drugs used in cell-based screening is mostly determined by practicability and not by the effective or toxic concentrations of the individual compounds. The majority of compound libraries supplied compounds in solution at a fixed 1 or 2 mg/ml concentration in the past and nowadays, compounds are offered, mostly at 10 mM concentration in dimethyl sulfoxide (DMSO) [49]. The majority of the libraries are available in 96- or 384-well microplates, deep well plates or microtube racks. To simplify processing, dilutions of the compounds are best prepared at the same concentration for all drugs. While it would make more sense to use each clinical compound at a clinically relevant, effective concentration, equimolar concentrations are used most often to simplify and speed up the dilution steps. Compounds are usually screened at a concentration between 1 and 10 μmol/l, which might present dilution problems and may cause toxicity. Since the amount of DMSO must be limited as much as possible, dilutions of the drugs may be prepared in water-based solutions for cell-based assays and drug precipitation may occur as a result of poor water-solubility during dilution. Compound libraries are usually stored at −20°C in an upright position and compounds may settle down in the bottom of microtubes or wells during freeze-thaw cycles and some of the drugs may also precipitate. As a result, the cells may be treated with lower concentration of drugs than expected either due to incomplete mixing or dissolution of drugs. Also, compounds may lose activity during freezing and thawing, or due to an oxidative reaction with DMSO, which will also have various effects on the cells and may interfere with the assay [50]. Thus, hit compounds, if a fresh resupply is used in the confirmation studies, may show similar activity at a lower concentration than in the primary screen or even display higher activity.

Drug combinations may provide a further option to reduce the number of assays to be run [51, 52]. If you expect that the number of active compounds is low, you may consider pooling multiple compounds and testing them in combination. The number of assay runs may be reduced by an order of magnitude, if 8–12 compounds are pooled and only low number of "hits" is expected. Re-testing of the individual compounds will be necessary for each of the initial hits but since the expected hit ratio is low, it may not present excessive follow-up work. However, potential toxicity of the compounds needs to be considered: a toxic compound can mask the cytoprotective effect of an active compound if the cells are simultaneously treated with both drugs. Thus, the number or ratio of cytotoxic drugs might be the determining factor whether drug pooling is possible or not in a cellular model. On the other hand, this approach may allow us to search for potentiating compounds in a model if the test compounds are used in combination with a drug that provides limited protection [20, 53, 54].

4. Mammalian cell culture

Cell-based screening requires adaptation of cell culture techniques to higher throughput than what is typical in cellular assays and may present unexpected problems associated with the specific cell types or models [55]. The majority of these issues could have been sorted out by automation but the cost of robotic systems that handle cell cultivation remains extravagant. The first automated cell culture stations appeared in the market a decade ago, but these monstrous instruments remained outrageously expensive and very few units have been sold [56, 57]. Highly complex, cell-based models often include procedures that may not be automated and will require manual handling for specific steps. In this case, purchasing an expensive instrument that cannot fully replace the laboratory technician may increase the overall costs. Whereas training technicians specifically for higher throughput cell culture may be advantageous: provides flexibility, predictable costs and allows the introduction of newer models when needed.

Choosing the right cell type for the primary screen might be the key for discovering novel drugs or drug actions. Primary cells are often propagated for cell-based screening projects, assuming that they more closely mimic the processes of the cells in whole organisms than cell lines. However, primary cells are usually more difficult to grow and their division potential is limited to a few passages, during which they may not maintain their original characteristics. Whereas cell lines are well-characterized, immortalized cells that are easy to maintain and can be resupplied at reasonable costs, if necessary. The reagent and cell maintenance costs are lower for cell lines, as they can be cultured in classic cell culture media and typically grow faster. Human cells may be better suited for drug discovery, since the potential drug targets may not have orthologs in other species or the dissimilarity between the representative proteins may change the binding of compounds to the target [5]. On the other hand, many of the cell-based models use non-human cells either because the preferred cell-line originates from rodents or because the primary cells cannot be freshly isolated from humans. Either way, choosing the best model is more important than the use of human cells, since it is better to loose a few hits because of the differences in orthologs than to identify hits in an irrelevant model. If there are multiple options to choose from, it is worth keeping the lower cost model for the primary screening and use the other cell type as secondary model to confirm the action of hit compounds [58, 59].

Assay miniaturization is necessary to reduce the costs of cell culture and the assay-associated expenses [60]. Although, the ultimate goal is to minimize reagent costs, the power of testing treatments in larger cell populations will remain high and may be preferential depending on the model or assay type [61, 62]. Thus, 96-well cell culture plates are often used in more complex assays, while 384- and 1536-well plates can be used for simple assays when the assay readout is expected to change considerably with the treatments. In a simple cell viability assay, if the cellular injury reduces the viability by 50% and we expect that our drug treatment may provide a partial protection against the injury (e.g. 20% survival benefit) then the pipetting error and imprecision of the measurement may not allow us to downsize the assay. It should be noted that in cell culture, miniaturization involves a more complex adaptation of the cell cultivation process than proportional scaling down of the number of seeded cells and all the reagents. Differences in aeration and temperature fluctuations occur between the wells based on their respective position on a plate: typically the edges are exposed to excessive

temperature changes, evaporation and better oxygenation, while the inner wells are more homogeneous in this respect but they do not have comparable gas exchange and the cells may grow slightly slower in them. As a result, the outer wells may be excluded from analysis, leaving only 60 wells on a 96-well plate or 308 wells on a 384-well plate, thus 5-times higher number of treatments might be tested on a 384-well plate.

Contamination is a major issue in cell-based screening, because of the higher throughput of assays and steps that may not be carried out in an aseptic environment [63–65]. Compound libraries are often provided in solutions that are not sterile, though the drugs are dissolved in DMSO and there is little chance for bacterial contamination. The dilution step of compounds may be associated with contamination risk, partly because the storage plates often use tube caps that require manual handling and cannot be removed by a robotic system or because the pipetting station used for the dilution step does not fit in a biosafety cabinet. Also, experimental devices that are necessary to induce cellular injury may be utilized in some models and these instruments may exceed the size limits of a laminar flow cabinet [59]. While contamination risk may be minimized by careful planning of experiments, it is often impossible to eliminate all sources of bacterial contaminants. If the cell cultivation procedure is longer because the cells require longer differentiation steps, or the assay investigates the long-term survival of cells, the risk of contamination will also increase. In general, assays terminated within 3–4 h following the injury or drug treatment, may allow that some of the steps are carried out on the benchtop and may not require to work in a biosafety cabinet, since the bacterial growth becomes exponential after a lag phase and within this time-frame the low level of contaminants impose little risk of interfering with the assay [66]. However, longer exposures or assay runs requisite that all the steps are performed in a biosafety cabinet using sterile technique. It is essential to plan regular decontamination and sterilization of the instruments to avoid generating false data because of contaminants [67].

5. Assay-related issues

The readout of the screening assay that may be a viability assay or another functional test plays an important role in the identification of hit compounds and it is a key contributor to the expenses of the project. A high sensitivity, low cost assay is the obvious choice for primary cell-based screening of compound libraries [68, 69]. High sensitivity does not mean that we need to use luminescence measurements, because it is not the detection sensitivity what is important but the sensitive detection of the changes in cellular function or viability. A simple, colorimetric lactate dehydrogenase (LDH) assay may be capable to measure minor changes in cell viability that costly methodologies cannot detect. Reagent cost makes a huge difference if the number of runs is high, thus a less expensive assay may be preferable. A custom-tailored assay with "home-made" reagents may be a better choice than a commercial assay, depending on the model, and may save on supplies.

Specificity of the assay and the instrumentation necessary to read the plates are of similar importance but reproducibility and higher sensitivity may allow more flexibility in these parameters. Since we expect that the number of cytoprotective compounds is low in most models, it is better to have fewer false negatives and more false positives among the initial hits, thus

sensitivity may overcome the limitations of specificity. Endpoint assays are typically preferred, since the measurement time can severely limit the number of runs in kinetic assays. If a plate is read for 30 min in a kinetic assay, and the endpoint measurement lasts 1 min, it will take more than 2 days to read 100 plates kinetically, while the endpoint measurement can be finished within 2 h. (And no more than 6000–8000 compounds are tested on 100 plates if 96-well plates are used and the outer wells are excluded.) Thus, if an endpoint assay can replace a kinetic read with no or little loss in sensitivity, it remains the preferred choice in cell-based screening.

Viability assays are obvious choices for identification of protective compounds in a cytotoxic injury and many simple assays are available to choose from [70–72]. The most commonly used assays depend on substrates that are converted to easily detectable products by metabolic enzymes in living cells and the results are linear with the number of viable cells within a limited range [73–75]. The least expensive options are colorimetric or fluorescent tests that requisite less costly plate readers. However, assay interference is a common problem with these methods: many of the test compounds are colored or fluorescent substances and they may produce false results. The metabolic pathways or enzymes may be up or down-regulated in the studied injury model, thus careful selection of the assay is necessary [53, 71, 72]. Homogeneous assays may be preferred, since fewer steps allow less processing error, and easier automation, robotic integration of the assay.

Simultaneous use of multiple viability assays can help compensate for the weaknesses of individual tests but makes the data analysis more complex [53, 58, 71]. For example, in a cellular injury model, when the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay is the primary viability assay, LDH release may be used as a secondary measurement from the simultaneously sampled cell culture supernatant [20, 53, 71, 72]. Thus, cytoprotection (increased viability) will be detectable as an increased signal in the MTT assay and also as a reduced LDH signal (diminished cell death) in the respective supernatant sample. Whereas the simultaneous increase or decrease of both the MTT and LDH signals indicates a false positivity in one of the assays and may not be of further interest.

In cell-based assays, differences occur in the assay results depending on the respective position of individual wells, since the activity of metabolic enzymes is affected by the temperature fluctuations, inhomogeneity of gas exchange and potential pH variations. These effects may affect the results considerably in many injury models and corrections may be necessary to compensate for their impact. But even with the best compensations, the chances to find protective compounds in middle or outer wells may not be equal [53]. It is worth testing, that with the use of a positive control compound, our assay can similarly detect protection in all wells of the plate. Alternatively, we may confirm that mock-treated or vehicle treated (negative control) wells show similar results throughout the whole plate. If the measurements show larger variation, it may be necessary to run measurements in duplicates. In this case, duplicates need to be allocated to various positions and not to neighboring wells on a plate, so that the average protection will be similar for all test positions [53].

Independent repeats are expected in all cell culture experiments but running cell-based screens repeatedly several times would unnecessarily increase the expenses [76–79]. We can assume that only a handful of drugs may protect the cells and the majority of the compounds

will have little effect in the injury model. It is unnecessary to confirm the lack of effect for those drugs that exert no protection. The goal of the screen is to find potentially active drugs, thus screening a compound library needs a single measurement, even if we use a cell-based assay. The primary screen will not provide necessary data to state that the initial hits exert significant protection, we need to confirm the protective effect of potential hit compounds in repeated experiments to conclude that the effect is significant [77–79].

6. Data analysis

Hit identification is the main aim of data analysis in cell-based screening and this process may not follow the rules of statistics to the letter. In high-throughput screening, Z-factor has been used as the gold standard method to analyze the assay and data set quality [80]. This methodology assumes that the identification of hit compounds requisite good separation of positive and negative controls. However, the power of this methodology was questioned later, since higher background or noise values are typically present in several assays, and poor signal-to-background or signal-to-noise separation still allow the identification of "true hits" [81, 82]. Larger variation occurs in a cell-based assay than in biochemical assays, even so cell culture experiments have been successfully used to study the effects of drug treatments. Thus, the variable damage induced in many injury models is an acceptable feature of the experimental methodology but may require more flexibility during data analysis.

The ultimate goal is to identify compounds that provide protection against an injury, which means less damage or increased viability of the cells depending on the specific model. A Z-score, the number of standard deviations from the mean vehicle treated wells undergoing the injury, may be calculated using the whole dataset and it easily shows the outliers [83]. Based on the assumptions that (1) the wells treated with various ineffective drugs will show similar response to the vehicle-treated wells and (2) there are very few active drugs, the compound-treated wells can quasi replace the negative control wells in the analysis. However, if the dataset shows larger variation, the Z-scores will be smaller values, thus meaningful cut-off values should be chosen individually for the selection of hits in each experimental model [54, 58, 59]. Apart from the more complex procedures, like Z-score calculation, there are various other options to identify cytoprotective compounds. Selection of the wells, in which diminished cell death occurred, is often easier on a single plate than in a large dataset, thus it might be useful to establish cut-off values based on the results of each plate separately. Data may be categorized immediately at plate reading and the outliers can be labeled as potential hit compounds or toxic molecules depending on the changes in viability. In this way, you can start up the hit confirmation before the completion of the primary screen, which may be preferable if the primary screen of a larger compound library requisites longer time.

Hit selection may be overly complex if dual or multiple readouts are used to identify active compounds. In the simplest case, one of the parameters is used only at a fixed cut-off value and the second parameter is ranked according to the activity to find the protective compounds. In a cell injury model, viability values may be used for preliminary classification of drugs as toxic and non-toxic compounds, and the other parameter (e.g. ROS production or

an inflammatory signal) may be used to determine the protective effect of test compounds [54, 58]. Alternatively, the measurement values may be ranked separately for the individual parameters and cut-off Z-scores may be established for each of them. If a test compound performs above the cut-off values for each of the parameters, we may classify it as a hit that proceeds to the confirmation step. A further option is to streamline the decision making to the usual single parameter analysis: a new factor may be derived from the two primary measurement values, and the newly generated parameter can be ranked by the Z-scoring method.

Hit confirmation is the next step, following the identification of preliminary hits [83]. This procedure can take the usual steps of cell culture experiments. Independent repeats are needed and data analysis should be performed following the generally accepted rules of *in vitro* assays [79]. If more assays are available to test the activity of potential hits, it is worth including them at this step as secondary assays [83]. The cellular target of confirmed hits often represents new challenges, since the identification may not be a straightforward process in many of the disease models. However, the new targets are of high value for structure activity relationship (SAR) analysis and may shed new light on the mechanisms of disease development and progression.

7. Conclusion

Recent tendency in drug discovery suggests that target-based research will be complemented with target-agnostic approaches in the future. As opposed to classical target-based drug discovery approaches phenotypic assays may be necessary to identify novel compounds that show activity in orphan diseases or in common medical conditions that currently lack effective therapeutics. Cell-based models are often used to study various aspects of illnesses and many of these may be modified for powerful tools in drug discovery. The use of these models for cell-based screening may allow identification of potential drug candidates and chemical genomics approaches can promote reverse identification of novel drug targets [84].

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Chapter 8

Bioinformatics Discovery of Vertebrate Cathelicidins from the Mining of Available Genomes

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Abstract

Due to the worrying increase in antimicrobial resistance to conventional antibiotics, the search for alternatives is becoming increasingly important. Antimicrobial peptides (AMPs), originating from natural resources, have been recognised as a novel class of antibiotics. An advantage of peptides over antibiotics is that the resistance is more difficult to attain than for conventional antibiotics. With the increasing number of genomes sequenced and available in the public domain, one alternative methodology to obtain novel AMPs is to analyse genes and proteins from genomic databases to predict and identify amino acid sequences that share similarities and molecular features with natural bioactive antimicrobial peptides. In this chapter, we summarise some of our recent results on the production of antimicrobial peptides, particularly, how we managed to identify a family of antimicrobial peptides: cathelicidins, through bioinformatics tools, from the genomes of two lower vertebrates (a reptile and a bird) available in public databases. We hope that our preliminary investigation with these novel peptides could be useful for the design of future strategies that pursue the production of antimicrobial peptides through biotechnology.

Keywords: cathelicidin, antimicrobial, peptide, antibiotics, genome mining

1. Introduction

Conventional drugs for the treatment of human diseases can be classified as small molecules, proteins and peptides. The cost of goods, products quality and scalability are always major factors when considering the development of any new class of drugs. Generally, in the drug development process, small molecules are still preferred over peptides primarily due to their ease of production, simplicity of administration (as oral pill), and superior pharmacodynamics properties. In addition to stability and delivery, these factors have historically been seen as hurdles for peptide drug development versus their small molecule counterparts [1].

In the case of antibiotics, due to the worrying increase in antimicrobial resistance to conventional antibiotics, together with the long-term decline of investment by pharmaceutical companies, the search for alternatives is becoming increasingly important [2]. Traditionally, soil bacteria (especially actinomycetes), fungi and higher plants were main sources for drug discovery. However, no new class of antibiotics has been discovered since 1987 [3]. Antimicrobial peptides, originating from natural resources, have been recognised as a novel class of antibiotics [4]. Cationic antimicrobial peptides neutralise target pathogens through deleterious interactions with membrane components, which relies on the difference in lipid composition between normal eukaryotic and prokaryotic (or transformed eukaryotic) cell membranes [5]. Therefore, microbial resistance to antimicrobial peptides is rare. Moreover, peptide antimicrobials are active against both metabolically active and non-metabolising microorganisms, whereas most antibiotics have specific targets that define their mechanism of action and are active only against replicating microbes. Antimicrobials with novel *modi operandi* are consequently in high demand in a world faced with an increasing number of multidrug resistant pathogens, as well as a changing panorama of infections [4, 6].

Our emphasis in this chapter will be on the discovery of naturally occurring antimicrobial peptides for use in development of new antimicrobial drugs. Discovery of natural bioactive molecules is, of course, only the first step in the biodiscovery pipeline. The spectrum of available candidate peptide drugs is by far not limited to the human peptide pool. Through evolution, numerous peptides have evolved to exhibit their natural bioactivity outside of the producing organisms [5]. The cationic peptides polymyxin B and gramicidin S have been used in the clinic as topical over-the-counter medicines for a long time, and the cationic lantibiotic nisin is used as an antimicrobial food additive. In general, antimicrobial peptides in different species are surprisingly different. For example, magainins found in the skin of the frog *Xenopus laevis* were initially thought to be universal antimicrobial agents. However, it turns out that every frog investigated has its own peptide antibiotics [7]. It has been argued that that the immense diversity of cationic peptides arises from their antimicrobial function as well as the different pathogenic microbe challenges they face in each host organisms. We believe that nature still harbours a virtually infinite array of potential peptidic medications that await human pharmacological characterisation. Particularly, organisms living in germ-filled environments could be an abundant source of antimicrobials and can likely provide us with superior templates for use in development of new antimicrobial drugs to help solve the medical problems that exist today [8].

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With the advent of facile genome sequencing, new antibiotics are being found by the techniques of genome mining, offering hope for the future [9]. The aim of this study is to illustrate the identification of novel antimicrobial peptides from exotic vertebrates through refined computational methods. Genome mining involves looking at the sequenced genome of organisms to determine if gene cluster involved in the production of new antimicrobial peptides can be found in these organisms. The cathelicidins are a family of antimicrobial peptides encoded in the genome of vertebrates. These peptides are characterised by being formed by a prepropeptide, which in neutrophils is processed by the enzyme elastase to originate the active peptide. There is a great variability between the sequences of the active peptides of cathelicidins [10]. However, the pre-pro peptide is highly conserved, which is very useful for the mining of vertebrate genomes to identify putative genes of this family of antimicrobial peptides. The genes coding for cathelicidins typically consist of four exons, the fourth exon being the one that codes for the active peptide and, therefore, the least conserved. The number of cathelicidin genes is different among species. For example, cows have 11 genes, humans have only one, and sheep have eight [10, 11]. This structural and/or functional diversity within the cathelicidin family could reflect the spectrum of microbicide and immunomodulatory activities of cathelicidins in different species exposed to different microbial load [12].

We have previously described the characterisation of peptides derived from the genomes of mammals [13]. We here illustrate a simple method for the identification of two cathelicidin genes in the genome of lower vertebrates (birds and reptiles) living in different environments, and thus surrounded by different pathogens. We also perform the bioinformatic analysis of the corresponding active peptides to detect possible antimicrobial properties and describe the functional characterisation of the two identified cathelicidins against a panel of reference microorganisms. Finally, we describe our current attempt for the biotechnological production of these peptides in microalgae.

2. Materials and methods

2.1. Obtaining of the cathelicidin sequences

Access to the genomes of the different species studied was carried out in September 2012, through the genebank public databases (http://www.ncbi.nlm.nih.gov/genome). At these dates, different genomes were still assembled in contiguous, or contigs, that were accessible for tracing by nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For the screening of animal genomes, a nucleotide sequence of 83 base pairs (bp) was used, based on a highly conserved region between bird cathelicidins, which corresponds to the following sequence:

cccaggctgtggactcctacaaccaacggcctgaggtgcagaatgccttccggctgctcagcgccgaccccgagcccggcccg

P R L W T P T T N G L R R M P S G C S A P T P S P A

The search for sequences homologous to this sequence was made using the BLAST application, using the values of the default parameters, which returned us the contiguous (contigs), or scaffolds, of genomic DNA where sequences homologous to our sequence and, therefore, probable coding genes for cathelicidins.

2.2. Prediction of cathelicidin genes

Once the different contigs of homology were obtained with this sequence, we proceeded to study if they contained genes corresponding to cathelicidins. For this, two types of programs were used: GenScan (http://genes.mit.edu/GENSCAN.html) and GeneMark (http://exon.gatech.edu/GeneMark/), with the default values of the different parameters. Both programs predict putative genes within these contigs or scaffolds of previously selected sequences. The criteria adopted for the identification of cathelicidins in these contigs was that the predicted genes contained four exons and that in addition the amino acid sequence of the first three exons was homologous to the already known cathelicidins, with a percentage of residue identity of more than one 30%, provided that the position of some key residues of the pre-pro-peptide, such as cysteines, essential for the maintenance of its structure, is preserved.

2.3. Prediction of antimicrobial peptides

The fourth exon of the predicted cathelicidins was then translated into amino acids and, to identify the possible cleavage site by the neutrophil elastase enzyme, the amino acid sequence of the fourth exon was subjected to the online Peptidecutter tool (http://web. expasy.org/peptide_cutter/), taking as an active peptide that generated after the theoretical cut by this enzyme in its leftmost position, towards the C-terminal end.

2.4. Bioinformatic analysis of the cathelicidin sequences

The percentage of hydrophobicity and the Boman index of the derived cathelicidins were calculated by the ADP3 tool (http://aps.unmc.edu/AP/prediction/prediction_main.php). The Boman index estimates the potential of a protein to bind to another protein. Thus, if a protein has a high Boman index, it means that said peptide would be multifunctional and, once inside the cell, it would have more or less capacity to bind to several proteins and perform different functions within the cell, given its capacity to interact with a wide range of proteins. The antimicrobial activity of the peptides was evaluated in silico with the AMPA tool (http://tcoffee.crg.cat/apps/ampa/do) described in [14] Torrent et al. and the APD3 tool (http://aps.unmc.edu/AP/prediction/prediction_main.php), described in [15], using the parameters that both have defined by default. The secondary structure of the peptides was calculated using the PSIPred tool (http://bioinf.cs.ucl.ac.uk/psipred/). The alpha-helicity of the regions present in the peptides was visualised in 2D by the helical projection tool available on the website http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.

2.5. Peptide synthesis

After performing the bioinformatic study, peptides were synthesised (Caslo, Lyngby, Denmark) on a 10 mg scale and with a purity of more than 90% (analysed by HPLC). To avoid

degradation of peptides during storage, their N-terminal end was acetylated and their C-terminal end amidated. For the performance of the different assays, aliquots of each of the polypeptides (2 mg) were dissolved in pure water to a stock concentration of 100 μM.

2.6. Antimicrobial activity

The *in vitro* antimicrobial activity of the polypeptides was tested against the following pathogenic microbes, including bacteria and fungi*: Staphylococcus aureus* ATCC 6538, *Salmonella* sp. CECT 456, *Klebsiella pneumoniae* ATCC 23357, *Escherichia coli* ATCC 9637, *Bacillus cereus* ATCC 21772, *Proteus mirabilis* CECT 170, *Enterococcus faecalis* ATCC 29212, and *Candida albicans*. The minimum peptide concentration necessary to inhibit the growth of the tested microorganisms was evaluated by MIC assay, as described elsewhere [16]. Pathogenic microorganisms tested to evaluate the antimicrobial activity of the peptides were preserved at a temperature of −80°C in Luria-Bertani medium (LB) supplemented with 20% volume/volume glycerin. To determine the MIC, the peptides were solubilised in sterile milli-Q water to a final concentration of 100 μM, after which serial dilutions were carried out. 50 μl of each dilution of each polypeptide was mixed with 50 μl of the corresponding bacterial pointer suspension in Mueler-Hilton medium to a total volume of 100 μl in each well of a microplate. Thus, the effective concentrations to which the bacterial suspensions were subjected were 50, 25, 12.5, 6.5, 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 μM. Two replicates were carried out for each strain, concentration and peptide under test. As positive controls water was used in place of the specific polypeptide, and as negative controls each of the polypeptides was used without the corresponding bacterial suspension. In addition, two non-antimicrobial peptides were used as negative controls. Such peptides had a structure similar to the peptides of the invention (α-helix), but their sequence is totally different and shows no antimicrobial activity. The microplates were incubated at 37°C for 12–16 h with a shaking of 20 s prior to the measurement of the absorbance. To evaluate microbial growth, the optical density at 600 nm was measured using an EZ Biochrome plate reader. Each experiment was performed twice. As MIC, the lowest peptide concentration was taken at which no bacterial growth occurred at the end of the experiment.

2.7. Haemolytic activity

The haemolytic activity of the peptides is an indicator of the toxicity of peptides in eukaryotic cells, and a characteristic that is generally determined for those compounds that may come into contact with the human body. A lower percentage of haemolysis could be correlated with a lower cytotoxic activity of the peptide. Briefly, the haemolytic activity of the peptides was evaluated by determining the release of haemoglobin that is produced by contacting a solution comprising each of the peptides with a suspension of 10% volume from fresh human blood. Collection of blood samples was performed under aseptic conditions using a Vacutainer® K2E (Belliver, Great Britain) system with EDTA, and stored in Alsever medium at 4°C. When the assays were performed, blood was collected centrifuged at 6000 g for 5 min to separate the erythrocytes. After the erythrocytes were collected, they were washed three times with PBS buffer and resuspended in PBS buffer until a suspension containing 10% by volume of erythrocytes was obtained. In parallel, the peptides were solubilised in PBS buffer to obtain a battery of concentrations for each of them ranging from 12.5, 6.25, 3.13, 1.56, 0.78, 39, 0.2, 0.1,

0.05 and 0.02 μM. Three replicates were used for each peptide and concentration tested. Briefly, 50 μl of the 10% by volume erythrocyte suspension was mixed with 50 μl of the aliquots comprising each of the different concentrations of the peptide to be tested in each well of a 96-well plate, and the mixture for 45 min at 37°C under agitation. The plates were then centrifuged at 3500 g for 5 min, and 80 μl aliquots of the supernatant were transferred to 100-well flat bottom microplates, which were diluted with 80 μl of milli-Q water. The degree of haemolysis was determined from the absorbance at 540 nm with a plate reader. As a positive control for haemolysis assays, a PBS buffer solution containing 1% Triton X-100 (Sigma-Aldrich, Spain) was used. The percentage of haemolysis (H) was determined using the equation: $H = 100 \times [(Op - Ob)/(Om - Ob)]$ where Op is the optical density measured for one of the concentrations of the peptide tested; Ob is the optical density of the buffer solution; and Om is the optical density for the positive control with Triton X-100.

2.8. Molecular cloning of cathelicidins

Synthetic DNA (Genscript, Piscataway, NJ, USA) corresponding to amino acid sequence of the peptides was designed so that they contained the signal peptide from the Chlamydomonas aryl sulphatase protein, the codon optimisation for *C. reinhardtii* and they incorporated restriction sites for correct positioning into the multiple cloning sites (MCS) of the vector pChlamy_4. (Thermofisher, Madrid, Spain). For the transformation of the cyanobacteria *S. elongatus*, primers were designed to incorporate restriction sites for the correct positioning of the amplified inserts into the vector pSyn_6 (Thermofisher). The TAP and BG11 media and all restriction enzymes and primers were purchased from Thermofisher. The correct cloning of the inserts was verified by PCR (for colony screening) and by sequencing (Secugen, Madrid, Spain).

2.9. Transformation of microalga

For the cloning and transformation of the eukaryotic and prokaryotic microalga *C. reinhardtii* and *S elongatus*, we followed the recommendation of the manufacturer (Thermofisher). Antibiotics zeocin and spectinomycin were purchased from Thermofisher. The anti-V5 epitope antibody used for the immunodetection of recombinant peptides was purchased from Invitrogen.

3. Results

3.1. Identification of cathelicidins

Cathelicidins are found in varying numbers in numerous different species, including reptiles and birds. A remarkable degree of molecular diversity has been noted within this gene family. However, a well-conserved feature across evolutionary distant species is an N-terminal cathelin-domain. Using this domain to search into genome databases, we have found two novel cathelicidins from the genome mining of a reptile (painted turtle) and a bird (budgerigar). Predicted cathelicidins were identified by screening genome databases using the BLAST tool for DNA sequences. The painted turtle cathelicidin of 154 amino acids was found in contig number 974.21, whereas a bird cathelicidin of 151 amino acids was found in contig 900159920384 of the

newly sequenced genomes of these species, which from September 2012 were available in the genome database (http://www.ncbi.nlm.nih.gov/genome/).

The complete sequence of cathelicidins from these two species was assembled by joining the four exons identified in abovementioned contigs using GeneScan and GeneMarK programs and located in the following positions:

Species	Exon	Begin	End	Length
Turtle	1	799	933	135
	2	2556	2663	108
	3	3485	3568	84
	4	4442	4579	138
Bird	1	125	298	174
	2	499	606	108
	3	712	792	81
	4	877	969	93

The DNA sequence thus assembled was translated into amino acids and the resulting protein was subjected to PSI-BLAST to confirm that this protein was indeed a cathelicidin (not shown). The cathelicidin of the painted turtle, thus obtained, had the following sequence:

LRKDVLSFRDFPAEIPDEPPLVTPTDPSRCRVGSNLGPQRMSLYCDMTSNPRQELKFMV-
KETVCPVSENLNGTECDFRDNGVVRDCSGFFSTQQESPIVIINCNTVTKED-
PHIRRSRSPRRSRWPRRWYLPGSYTLIAHGGGKGKGKGSRLQMA

Whereas for the bird, the sequence of the corresponding cathelicidin was the following:

MPSSWALVLVVLGGACALPAPAPLAYTQALAQAVASYNQRPEVQNSFRLLSADPEPAP-
SIQLSSLQLLNFTIMETQCPARARIHPDACEFKEDGLVKDCSAPVPQHGGPVLGVTCVD-
STADPVRVKRFWPLLVTAIRTVAAGVSMFKSSKG

The coding sequence of exon 4 (in blue) is that which corresponds to the active peptide, while the remaining portion corresponds to the conserved pre-pro-peptide.

The amino acid sequence of exon 4 from both reptile and bird cathelicidin was then subjected to the prediction of the cut site for neutrophil elastase and it generated the following antimicrobial peptides:

Cc_SP-37: SPRRSRWPRRWYLPGSYTLIAHGGGKGKGKGSRLQMA

Mm_KR-26: KRFWPLLVTAIRTVAAGVSMFKSSKG

3.2. Bioinformatic analysis of peptides

Peptides Cc_SP-37 and Mm_KR-26 were subjected to *in silico* analysis of their antimicrobial activity using several bioinformatic tools. For example, the online tool APD3 calculated the different parameters related to the possible antimicrobial activity of the peptides. The APD

database has a unique peptide prediction program. For example, the program will calculate select properties of the peptide (e.g., net charge, length, percentage of hydrophobic residue and amino acid composition). The *in silico* structural analysis of the peptides indicated that both of them were alpha helical and had a positive net charge. However, the cathelicidin Mm_KR-26, from the bird, had a lower net charge, higher hydrophobicity and lower Boman index than the cathelicidin Cp_SP-37, from the reptile (**Table 1**).

To predict and calculate the regions of the protein with possible antimicrobial activity, the AMPA bioinformatics tool was used, which predict antimicrobial regions on any protein by assigning an antimicrobial index to each residue (**Figure 1**).

The AMPA algorithm also uses an antimicrobial propensity scale to generate an antimicrobial profile by means of a sliding window system. The probability values displayed correspond to misclassification probability that is the probability to find the predicted stretch in a non-antimicrobial protein. Using this tool, a single stretch, spanning residues 2–15, was found for the turtle cathelicidin Cc_SP-37, which confirmed the possible antimicrobial character of the predicted cathelicidin. No stretch was found for the bird cathelicidin Mm_KR-26 (**Figure 2**).

Peptide	Specie	Net charge	Hydroph	Boman index
Cp_SP-37	*Chrysemys picta bellii*	+9	24%	2.59 kcal/mol
Mm_KR-26	*Melopsittacus undulatus*	+5	50%	0.65 kcal/mol

Table 1. Structural analysis of genome-derived cathelicidins from painted turtle (*Chrysemys picta bellii*) and budgerigar (*Melopsittacus undulatus*).

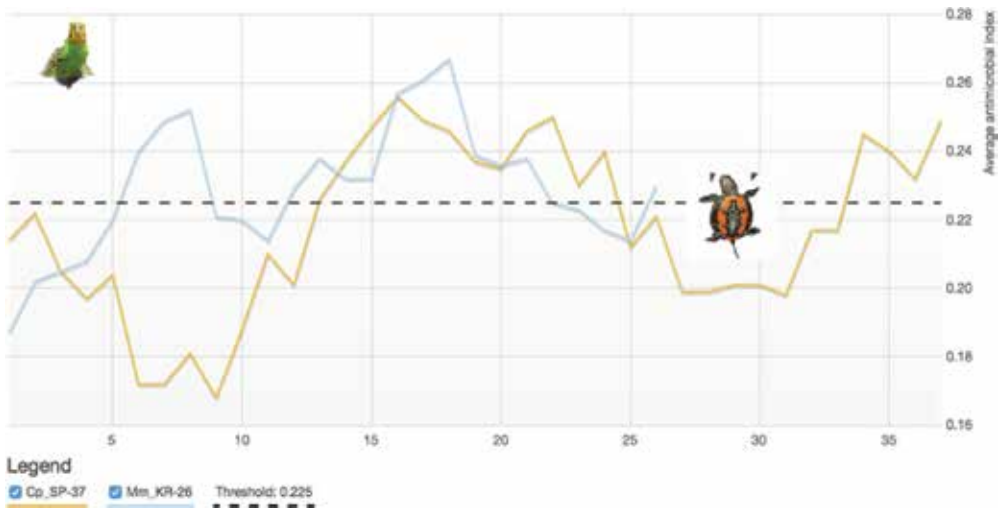

Figure 1. Graphical result of the in silico study of the cathelicidin sequences Mm_KR-26 (blue line) and Cp_SP-37 (yellow line), using the AMPA bioinformatic tool. The calculated average antimicrobial index is represented along the amino acid sequences. The threshold value of this index is indicated by a dashed line.

Figure 2. Result of the in silico study of the cathelicidin sequences, using the AMPA bioinformatic tool. The antimicrobial stretches are shown with a mean and a propensity and probability value.

The secondary structure of the bird and turtle cathelicidins was then computed with the PSIPred tool, which for the bird cathelicidin Mm_KR-26, predicted a single helical region comprised between residues 2 and 23 (**Figure 3**). However, no helical portion was predicted for the turtle cathelicidin Cc_SP-37 (not shown).

To further study the amphipathic character of the helices predicted by the PSI-Pred tool, these residues were represented in helical projection (**Figure 3**). For the helix of the peptide Mm_KR-26, comprised between the residues 2 and 23, it was observed that the two positively charged residues (R12 and K22) were disposed towards the same side, thus generating an amphipathic helix, characteristic of the antimicrobial peptides, with a hydrophobic moment of 4.37 units at 14.1°C.

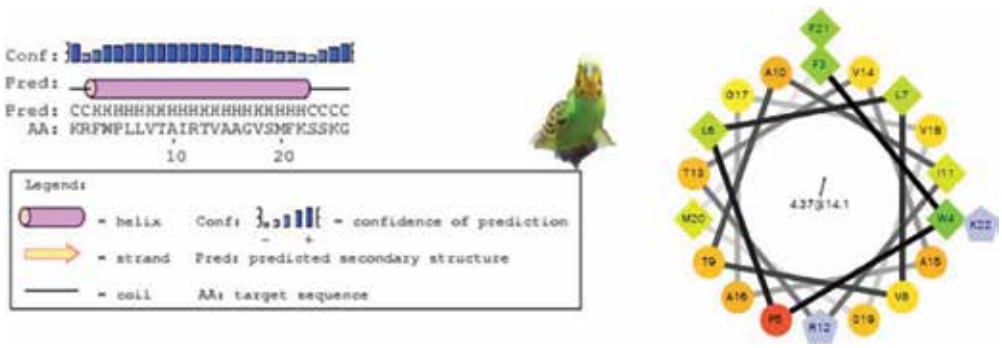

Figure 3. Result of the in silico study of the cathelicidin Mm_KR-26, using the PSIPred bioinformatic tool for the prediction of the secondary structure. The predicted helical content of the bird cathelicidin Mm_KR-26 was then subjected to a helical projection and calculation of the hydrophobic moment, using the on line viewer available at http://rzlab.ucr.edu/scripts/wheel/wheel.cgi. Hydrophilic residues are presented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is colour-coded: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. The scalar value of the hydrophobic moment of the helix and the angle of its direction are shown inside the helix.

3.3. Antimicrobial activity of peptides

For the analysis of the antimicrobial activity of cathelicidins, we used the minimal inhibitory concentration assay over a panel of different microorganisms of reference (**Table 2**). For simplicity, we show some figures with the microorganisms *B. cereus, E. coli, E. faecalis, C. albicans, P. corrugata and Salmonella* sp. In these experiments, it was found that both the bird peptide Mm_KR-26 and the painted turtle Cc_Sp-37 were particularly effective at a low dose to inhibit the growth of *B. cereus*, with MIC values of 1.6–3.1 and of 3.1–6.3 μM, respectively. However, both peptides were not effective, at the higher dose tested, to inhibit the growing of *S. aureus* and *P. mirabilis* and both were not very effective against *C. albicans*, with MIC values of 25–50 μM (**Table 2**).

To visualise the relative efficacy of the inhibition of the growing of microorganism, we estimated the percentage of inhibition of the growing of microorganisms at different concentrations of peptide, as well as the effective concentration of peptide to inhibit at 50% the growing of the microorganism, the EC_{50} values. **Figures 4** and **5** (top and bottom) show these values obtained with a few representative microorganisms for the bird peptide Mm_KR-26 and the painted turtle Cc_Sp-37, respectively.

As can be seen from the results shown in **Figure 4**, the cathelicidin Mm_KR-26 was particularly effective against *E. faecalis*, which required less concentration of peptide for inhibition of the growth of this microorganism, (with an EC_{50} value of 1.3 μM) and, in less extent, against *C. albicans* with an EC_{50} value of 34.6 μM (**Figure 4**).

As can be seen from the results shown in **Figure 4**, the cathelicidin Mm_KR-26 was particularly effective against *E. faecalis*, which required less concentration of peptide for inhibition of the growth of this microorganism, (with an EC_{50} value of 1.3 μM) and, in less extent, against *C. albicans* with an EC_{50} value of 34.6 μM (**Figure 4**).

Similarly, the painted turtle peptide Cc_SP-37 was particularly effective against *E. faecalis*, which required less concentration of peptide for inhibition of the growth of this microorganism, (with

Reference microorganism	Mm_KR-26	Cc_SP-37	Kanamycin
S. aureus (ATCC6538)	>50 μM	>50 μM	0.8–1.6 μg/ml
Salmonella sp. *(CECT456)*	3.1–6.3 μM	12.5–25 μM	0.4–1.6 μg/ml
K. pneumoniae (ATCC23357)	1.6–3.1 μM	3.1–6.3 μM	0.4–0.8 μg/ml
E. coli (ATCC9637)	3.1–6.3 μM	6.3–12.5 μM	0.4–1.6 μg/ml
B. cereus (ATCC21772)	1.6–3.1 μM	3.1–6.3 μM	0.8–3.1 μg/ml
P. mirabilis (CECT170)	>50 μM	>50 μM	0.4–0.8 μg/ml
E. faecalis (ATCC29212)	0.8–1.6 μM	6.3–3.1 μM	0.4–0.8 μg/ml
C. albicans	25–50 μM	25–50 μM	–
P. corrugata	6.3–12.5 μM	25–50 μM	–

Table 2. Minimum inhibitory concentration of the bird peptide Mm_KR-26 and the painted turtle Cc_Sp-37 compared with the antibiotic kanamycin.

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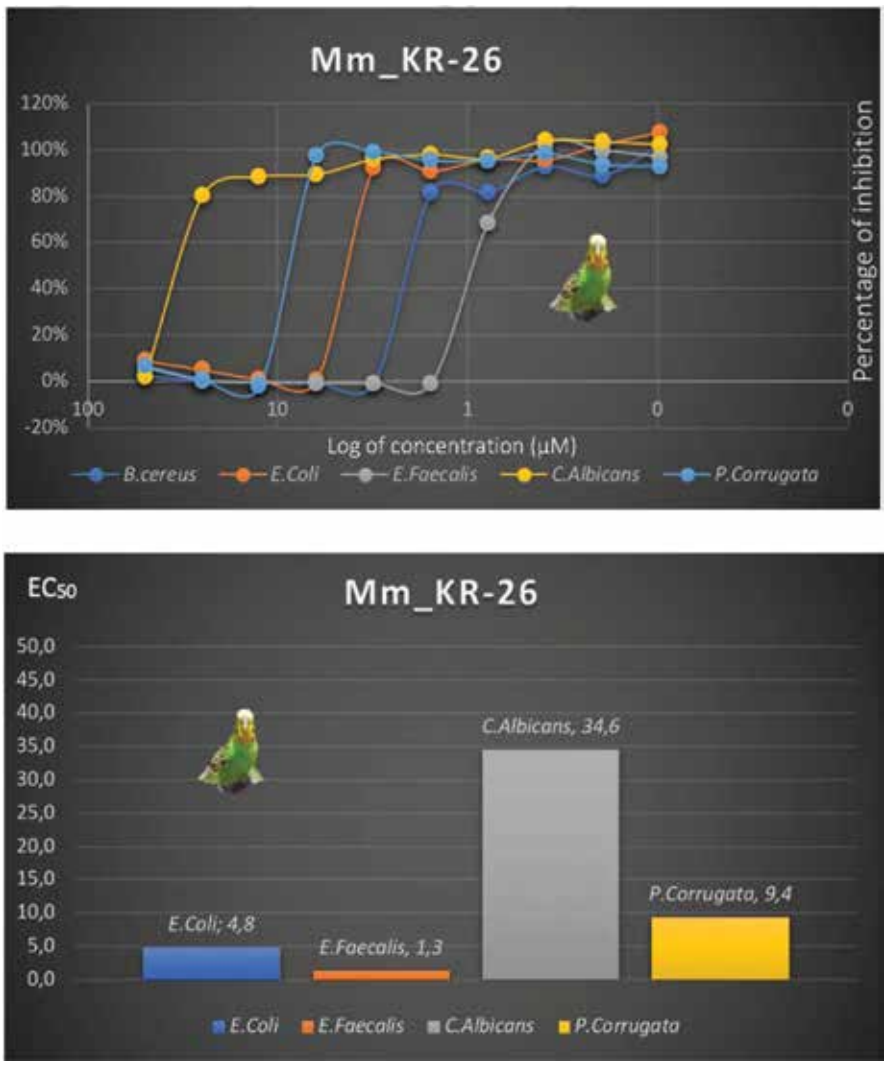

Figure 4. Analysis of the in vitro relative antimicrobial activity of the bird peptide Mm_KR-26 against a panel of representative microorganisms, showed as the percentage of inhibition (top) and calculating the effective dose for the inhibition at 50% (EC50), showed bottom.

an EC_{50} value of 5.7 μM) and, in less extent, against *C. albicans* and *P. corrugata* with EC_{50} values of 46.8 and 40.8 μM, respectively (**Figure 5**).

For both cathelicidin peptides, we tested the haemolytic activity at concentrations ranging from 12.5 to 0.2 μM, concentrations at which the peptides were able of inhibiting the growth of pathogenic microorganisms. The haemolytic activity of both peptides was always less than 35%, indicating that the peptides at these concentrations do not show toxicity for human erythrocytes (data not shown).

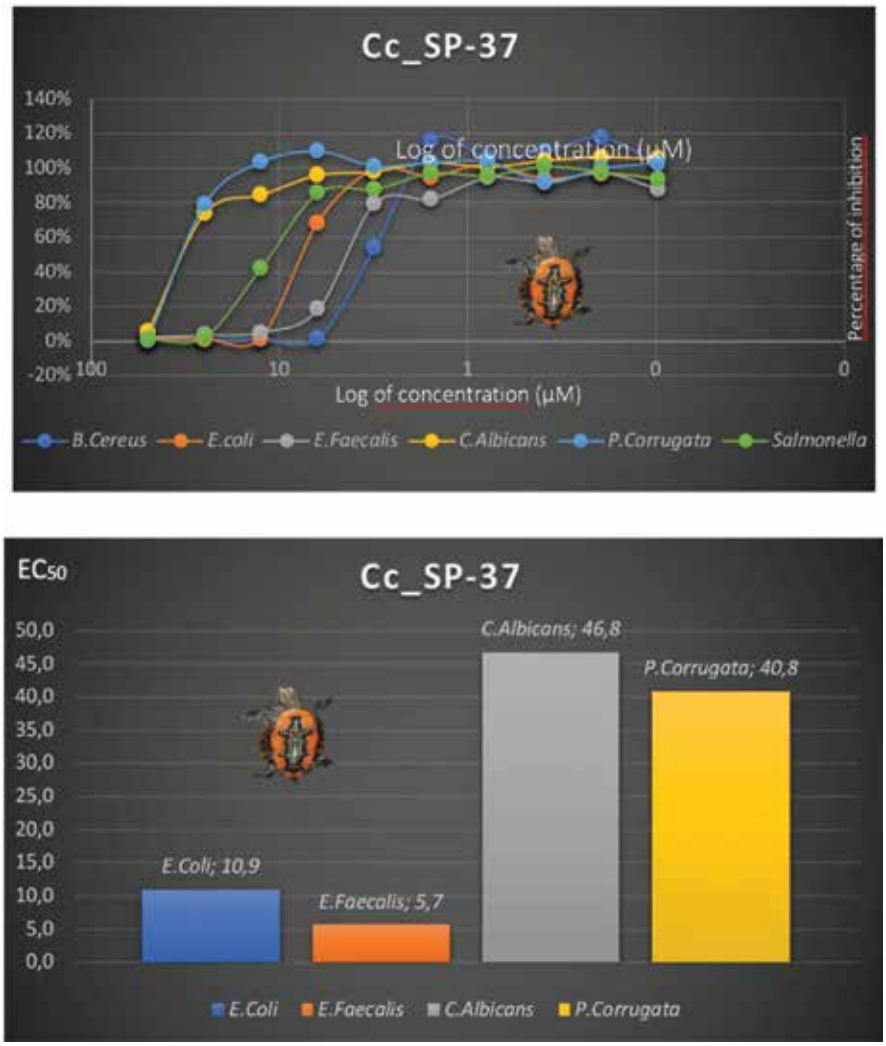

Figure 5. Analysis of the in vitro relative antimicrobial activity of the painted turtle peptide Cc_SP-37 against a panel of representative microorganisms, showed as the percentage of inhibition (top) and calculating the effective dose for the inhibition at 50% (EC50), showed bottom.

Once we tested the antimicrobial activity of the peptides, we next initiated the recombinant production of peptides in the eukaryotic microalga *Chlamydomonas reinhardtii* and in prokaryotic cyanobacteria *Synechococcus elongates,* given the advantages of using these microalga as biofactories [17]. For the eukaryotic microalga, in order to obtain a secreted product, we considered the introduction of the signal peptide of the aryl sulphatase, as previously described [18], and the synthetic DNA with the codon optimisation for this particular microalga. We also added restriction sites for the correct cloning of the synthetic DNA into the vectors pChlamy_4 and pSyn_6. Both expression systems, prokaryotic and eukaryotic microalga have strong promotors and can introduce 6xHis tags for the purification of the recombinant proteins (**Table 3**).

Vector features	pChlamy_4	pSyn_6
Promoter	HSP70-rbcs2 (strong, endogenous)	psbA1 (strong, endogenous)
Selection	Bleomycin/Zeocin	Spectinomycin
Epitope tags	TEV-cleavable N-terminal 6His, or C-terminal V5-6His	
Gene integration	Stable (random)	Stable (targeted)
Growth media	TAP	BG-11

Table 3. Comparison of the microalga vectors pChlamy_4 and pSyn_6 (Thermofisher) used for the recombinant expression of cathelicidin antimicrobial peptides into the microalga *C. reinhardtii and S. elongatus,* respectively.

Figure 6. Transformed cyanobacteria *S. elongatus* growing in BG-11 agar plates containing spectinomycin (5 μg/ml). Negative control with no insert (left) and containing the bird cathelicidin peptide Mm_KR-26.

Using the commercially available kits for microalga expression, two microalga (*C. reinhardtii and S. elongatus*) were transformed for the recombinant expression of the bird and painted turtle cathelicidins. As an example, **Figure 6** shows the transformation of *S. elongatus* with the DNA coding for the bird cathelicidin peptide Mm_KR-26.

Current experiments are underway in order to optimise the recombinant production of these cathelicidins in a higher culture volume and to study the yield of the peptide production using microalga as biofactories.

4. Discussion

Currently, approximately 15 therapeutic agents based on AMP peptides are found in clinical trials of anti-infective or anti-inflammatory indications, generally limited to topical applications

[19]. One of the main questions to bring this type of molecules to the clinical application refers to their toxicity. To date, most clinical trials focus on topical use. Although, many peptides have activity on eukaryotic membranes, this activity is much lower compared to prokaryotes. This may be due to the absence of negatively charged lipids on the surface of eukaryotic membranes, the lower membrane potential in most cells and the presence of cholesterol in eukaryotes. However, despite the large number of peptides described in various organisms (more than 2000) and the advances to take advantage of its clinical potential, there is currently no peptide approved for use in humans by the Food and Drug Administration (FDA) [20].

One of the most widely used strategies for the biotechnological production of proteins and recombinant peptides is their expression in microorganisms, such as *E. coli* or yeast. Among the advantages of these expression systems is the well-known physiology of these microorganisms and the existence of specific and well-characterised strains to achieve high levels of expression, the existence of numerous protocols for the transformation of *E. coli* and/or yeasts and the availability of vectors that contain a great diversity of promoters and selection markers, and so on [21]. However among the disadvantages presented by this antimicrobial peptide, production system is, obviously, the potential susceptibility that the producing microbes could have to the harmful antimicrobial peptides, in case these are exposed to. Another disadvantage, in case the peptide is trapped as insoluble form in inclusion bodies, implies the need to further purify these peptides from the extract, and, according to the potential use of the peptides, also the removal of endotoxins. Synthetic biology techniques facilitate the availability of the genetic material that codes for antimicrobial peptides with the codon optimisation of the host and it also allows the incorporation of signal peptides, epitopes for the detection of the final product, or the inclusion of appropriate targets for proteolytic enzymes, tags or tails to facilitate purification [22].

An alternative for an expression system of antimicrobial peptides without contaminating endotoxins could be the transformation of eukaryotic cells from animal or human origin. In this case, it may not be necessary to purify or decontaminate the extracts, since the culture would be free of pathogens. However, the cost of producing recombinant proteins would be considerably higher than using conventional microorganisms. Therefore, the economic yield of these clean expression systems is lower than the microbial ones [23].

We here propose that a production system based on microalga could be an alternative to the conventional fermentation system, which uses bacteria or yeast and/or mammalian cells [24]. First, the antimicrobial peptide is less likely to be toxic to this production system. Second, this production system differs from the others due to the greater volume of scaling that these cultures allow, since it can be spread over large areas, in microalgae production systems. This fact makes these production systems very profitable. Although they can be less efficient, the cost/benefit ratio could be higher for the large-scale production of these peptides. Another advantage of these green production systems is that some microalgae are considered generally regarded as safe (GRAS). Hence, in these cases, depending on the type of application expected for the antimicrobial peptides, purification processes may not be necessary, for example, applications for cosmetics, topical medicines, pesticides for agriculture, food conservation, and so on.

There are already numerous tools for the efficient transformation microalgae. For example, in the case of the microalga *Chlamydomonas reinhardtii*, it is possible to transform the three genomes: nuclear, chloroplastic and mitochondrial. Once transformed, microalgae can be grown in photobioreactors, in controlled conditions and free of pathogens. The microalga used as biofactories of recombinant drugs, on the other hand, could also be grown under controlled conditions to avoid escaping into the environment and to improve the biosecurity of the final product [25].

5. Conclusions

Antimicrobial peptides, originating from natural resources, have been recognised as a novel class of antibiotics. We have shown that genome mining is an effective method for finding new antimicrobial peptides, such as cathelicidins, from the genomes of a turtle and a bird. The derived peptides were tested for their *in vitro* antimicrobial activity showing that these antimicrobial peptides were particularly effective against bacterial growth, both Gram+ (*E. faecalis*) and Gram− (*E. coli*). In addition, these peptides had low haemolytic activity in human erythrocytes, which makes them suitable for use in different formulations and/or pharmaceutical, cosmetic and/or phytosanitary compositions, among others. Using synthetic constructions, current experiments are underway to produce these peptides as recombinant chimeric proteins in eukaryotic microalga and cyanobacteria. We hope that our preliminary investigation with these novel peptides could be useful for the design of future strategies that pursue the production of antimicrobial peptides through biotechnology in order to provide novel treatment opportunities based on antimicrobial peptides.

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Conflict of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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