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Unique Aspects of Anti-cancer Drug Development

Edited by Jolanta Natalia Latosinska and Magdalena Latosinska

UNIQUE ASPECTS OF ANTI-CANCER DRUG DEVELOPMENT

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and **Magdalena Latosińska**

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Meet the editors

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Contents

Preface

The evidence of cancers in humans, animals and plant species, found almost on every continent in every epoch, suggests that this disease is as old as multicellular life on Earth. In spite of 5000 years of more or less extensive research and a huge number of different documents related to this area, written (papyri, epic poems, travelogues, manuscripts, published books and papers, theses, reports and numerous conferences) or graphical (paintings, sculptures, drawings, photographs, X-ray or MRI scans, USG images, thermograms) as well as collected by computers (databases of human genome, cancer genotype, drug targets, images), the question of cancer is still unsolved. Cancer is considered as a highly complex and not fully understood systemic disease. This unique disease can attack any human or animal organ from the skin to the brain and any part of plant and has so many variations that go far beyond any standards and attempts of classification. It can affect anyone, irrespective of occupation, religion or gender.

Why is it so difficult to understand, to diagnose and, most importantly, to overcome that the greatest minds bow down before it? Maybe, because it begins from the organism's own mutated single cell, which breaks out of control, and is focused on its own survival and spreads at any costs. There is no doubt that a multiplicity of factors can trigger a cancerous mutation. Factors that increase the risk of cancer range from those related to the body like inherited genes, hormonal imbalances or immune system disorders, through environmental, chemical (hazard substances including drugs and food), biological (viruses, bacteria, parasites, fungi and tumour cells) or physical (different forms of electromagnetic radiation) to psychological like stress, which alters the levels of hormones in the body and strongly affects immune system (natural defence mechanism).

Even though we live more comfortable, healthier lives (diseases like flu, measles or tuberculosis do not kill us) and definitely with each generation living longer, civilization comes at a price. The risk of a mutation undefeated by immune system, thus "successful" for cancer development, increases significantly with age, high level of everyday stress and severe rise in environmental pollution and progress in technology. Although it would be naive to expect that cancer could be ever entirely eliminated, there is still hope for finding successful prevention methods, efficient screening tests, precise diagnostic tools and finally effective treatments.

The goal of this book is to give a view of the unique aspects of cancer across a few different areas of interest like cancer uniqueness, documentation of its spread in nature, the molecular mechanisms of anticancer drugs based on Chinese herbs, novel promising targets of annexins and kinases and the search for lupin-based anticancer drugs and astonishing immunotherapy applications. It is our hope that every reader will find in this book interesting, inspiring and stimulating information concerning cancer research.

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Chapter 1

Introductory Chapter: Having a Brain is Not Necessary to Get Cancer… but Indispensable to Fight It

Magdalena Latosińska and
Jolanta Natalia Latosińska

Additional information is available at the end of the chapter

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1. Introduction

Approximately one in eight deaths is caused by cancer, in fact one of more than 200 types of cancer, which is equal to the number of deaths caused by air pollution and exceeds the number of deaths being a result of two oldest known and two most common diseases ever, malaria and tuberculosis, combined.

According to the Globocan statistics in 2012, liver (19%), stomach (8.8%), colorectum (8.5%), breast (6.4%), esophagus (4.9%), pancreas (4%), prostate (3.7%), and cervix uteri (3.2%) cancers claimed a majority of victims. Almost 80% of cancer cases are diagnosed in people over 55 years old. The overall number of cancer incidences is almost 25% higher in men than in women. In 2012, there were 14.1 mln of new cancer cases, 8.2 mln cancer deaths, and 32.6 mln people were living with diagnosed cancer. On the basis of the statistics of the age-standardized rates of the incidents of all non-melanoma cancers, in 2012, almost 48% cases were detected in Asia, 24% in Europe, 13% in North America, 7.8% in Latin America and the Caribbean Islands, 6% in Africa, and 1.1 % in Oceania (**Figure 1**). The highest numbers of new cancers have been reported mainly in highly developed countries like Denmark, Australia, Belgium, Norway, United States of America, Ireland, Republic of Korea, the Netherlands, and France. As many as 43% of all cases of melanoma (cancer closely related to the exposition to excessive UV radiation) are detected in Europe, 32% in North America, 9.4% in Asia, 6.5% in Oceania, 5.9% in Latin America and the Caribbean Islands, and 2.9% in Africa. Most of them are confirmed in New Zealand, Australia, Switzerland, the Netherlands, Denmark, Norway, Sweden, Slovenia, the United Kingdom, and the United States of America. A surprisingly high risk of the melanoma cancers in the Northern part of Europe is believed to be closely related to frequent foreign travels by light-skin people to the sunny south.

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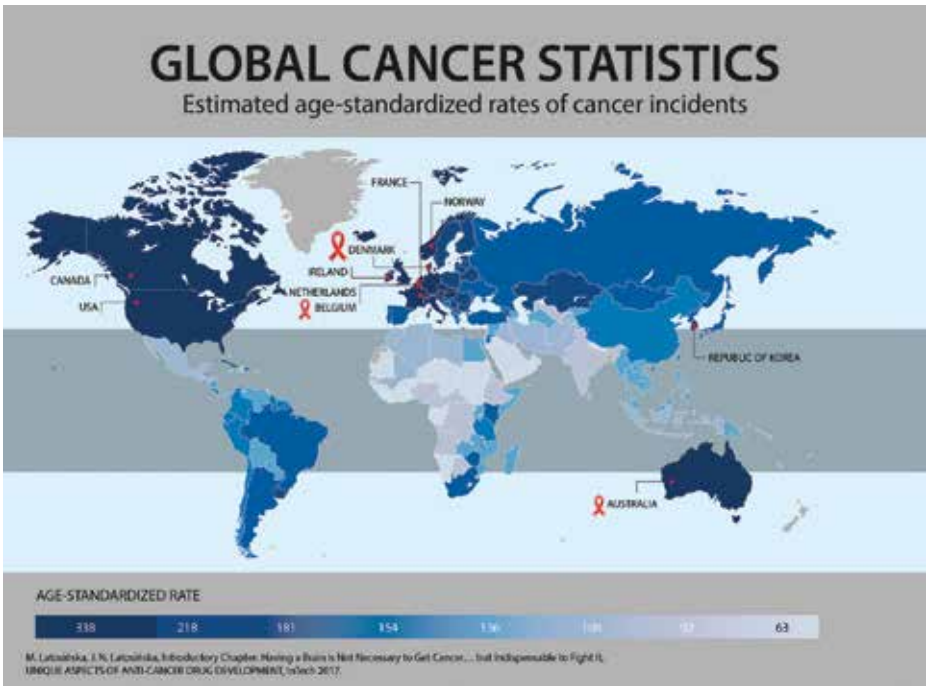

Figure 1. Non-melanoma cancer cases around the world in 2012 (statistical data: Globocan).

2. Cancer is as old as multi-cellular life on the Earth

It was long believed that cancers, especially malignant ones, are the domain of our time, because cancer cases have been scarce in archaeological excavations. But in fact, cancer has been known forever and has not necessarily been related to civilization or geographical location and definitely not limited to humans [1]. It may well appear in animals as well as in plants.

The oldest evidence of cancer dates back to about 60–70 million years ago and has been found in fossilized remains of a dinosaur in Wyoming. In the human fossils [2, 3], to this day, only about 200 cancer cases have been found, which probably count for only a small fraction of all cancers. Most of them are bone tumors either originating in skeletal tissue or being a result of metastasis. According to contemporary statistics, about 90% of neoplasm cases are carcinoma, which is formed from the epithelial tissues inside the organs or body cavities, that is, soft tissue. The natural body decay limits the possibilities of finding cancer cells to the remaining 10% in the skeletons or mummies. Although primary bone cancers are very rare, metastatic ones are very common and allow detection of traces left by primary cancer. Until 2016, the oldest specimen of cancer has been considered the hominid malignant tumor (probably Burkitt's lymphoma) found in 1932, in Kenya, in the remains of a body (mandible) of either *Homo erectus* or an *Australopithecus*. In 2016, a much older, 1.7-million-year old, hominin primary malignant cancer osteosarcoma from Swartkrans Cave, South Africa [4], and a benign bone tumor osteoid osteoma in the hominin

Australopithecus sediba from Malapa, South Africa, dated to 1.98 million years ago [5] were discovered. Both the abovementioned bone tumors often occur in young people and are not correlated with factors related to the modern lifestyle. Newer evidence covers three primary bone cancers—osteosarcomas—dated to Neolithic (Bassa Padana, Italy) [6], 1500–1070 BC (Egypt) [6], and 800–900 BC (Münsingen, Switzerland) [7]. Secondary bone cancers are much more frequent. The oldest evidence of one of the most aggressive cancers (primary cancer of the prostate gland) has been found in the form of osteoblastic proliferation in the skeleton of a mature male in Neolithic mass burial (ca. 5000 years BC). In 2015, a 4200-year-old skeleton of an Egyptian woman, whose breast cancer spread to her bones (metastatic cancer), was found in a tomb in the Nile Valley, Sudan. Osteoblastic and osteoclastic lesions, being an evidence of metastasis, have been found in the skeleton of a 40–50-year-old Scythian king, who lived 1700 BC in Southern Siberia (Russia) [8]. Excavations in Amara West, Sudan, revealed the spread of primary cancer to the collar bones, shoulder blades, upper arms, vertebrae, ribs, pelvis, and thigh bones on the skeleton of a 25–35-year-old male dated to around 1200 BC [9]. Apart from the abovementioned cases, multiple myeloma dated to 4000 BC (Mauer, Austria), nasopharyngeal, 3000 BC (Giza, Egypt), and 2300–1800 BC (Naga-ed-Deir, Egypt), metastatic carcinomas, 2200–800 BC (Czech Republic) and 1500 BC (Russia), tumor in pelvic bone in mummy, 2000 BC (Alexandria, Egypt) have been discovered. Newer evidences of fossils with metastasis are dated to the first century AD, Italy [10], Hungary [11], medieval times Switzerland [12], Hungary [13], Denmark [14], England [15–17], and Persia and Peruvian Incas in pre-Columbian America (around 1400 AD) [18]. Clearly, the majority of evidence comes from Europe and Egypt, which results from extensive excavation works and excellent preservation of mummified and skeletal human remains, but individual cases originated from Australia, North and South America are also known.

The evidence of cancers comes not only from fossils but also from written records dating back to ancient times (3000–1500 BC, Mesopotamia and Egypt). The symptoms, differentiation between benign and malignant neoplasm, and the surgical methods of treatment have been described in Egyptian Papyruses including the Edwin Smyth (2500 BC), Leyde (1500 BC), and George Ebers (1550 BC). The first description of cancer *per se*, in fact breast cancer, and information about the lack of treatment, comes from the Smyth papyrus (the so-called case 45), which nowadays is assumed to originate from Imhotep, a great Egyptian physician who lived around 2625 BC (Old Kingdom). The Code of Hammurabi, Babylonian law code of ancient Mesopotamia dated to 1750 BC, set up the standard fee for surgical tumors removal. The Rites of the Zhou Dynasty, a collection of social forms, governmental system, and ceremonial rites, written from 1046 to 256 BC, mentioned the treatment of the cancer stages (swellings and ulceration, necrosis, and ulceration). The first medicine, in the form of arsenic paste, is mentioned in the Hindu epic poem, the Ramayana, dating back to 500 BC. Different benign and malignant types of tumors were described in the fifth century BC by Hippocrates of Kos famous Geek physician. He has found blood vessels around a tumor resembling a crab in its shell and introduced the name of this disease *karkinos* (crab in Greek). The same associations had Marco Polo, who in the *Travels of Marco Polo*, a thirteenth century travelogue, described a female reproductive organs disease called "the crab" (a woman died after rupturing it in her abdomen). Hippocrates also formulated the humoral theory of cancer genesis. The story of the first successful excise of breast cancer of Atossa, the queen of Persi,

performed by Democedes, Greek Slave, is described in *Histories*, written around 440 BC by the Greek historian Herodotus from Halicarnassus. The Chinese *Huangdi Neijing* (*the Inner Cannon of the Yellow Emperor*), the oldest known medical book, dated to (475–221 BC) and (206 BC–220 AD) contained the first description of tumors including their progression, metastasis, and death but also therapies: spiritual, pharmacological, diet, acupuncture, and treatment of respiratory diseases. Nearly 400 years after Hippocrates, Cornelius Celsus described the first surgeries on cancers and introduced the well-known Latin term *cancer*, which is used to describe malignant tumors and became the root of terms *carcinogen* and *carcinoma*. Claudius Galen, the most famous Roman Empire physician in the second century, accepted Hippocrates' ideas, but left a comprehensive description of many neoplasms and introduced a special term *oncos* (Greek). Nowadays, this term is a part of the name *oncology*—the branch of medicine that deals with cancer. Galen believed that cancer was incurable; however, some tumors were removed surgically. Between the fifth and fifteenth century, European medicals used surgery and cautery on smaller tumors and caustic pastes, usually containing arsenic, on large cancers. Apart from this phlebotomy, diet and herbal medicines were applied. During the Islamic Golden Age (eighth to thirteenth century), many of the famous Arab physicians studied classic Greek medical texts, mainly Hippocrates and Galen's ones. Rhazes and Avicenna identified several cancer types, including eye, nasal, tongue, stomach, liver, the urinary system, kidney, testis, and breast, and spleen and nerve tumor. Rhazes, in 925, and Avicenna, in 1037, described cancers as extremely difficult to treat, but curable in the earliest stages of development. Avicenna discussed the combined effect of diet and medicines on cancer progression. The properties of the herbal drug "Hindiba" (*Cichorium intybus* (L.)) used by him in cancer treatment have been confirmed about 900 years later. Albucasis (Alzahrawi), a Muslim surgeon, conducted the first breast cancer surgeries using 200 different instruments invented by himself. He described his surgical equipment in *At-Tasrif*, which quickly became a standard reference, also in Europe. In 1131, Ibn Zuhr provided the first accurate description of esophageal and stomach cancers based on autopsies. In the thirteenth century, Europe autopsies were not popular, but sometimes performed to check the internal cancers growth. However, some mysterious death has been waiting for explanation till date, for example, the fifteenth century king Ferrante I of Aragon. Autopsy of his mummy revealed a case of adenocarcinoma in the muscles of his small pelvis [19]. Some protocols of autopsy have been developed by Antonio Benivieni, Florentine physician, who pioneered their use to understand the cause of death. *De Abditis Morborum Causis (The Hidden Causes of Disease)* written by him in 1507 contains the first "printed" case report of cancer. However, the major medicinal text on cancers was that of Galen, which influenced cancer treatment until the seventeenth century and *De Humani Corporis Fabrica* written in 1543 by Andreas Vesalius. In the seventeenth century, the Dutch surgeon, Adrian Helvetius, performed lumpectomy and mastectomy to cure breast cancer. The Italian, Bernardino Ramazzini, the founder of occupational medicine, was the first who linked lifestyle with the development of cancer. He described the health hazards of chemicals, dust, metals, repetitive or violent motions, odd postures, and other disease-causative agents encountered by workers in the 50 most important occupations in *De Morbis Artificum Diatrib* (*Diseases of Workers*) published in 1700. In 1761, the Englishmen John Hill published the first paper linking tobacco and cancer; 14 years later Percival Pott identified the first occupational carcinogen—soot. By the end of the eighteenth century, hundreds

of materials were recognized as carcinogens. In 1845, Virchow identified and published a description of the blood cancer disease and proposed the name leukämie (eng. *leukemia*); properly diagnosed rare chordoma tumor originated from the clivus, and later in 1888, during postmortem examination of Kaiser Frederick III body, he identified the epidermal cancer of larynx (hybrid verrucous carcinoma). Virchow also linked the cancers (e.g., mesothelioma, lung, prostate, bladder, pancreatic, cervical, esophageal, melanoma, head, and neck) with long-term inflammation [20], which was earlier suggested by the Dutch, Hermann Boerhaave. In 1842, Domenico Antonio Rigoni-Stern after the statistical analysis of cancer incidence and mortality in 1760–1839 in Verona concluded that cancer death rate is rising, increases with age, is less frequent in the country and is more likely among unmarried people.

In the past centuries, till half of the eighteenth century, the life spans hovered around 30 or 40 years, that is, was much shorter than 55 achieved in Europe only in 1920. People did not live long enough to get cancer, were killed during wars, murdered, poisoned, or were suffering from diseases other than cancer, for example, schistosomiasis, malaria, hookworms, cholera, plague, or tuberculosis. Cancer-related mortality grew by about 30% from 1900 to 1916, also due to the mass introduction of Roentgen's discovery, X-ray, to medicine and industry, which applied without any limitations and knowledge of doses resulted in cancers. Many scientists and doctors working with X-ray radiation suffered from radiation-induced cancers, including Roentgen, who died of intestinal cancer [1, 21]. But since 1926, cancer has become the second most common cause of death, just behind the heart disease.

Currently, cancer is considered as the second after heart disease as the most frequent natural cause of death among world leaders. Hatshepsut (1507–1458 BC), the fifth pharaoh of the eighteenth Dynasty of Egypt, died of bone cancer. Galerius (Roman Emperor, 305–311) likely died of bowel cancer. Napoleon Bonaparte (1769–1821) died from stomach cancer, which earlier killed his father Carlo. Among UK rulers, Edward I, Hammer of the Scotts (1239–1307) and Henry V (1387–1422) probably died of cancer of the rectum, Kenneth I (810–858) of neoplasm, Mary I (1516–1558) of ovarian cancer, George VI (1895–1952) of lung cancer, and Edward VIII (1894–1972) of throat cancer. The Queen Mother (1936–2002) battled the colon and breast cancers. Three among 266 popes died due to cancer: pope Clement VI (1291–1352) of tumor, Pius V (1504–1572) believed to have died due to undefined cancer, and Saint John XXIII (1881–1963) of stomach cancer. Saint John Paul II (1920–2005) has benign intestinal tumor removed in 1992. Henry Pu Yi (1906–1967), the last emperor of China before the Xinhai Revolution, died of kidney cancer, while the father of modern China Sun Yat-sen (1866–1925) died of liver cancer. While many USA presidents, vice presidents, and first ladies have been plagued with cancer, the only one USA president who has passed away due to cancer (throat cancer) was Ulysses S. Grant (1822–1885), a heavy smoker, while American first ladies Pat Nixon (1912–1993) died of lung cancer and Jacquelin Kennedy Onassis (1929–1994) died of lymphoma. Other presidents who died of cancers include Francois Mitterrand (1916–1996), the president of France, of prostate cancer, German president Paul von Hindenburg (1847–1934) of lung cancer, while Willy Brandt (1913–1992), the Chancellor of West Germany, of colon cancer, José Napoleón Duarte (1925–1990), the Junta leader and president of El Salvator, of stomach cancer, Corazon Aquino, the eleventh president of the Philippines, of colon cancer, and María Eva Duarte de Perón (Evita) (1919–1952), the first lady of Argentina, of uterus cancer.

Many British prime ministers died of cancer: Arthur Chamberlain (1869–1940) of bowel cancer, David Lloyd George (1863–1945) of prostate cancer, Anthony Eden (1897–1977) of liver cancer, Harold Wilson (1916–1995) of colon cancer, and Andrew Bonar Law (1958–1923) of throat cancer. Those who died of cancers include three Canadian prime ministers John Abbott (1821–1893) of brain cancer, a father of Canadian medicare, Tommy Douglas (1904–1986) of inoperable cancer, and one of the best Canada's prime ministers Pierre Trudeau (1919–2000) of prostate cancer.

According to the statistics among famous people (scientists, actors, writers, poets, etc.), lung (27%), leukemia (10.5%), pancreas (10.4%), prostate (7.7%), breast (7.4%), brain (6.9%), and stomach (6.2%) claimed a majority of victims. Surprisingly, heart diseases are much less popular cause of death. Cancer collects harvest among the most brilliant scientists like French mathematician/physician Blaise Pascal (1623–1662)—stomach tumor, James Clerk Maxwell (1831–1879), Scottish mathematician, physicist—abdominal cancer, Robert Oppenheimer (1904–1967), theoretical physicist and father of the atomic bomb—throat cancer, Enrico Fermi (1901–1954), Italian/US nuclear physicist—stomach cancer, Maria Skłodowska-Curie (1867–1934), the only person to win a Nobel Prize in two different sciences physics and chemistry—leukemia, Irène Joliot-Curie (1897–1956), her daughter—leukemia, Rosalind Franklin (1920–1958), the author of X-ray photographs, which proved that the DNA molecule is a helix—ovarian cancer, Francis Crick (1916–2004), who used Franklin's brilliant discovery and win Nobel Prize—colon cancer. Ada Lovelace (1815–1852), an English mathematician considered the first computer programmer, died of uterine cancer. Among famous persons who died on cancer are scientists representing all fields of study, for example, Elinor Ostrom (1933–2012), American economist—pancreatic cancer, Eli Lilly (1839–1898), American pharmaceutical chemists, Caro Lucas (1949–2010), Iranian Armenian Engineer, Edsger W. Dijkstra (1930–2002), Dutch computer scientists, Adam Ulam (1922–2000), Polish-American historian, political scientist and writer—lung cancer, Ernest J. Briskey (1930–2006), food scientist—leukemia, Hugh Latimer Dryden (1898–1965), American aeronautical scientist, Daniel Weinreb (1959–2012), computer scientist working in the Lisp environment, Gerrit Jan van Ingen Schenau (1944–1998), physicist, Allen Newell (1927–1992), computer scientist and cognitive psychologist. Suzanne Corkin (1937–2016), a pioneer in the field of cognitive neuroscience, died of liver cancer. Quantum physicist Deborah Jin (1968–2016), who obtained the first fermionic condensate, also died on cancer. Cancer reaches businessmen Steven Jobs, Apple Inc. – pancreatic cancer, actors (Paul Newman—lung cancer, Humphrey Bogart—throat cancer, Jack Lemon—colon cancer, Farrah Fawcet—anal cancer, Judy Holliday, Yul Brynner—lung cancer, Audrey Hepburn—colorectal cancer), poets (e.g., Arthur Rimbaud—bone cancer) and musicians (Duke Ellington, Samuel Barber—lung cancer, Joe Cocker—lung cancer, Robbin Gibb—colon and liver cancer, David Bowie cancer, Bob Marley—brain and lung cancer). Cancer reaches saints as well as gangsters (Bugs Moran—lung cancer, Jamie Daniel, Mark Chopper), American mafia bosses (John Gotti), serial killers (Ian Brady, Richard Ramirez), or nazists (Klaus Barbie, Aribert Heim—intestinal cancer). The last members of the most famous families into the darkness of history were knocked down by the cancer (Anna Maria Luisa de'Medici—breast cancer).

The evidence of cancers has taken sometimes surprising forms. Only in the twentieth century a few cases of surprising Renaissance methods of cancer documentation were discovered. The famous Michelangelo's marble statue "*Night*", located in the Medici tomb (Medici Chapel, Church

of San Lorenzo, Florence, Italy) and dated to 1520–1534, presents a female figure with owl—the night bird, symbol of feminine energies, the moon, magic and darkness, prophecy and wisdom. The left breast of this statue is completely different than right, and has abnormalities associated by oncologist with locally advanced cancer [22]. Another "clinical documentation" of the cancer stage contains "*La Fornarina*" by Rafael Santi painted in 1520. The left breast of "*La Fornarina*" is enlarged and deformed due to cancer; it is even possible to describe the stage of the breast cancer [23]. Niccolò Renieri Vanitas "*Allegory of Transience*", Keresztény Múzeum, Esztergom, Hungaryl, from 1626, also show cancer in advanced stage. A similar case of multifocal breast cancer was found in Lucas Vorsterman of a Ruben's painting after Titian's which shows a young woman dressed in a fur coat and a hat. Two lumps in the upper external quadrant of her right breast indicate a deep tumor [24]. Three Rubens paintings "*The three Graces*" ca. 1630–1635, "*Diana and her nymphs pursued by satyrs*", ca. 1636 and "*Orpheus and Euridice*", ca. 1636–1637, Prado Museum, Madrid, Spain, also indicate breast cancers [25]. In the "*The three Graces*" painting the tumor, between the left breast and the left axial, is exophytic, irregular, with inflammatory, while in two remaining show abnormalities suggesting the early stages of the breast cancer. Another example is Il Cerano painting "*The Madonna Delivers Milan from the Plague*" from 1631, located in the church of Santa Maria della Grazia, Milan, Italy, which depicts a young woman with ulcerating right breast cancer [26]. In 1654, Rembrandt Harmenszoon van Rijn painted his mistress Hendrickje Stoffels, later died after a long illness, as "*Bathsheba at her bath*". In 1967, the asymmetry and blue mark on her breast has been interpreted by Australian surgeons as a case of breast cancer [27]. The controversy whether Rembrandt indeed depicted cancer remains unsolved until today. Knowing that breast cancer has been responsible for the deaths of about 25 million women throughout history, it seems not surprising that this form of cancer was indeed documented by the artists. Nonetheless, old masters with photographic realism of painting introduced by Leonardo da Vinci can be considered as clinical photographers of their age.

Cancer is a common disease in the whole animal kingdom, with the average annual rate of all cancer types in dogs [28, 29], cats [29], horses, rates, mice [30, 31], or cattle close to that noted in humans. The cancer cases among the domestic animals are better documented than in wild ones, and suggest similar types of cancers developing in them and in humans [32]. Even aquarium fish develop bumps or lumps, internal tumors and cancers, much like humans and other animals. For example, Koi fish are susceptible to the reproductive organs tumors, goldfish to fibroma tumors and sarcomas, while gypsy-swordtail fish develop malignant melanoma skin cancers. The studies of cancers in wild populations deliver more interesting, unique data on the methods of the cancer spread as well as its origins. For example, the studies of Tasmanian devils reveal that Devil facial tumor disease, able to kill almost 90% of their population, has been spread as infectious tumors [33] similar to canine transmissible venereal tumor. This cancer cell line most probably appeared 200–2500 years ago in wolf, coyote, Siberian Husky, or Shihtzu dogs. The studies of sea animals like sea lions, which suffer from urogenital cancers [34], and beluga vales, which predominantly die due to intestinal cancer [35], indicated the role of organic pollutants in carcinogenesis. Also widely performed sharks studies revealed a lot of kidney [36] or melanoma [37] cancer cases, which put down the misleading conviction that sharks are cancer-free and that shark cartilage can cure cancer. However, a few species of animals, which seem cancer resistant and get cancer very rarely, have actually been discovered. It is known that taller people/larger dogs are slightly more cancer-prone than shorter people/smaller dogs. But surprisingly, this rule is not applicable

to elephants or whales. Elephants, which have trillion more cells than humans and reach 56 years in the wild, have much lower cancer rates than humans. Thus, evidently cancer prevalence is not correlated with body size, which is called "Peto's paradox." It has been discovered that elephant's genome is unique and contains 20 copies of a tumor-suppressor p53 (cancer-fighting gene playing the most important role in protecting against cancer) in contrast to other mammals, which have only one. A mutation of p53, which switches off this gene, found in 50% human cancers, allows abnormal cells to proliferate and form tumor. Bowhead whale species of family *Balaenidae* Gray, 1821 with over 1000 times more cells than humans, the longest-living animals that can live over 200 years, also have low cancer incidence as compared to humans. It has been found that they possess special adaptive genetic changes—genes involved in DNA repair, cell-cycle regulation, cancer, and aging. The naked mole rat (*Heterocephalus glaber* Rüppell, 1842), which is not naked, is not a mole, is a rodent but not a rat and lives up to 30 years, has been announced unique cancer-free species and a proclaimed animal of 2013. Extensive studies have revealed that its fibroblasts secrete extremely high-molecular-mass (five times larger than human) hyaluronan, which mediates the cancer resistance [38]. But in 2016, it has been discovered that these animals also may have cancer [39, 40]. Cancer has been diagnosed even in invertebrates like flies and worms [41] as well as very primitive animals like spoons [42], cnidarians [43] or freshwater hydra [44], which do not have a recognizable brain. In-depth studies show that hydra tumors share several features similar to human tumors. All this evidence suggests that cancer may be as old as animal organisms.

In human and animals, cancer develops as a result of deregulation of cell growth which is often triggered by mutation. Plants can also experience cancer which is called canker (necrosis of the bark and cambium on stems, branches, or twigs) and brought on via infections by fungi, bacteria, viruses, or insect infestation. Some tree species (cultivated or wild) are especially prone to canker development. For example, *Nectria cinnabarina* (Tode) Fr. genus of *Ascomycete* (Berk.) Caval.-Sm. fungi is a pathogen of tree species including apple (*Malus* (Mill.)), ash (*Aesculus* (L.)), birch (*Betula* (L.)), golden rain tree (*Koelreuteria paniculata* (Laxm.)), honey locust (*Gledetsia triacanthos* (L.)), maple (*Acer* (L.)), mulberry (*Morus* (L.)), and oak (*Quercus* (L.)); *Leucostoma* Meigen, 1803 and *Valsa* Fr. cause canker diseases in maples (*Acer* (L.) spp.), plums and peach (*Prunus* (L.) spp.), poplar and cottonwood (*Populus* (L.) spp.), willow (*Salix* (L.) spp.) elm (*Ulmus* (L.) spp.), and spruce (*Picea* (A. Dietr.) spp.); while *Phomopsis arnoldiae* (B. Sutton) is responsible for Russian olive (*Eleagnus angustifolia* (L.)) cankers. *Leucostoma persooni* (Nitschke) Höhn. and *Leucostoma cinctum* (Fr.) Höhn. are some of the most destructive pathogens on peach and ornamental plums (*Prunus* (L.) spp.). *Pseudomonas syringa pv. Syringae* Van Hall, 1904 and *Pseudomonas syringa pv. Morsprunorum* (Wormald) are responsible for the most cankers of many fruit trees like cherries (*Cerasus* (L.)), apricots (*Prunus* (L.)), and peaches (*Prunus persica* (L.) Batsch 1801). Thus, the majority of plants are unique host species for selected canker-causing organisms. However, plants are a bit less vulnerable to the cancer phenomenon and cancer effects than animals or humans. The walls of their cells are thick, thus the cells, including canker ones, are well separated. Therefore, the cancer can grow in separate parts of plant, but is unable to metastasize. Furthermore, plants lack the vital organs which after the cancer attack would quickly lead to their death. But the diffuse trunk cankers of chestnut (*Castanea dentata* (Marshall) Borkh.) caused by *Cryphonectria Murrill* Barr. parasitica or read oaks like Coast live oak (*Quercus agrifolia* (Née)), California black oak (*Q. Kelloggii* (Newb.)), and Shreve oak (*Quercus parvula var. Shrevei* (Greene)) cankers being a result of *Phytophthora ramorum* (Werres, De Cock & Man in 't Veld) and trunk and stem canker of coffee

trees incited by *Ceralostomella fimbriata* (E. et H.) Elliott are still able to kill a tree in a short time. The studies of cankers are important because they confirm a relation between fungi and cancer. In fact, mycotoxins (e.g., alfatoxin) belongs to carcinogens [1], able to cause p53 mutations. External stimuli such as fungal attack (eg. *Xanthomonas campestris pv. Viticola Nayudu* (Dye)) and UV-radiation activate the stilbene synthase genes in the grapes of *Vitis vinifera* (L.), *Morus* (L.) or *Arachis hypogaea* (L.) to produce resveratrol, which fights the fungus. In the human body resveratrol is converted by Cytochrome P450 1B1 (CYP1B1) to piceatannol an anti-cancer agent that can selectively kill cancer cells. Tutankhamun or Jagiellonian curse, responsible for many death cases after opening old tombs, turned out to be the responsibility of misbehavior of mycotoxins. Therefore, it is no wonder that some scientists, like Max Gerson or Tullio Simoncini, formulated the hypothesis that human cancer is a fungus, which form colonies and spread throughout the host area. Maybe the species from plant and animal kingdoms are attacked by the species from other kingdoms?

Anyway, the human, animal, and plant studies suggest that cancer may be extremely old, as old as multi-cellular life on the Earth.

3. Cancer is unique

The cancer uniqueness begins from the single cell, which after mutation is focused on survival and thus acquires very special features. Contrary to the healthy cells, cancer ones contain abnormal asymmetrically shaped nuclei, remain undifferentiated, and constantly divide, but also have the ability to form tumors by building layers on the top of each other (proliferation). Their altered metabolism based on glucose instead of oxygen and the ability to grow new blood vessels (angiogenesis) ensure continuous delivery of components necessary for cancer life. The implemented special "pro-survival" mechanism turns off apoptosis (programmed cell death). Cancer has not only limitless replicative potential but also invasive character with its ability to migrate and spread to other organs and tissues. It is in fact oriented toward its own survival exclusively, but not to the survival of the organism in which it develops. As cancer is characterized by the abnormal invasive growth of singe mutated cancerous cell, resourced by the life-sustaining chemical transformations guaranteeing uninhibited growth and immortality but also ability to metastasize and spread to other parts of the body, it is understood why it is so difficult to overcome.

The differences in individual genotype, random nature of DNA mutations inherited or developed, cumulating year by year in the organism and responsible for each of 200 types of biologically different cancers, make the cancer disease extremely complex in identification, diagnosis, and treatment. Identification of the behavior, spread, or damage made by cancer is difficult. The difficulty in fighting cancer lays in the fact that we have no idea by what exactly it is triggered, because of the number of possible factors associated with it. It can be the factors related to the body (inherited genes, hormonal imbalances, or immune system disorders), environmental factors (chemicals—hazard substances including drugs and food, biological oncogenes—viruses, bacteria, parasites, fungi, and in rare cases tumor cells, and physical—different forms of electromagnetic radiation), and psychological ones, for example, stress which alters the levels of hormones in the body and affects the immune system (**Figure 2**).

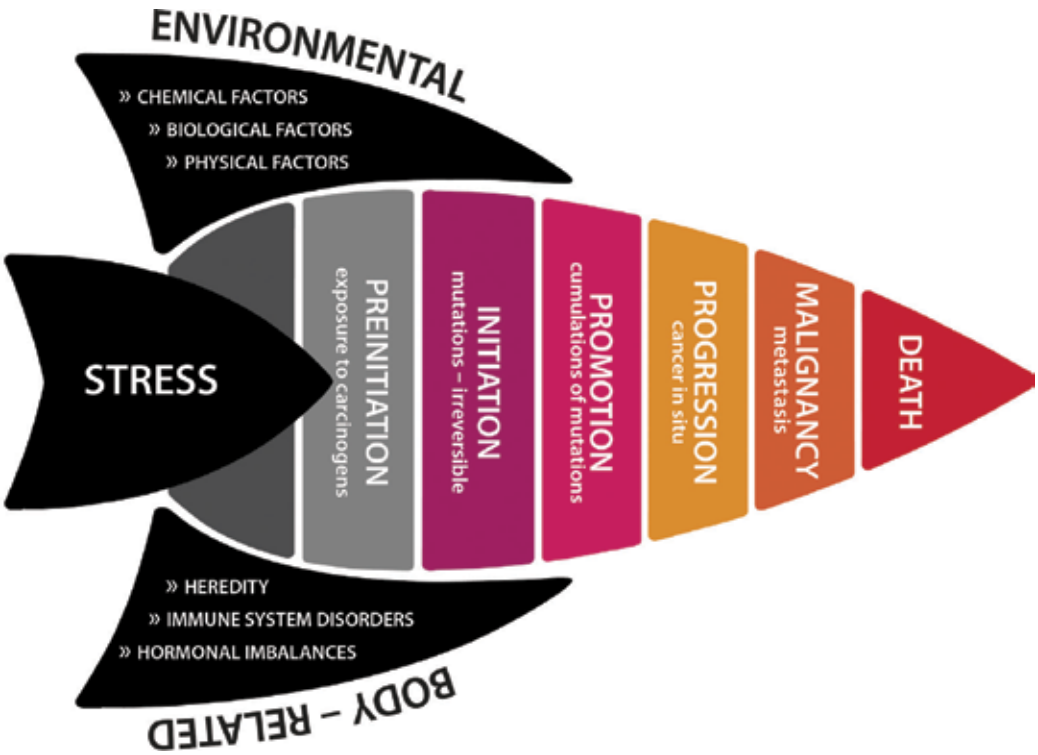

Figure 2. Multi-step process of carcinogenesis.

The problems with fast and accurate enough diagnosis at the early stage, deficit of effective anticancer drugs with no side effects as well as difficulties in access to certain parts of the body like the brain, are serious obstacles. But with insufficient knowledge, the effective treatments are hard to develop.

4. Final remarks

Matthew Neely, the Senator of West Virginia, was the first public figure who highlighted the necessity of studies on cancer. In 1928, he said that cancer is "*a monster more insatiable than the guillotine*." Shortly after the discovery that cancer had become the second most common cause of death, two large organizations in USA and Europe were established to lighten humanity's ever-growing burden of cancer. The National Cancer Institute (NCI) was established by the National Cancer Act of 1937 signed by USA president Franklin Delano Roosevelt and The International Agency for Research on Cancer (IARC), World Health Organization (WHO) agenda, was founded by the president of France, Georges Pompidou (who later died on lymphoplasmacytic lymphoma). The NCI coordinates the National Cancer Program and conducts and supports research and other activities related to the causes, prevention, diagnosis, and treatment of cancer. IARC till today published 118 volumes containing a total list of carcinogens

with 1162 entries, and provides statistics on cancer incidents and mortality around the World. In 1971, Richard Nixon, the USA president, announced "*war on cancer*" and signed the National Cancer Act Program to more effectively carry out the national effort against cancer.

Since then, cancer has been the most widely described and the most extensively studied (174 k books, 365 k journal articles, 113 k dissertations) disease. But despite significant progress in knowledge (e.g., the Cancer Genome Atlas sequencing the genomes of human cancer cells), improved-methods of diagnosis (e.g., magnetic resonance imaging (MRI), X-ray and computed tomography (CT) scans, positron emission (PET) tomography, ultrasonography, or thermography and invasive as biopsy), methods of drug discovery (studies in silico), and delivery (nanotechnology) as well as treatment (anticancer drugs combined in whole therapy lines, dietary supplements), over the last 50 years, cancer with increasing numbers of incidents and mortality still remains a major cause of death in the world and our abilities to cure it are still very limited.

Cancer is the oldest known disease, probably as old as life on the Earth. Since nearly 5000 years, it is considered as complex systemic disease, which requires combined methods of treatment (surgical—common in all periods from ancient Egypt till today, radiotherapy, and chemotherapy introduced in the nineteenth century, immunotherapy discovered recently). But because the genes contributing to cancer progression come from the organism's own cells, with the increasing lifespan the risk of cancer increases. Thus, it is naive to expect that this disease ever could be entirely eliminated. However, there is no reason to lose hope for finding an effective treatment or methods of its prevention. Recently, it has been discovered that nearly 50% of all cancers is preventable by the elimination of different factors, in particular environmental ones. Furthermore, some types of cancers or their recurrence are known to be predictable using different types of screen tests (including panel of multiple gene tests for mutations).

Being a human or having a brain is not necessary to get cancer, but really indispensable to prevent or fight it.

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Chapter 2

Application of Computer Modeling to Drug Discovery: Case Study of PRK1 Kinase Inhibitors as Potential Drugs in Prostate Cancer Treatment

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Wolfgang Sippl

Additional information is available at the end of the chapter

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Abstract

Computer modeling of natural products (NPs) and NP scaffolds is increasingly gaining importance in drug discovery, particularly in hit/lead discovery programs and at the lead optimization stage. Even though industry had lost interest in the implication of NPs in hit/lead searches, recent reports still show that computer modeling could be a useful assert for the identification of starting scaffolds from nature, which could be further exploited by synthetic modifications. In this chapter, the focus is on some useful tools for computer modeling aimed at the discovery of anticancer drugs from NP scaffolds. We also focus on some recent developments toward the identification of potential anticancer agents by the application of computer modeling. The chapter will lay emphasis on natural sources of anticancer compounds, present some useful databases and computational tools for anticancer drug discovery, and show some recent case studies of the application of computational modeling in anticancer drug discovery, as well as some success stories in virtual screening applications in anticancer drug discovery, highlighting some useful results on the application of on lead discovery (including promising NP scaffolds) against an interesting anticancer drug target, the protein kinase C-related kinase (PRK1).

Keywords: anticancer, molecular modeling, natural products, virtual screening, QSAR

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1. Introduction

1.1. Cancer and natural products

Cancer is one of the most feared causes of death, as it represents several disease forms and treatments possibilities are still limited for late stages of the disease [1]. Among the known drugs for cancer treatment, camptothecin, vinblastine, vincristine, podophyllotoxin, and taxol are of natural origin [2]. Nature is known to be an immense repository of natural products (NPs), constituting the source of about half of the anticancer drugs currently in the market [3]. Inspite of the drop in interest for NPs in drug discovery projects, from an industrial point of view, recent reports still show that NPs could constitute a useful assert for the identification of starting scaffolds for further discovery [4–6]. The quest for anticancer drugs of natural origin or with NP scaffolds has resulted in the development of NP databases, the most promising one being the naturally occurring plant-based anti-cancer compound activity-target database (NPACT), with ~1500 NPs, including experimentally verified *in vitro* and *in vivo* biological activities (in the form of IC_{50}s, ED_{50}s, EC_{50}s, GI_{50}s, etc.), along with physical, elemental, and topological properties of the compounds, the tested cancer types, cell lines, protein targets, commercial suppliers, and drug-likeness classification for each compound [7].

1.2. Prostate cancer

Among the many diverse cancer forms, prostate cancer is the second leading cause of cancer deaths in men worldwide [8]. Two different types of prostate cancer were identified: androgen-dependent prostate cancer and androgen-independent prostate cancer [9]. Androgen hormones (testosterone or dihydrotestosterone) are known to activate the androgen receptors located in the cell nucleus. The main function of these receptors is to modify gene expressions, thus controlling several biological activities in the cells, including cell growth and differentiation, development, and function of male reproductive and accessory sex tissues [10, 11]. It was found that the androgen receptors signaling pathway plays an important role in the progress and development of prostate cancer. In the first stage of androgen-dependent prostate cancer, the survival and growth of the cancer cells are mainly dependent on androgen hormones [11]. The initial treatment of androgen-dependent cancer, based on androgen ablation, is called hormone therapy. This procedure aims to stop the cell growth of cancer cells, which, in most cases, respond to this therapy. The recurring prostate cancer cells from hormone therapy would further not respond to androgen ablation, thus leading to the development of androgen-independent prostate cancer, which can further progress to metastasis [11]. The molecular mechanism of tumor recurrence is not completely clear. For the second stage of prostate cancer, there is no efficient therapy available.

1.3. Protein kinase C–related kinase

Protein kinase C–related kinase (PRK1, also known as PKN1) is a serine/threonine kinase known to play a role in controlling the activity of androgen receptors in prostate cancer.

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It was shown that the activation of PRK1 stimulates the activity of androgen receptors and is involved in tumorigenesis [12]. In 2008, Schüle et al. showed that PRK1 phosphorylates histone H3 upon ligand-dependent recruitment to androgen receptor target genes [12]. The phosphorylation of histone H3 at threonine 11 (H3T11) increases demethylation of Lys-9 by Jumonji C (JmjC)-domain-containing protein (JMJD2C), which promotes androgen receptor-dependent gene expression and tumor cell proliferation [13]. Additionally, PRK1 may directly phosphorylate JMJD2C, thereby stimulating its activity [14]. Meanwhile, the role of PRK1 in androgen-independent prostate cancer is unknown. PRK1 can be activated by the Rho family GTPases, thus mediating several processes related to the migration and cancer cell invasion and consequently playing a major role in the formation of metastases [15, 16]. Thus, PRK1 is considered to be a promising therapeutic target, and the discovery of novel potent and selective inhibitors could supply a meaningful tool for the treatment of prostate cancer. On the other hand, the discovery of selective and potent inhibitors would provide a tool for understanding particular biological roles of PRK1. In spite of the importance of PRK1 in the targeted therapy of cancer, only a few known inhibitors have been identified. The known PKC inhibitors (**Figures 1** and **2**) include staurosporine (PubChem CID: 44259) and its analogue (Ro-318220; PubChem CID: 5083), bisindolylmaleimide I (BIM I; PubChem CID: 2396), and lestaurtinib (PubChem CID: 126565), as well as the nonselective Akt inhibitor GSK-690693 (PubChem CID: 16725726) and Pfizer's JAK nonselective inhibitor CP-690550 (tofacitinib; PubChem CID: 9926791) [17, 18]. In a previous work, several novel PRK1 inhibitors were identified containing different scaffolds, using a homology model, with varying potencies [19]. In the present work, the focus is to search for new small molecules and natural products, which could inhibit PRK1 by using the recently published crystal structures.

1.4. Structural analysis of PRK1

PRK1 kinase belongs to the protein kinase C (PKC) superfamily and was first identified in 1994 from a human hippocampal cDNA library [20]. Three isoforms are found in mammals (called PRK1, PRK2, and PRK3). They possess different enzymatic properties and are distributed among different tissues [21]. The PRK1 structure is divided into three conserved regions:

- an N-terminal lobe (which includes a regulatory region containing three homologous stretches and is rich in charged amino acids),
- an auto-inhibitory domain called C2-like region (which is sensitive to arachidonic acids), and
- a C-terminal lobe (which contains the catalytic domains or called kinase domain).

Both lobes are connected by a hinge region. The catalytic domain is located between both terminal lobes and shows high conservation and similarity to the PKC family kinase domain [21, 22]. Moreover, PRK1 and PKC are members of AGC kinase family [23]. The characteristic feature of these kinases is a C-terminal regulatory region (C-tail). The C-tails regulate the enzymatic activity and insert conserved phenylalanine residues into the ATP-binding site [23–25].

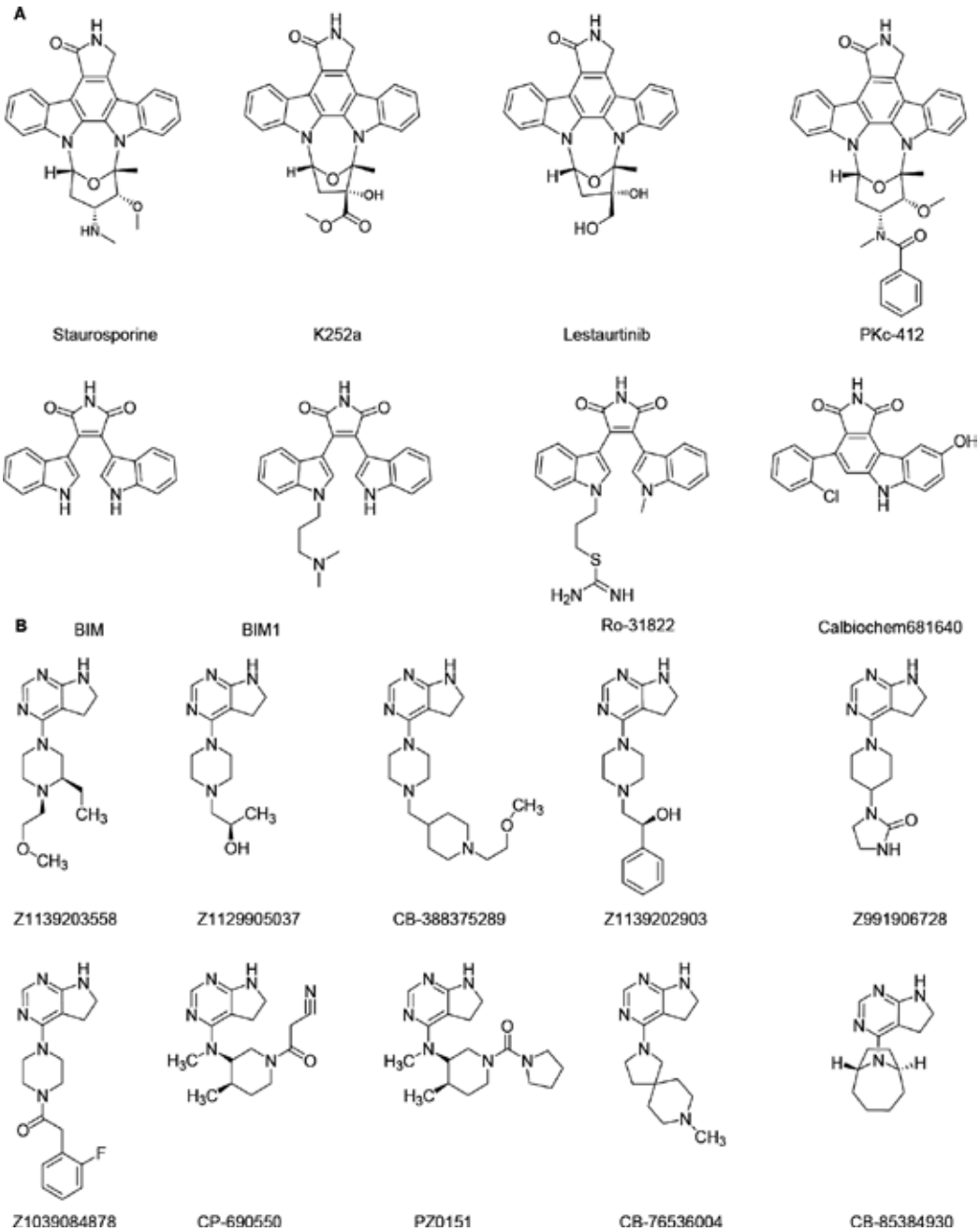

Figure 1. Chemical structures of PRK1 active compounds: (A) staurosporine and derivatives, (B) tofacitinib and other pyrrolopyrimidine derivatives [18].

1.5. Computational approaches in drug discovery

Virtual screening (VS) was first used at the end of the last century to refer to computational algorithms and techniques used to identify novel hit/lead compounds for biological target

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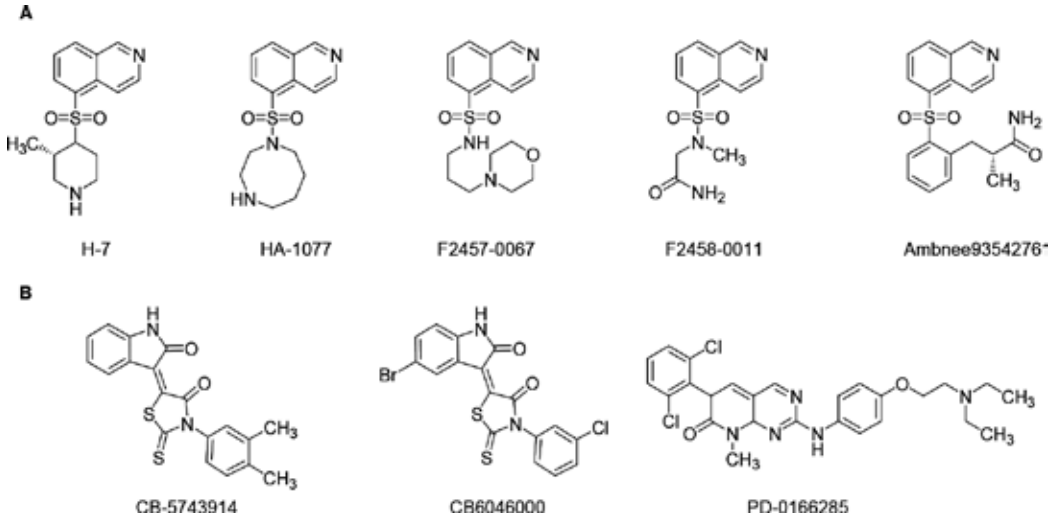

Figure 2. Chemical structures of more PRK1 active compounds. (A) Isoquinoline derivatives, (B) PD-0166285 and structurally diverse compounds identified by VS [18].

from large chemical libraries, depending on the known structure or the drug target or active ligands [26–29]. The VS approach is considered as a complementary approach to experimental or physical screening or high-throughput screening (HTS). VS has been fully integrated to most modern day drug discovery projects [30]. However, VS does not require the physical existence of the compounds to be tested. It is only based on computer models or the three-dimensional (3D) structures of the drug target, also known as structure-based VS (SBVS) or on the chemical structure of known active ligand(s), also known as ligand-based VS (LBVS) [31]. The fundamentals of the SBVS approach depend on the availability of the 3D structure of the biological target and a database of small molecules. The SBVS approach often uses molecular docking to generate the protein-ligand complexes of small molecules from a database into the target active site. The aim is to identify the compounds, which interact favorably with the target binding site [32]. Meanwhile, LBVS methods utilize chemical similarity analysis of structurally diverse or known active ligands, with the view of identifying novel small molecules, which could show similar biological activities [33–35]. However, both approaches have practical limitations. Therefore, researchers often combine SBVS and LBVS, with the aim that this might improve the efficiency of the VS results [36, 37]. Computational approaches for the prediction of biological activity also include quantitative structure-activity relationships (QSAR) analysis, which is aimed at finding a correlation between predicted and experimental biological activities of small molecules. This approach incorporates the influence of the molecular descriptors. QSAR models can be used initially to predict the activity of untested hits from a virtual screening campaign [38]. On other hand, QSAR modeling is useful tool during lead optimization, which is aimed at the improvement of the biological activities of the identified hits [39]. Recently, many crystal structures of the PRK1 drug target have been published [24]. Due to the flexibility of the biding site of the PRK1 structure, which adapts to different conformations, each protein-ligand complex was individually analyzed to reveal the important intermolecular interactions responsible for ligand binding that are useful for the

identification new hits. The aim of this research project is to analyze ligand binding to PRK1 and to identify novel-specific inhibitors that could block the activity of PRK1, using structure-based VS. In a preliminary study, the applied docking methods were validated and used to investigate its ability to predict the experimental conformations of the co-crystallized ligands. Second, several QSAR models were generated in order to find the significant correlations between the computational and experimental biological activity data. The constructed QSAR models contain different scoring functions, including computed binding-free energy (BFE) values for the protein-ligand complexes, in addition to molecular descriptors of the isolated ligands. To investigate the predictive ability of the generated QSAR models, internal and external methods were performed. Moreover, an enrichment study was performed, in order to assess the ability of the scoring functions to identify known binders or actives in large databases containing actives and inactives. By focusing on kinase data sets, GSK data sets 1 and 2 were screened to search for novel lead compounds, employing virtual screening methods. A NP library including compounds isolated from African flora was also screened. This chapter begins by presenting database tools (small molecule libraries) useful in VS campaigns, with a focus on small molecule libraries and NPs with anticancer properties. This is followed by some recent success stories on VS for the identification of inhibitors and/or modulators of some anticancer drug targets. The end of the chapter shows the case study of VS for the identification of PRK1 inhibitors.

2. Databases of small molecule libraries and some recent success stories in anticancer drug discovery using computer-based methods

2.1. Small molecule databases for virtual screening

A summary of small molecule libraries utilizable in virtual screening experiments has been provided in **Table 1**. Known cancer drugs have been included in several databases, including ChEBI [43], ChEMBL [44], DrugBank [46], EpiDBase [48], NANPDB [51], NCI-DIS, NCI-DTP, and NCI-FDA [52], along with SANCDB [54], SuperDrug [55], p-ANAPL [58], PubChem [59], and ZINC15 [60]. However, those with tested and proven *in vitro* activities against known cancer cell lines and/or with known *in vivo* activities only include CancerDR [41], OAA [53], and SYFPEITHI [56], along with the NP libraries: AfroCancer [40], CancerHSP [42], CHMIS-C [43], InPACdb [49], MAPS [50], NPACT [7], and TIPdb [57].

2.2. Some recent success stories

Computational modeling has been applied to understand drug-target interactions in several validated anticancer drug targets, for the identification of novel inhibitors from small molecule databases and/or for the elucidation of modes of action. We here present some few recent cases. A typical example is the recent discovery of new inhibitors of CXC chemokine receptor 2 (CXCR2) by applying ligand-based pharmacophore models [61]. It should be mentioned that CXCR2 and its ligand, CXCL8, are known to be implicated in a number of inflammation-mediated diseases, including cancer [62–65]. In the study, Ha et al. generated a pharmacophore

Database	Description	Web accessibility	Reference
AfroCancer	A data set of natural anticancer products from African flora	http://www.african-compounds.org/about/afrocancer/	[40]
Cancer drug resistance database (CancerDR)	A database of 148 anticancer drugs and their effectiveness against around 1000 cancer cell lines	http://crdd.osdd.net/raghava/cancerdr/#thumb	[41]
CancerHSP	An anticancer herbs database of systems pharmacology	http://lsp.nwsuaf.edu.cn/CancerHSP.php/	[42]
ChEBI	A database for chemical entities of biological interest	http://www.ebi.ac.uk/chebi/	[43]
ChEMBL	An open large-scale bioactivity compound database	https://www.ebi.ac.uk/chembl/	[44]
CHMIS-C	A herbal medicines database for cancer	http://sw16.im.med.umich.edu/chmis-c/	[45]
DrugBank	A resource that combines detailed drug data with comprehensive drug target information	https://www.drugbank.ca/	[46]
DUDE datasets	Benchmarking data sets of actives and decoys for diverse targets, including proteins, GPCRs and ion channels, clustered ligands, etc. drawn from ChEMBL	http://dude.docking.org/	[47]
EpiDBase	A database for small molecule epigenetic modulators	http://www.epidbase.org/	[48]
InPACdb	Indian plant anticancer compounds database	http://www.inpacdb.org/	[49]
MAPS Database	A database of phytochemicals, including the data of >500 medicinal plants	http://www.mapsdatabase.com/	[50]
NANPDB	A natural products database for compounds of Northern African origin, with a significant number of bioactive metabolites exhibiting anticancer activity	http://www.african-compounds.org/nanpdb/	[51]
NCI Drug Information System (DIS)	A searchable database of 3D structures (mainly organic compounds) from the NCI Drug Information System (DIS)	https://cactus.nci.nih.gov/ncidb2.2/ http://dtp.nci.nih.gov/docs/3d_database/dis3d.html	[52]
NCI-DTP database	Tested compounds from the National Cancer Institute (NCI) Developmental Therapeutics Program (DTP)	https://dtp.cancer.gov/databases_tools/data_search.htm	[52]
NCI-FDA	Several sets of FDA-approved anticancer drugs to enable cancer research	https://wiki.nci.nih.gov/display/NCIDTPdata/Compound+Sets	[52]
NPACT	A database for plant-based anticancer compounds	http://crdd.osdd.net/raghava/npact/	[7]
Oral anticancer agents (OAA) from Singapore	A database of 39,772 oral anticancer agents prescribed to 8837 patients in Singapore, with 55 clinically significant drug-drug interactions for the evaluation of drug interaction facts	https://www.ncbi.nlm.nih.gov/pubmed/22795926/	[53]

Database	Description	Web accessibility	Reference
SANCDB	A database of natural products from South Africa, containing also anticancer agents from the flora and fauna of this ecologically diverse country	https://sancdb.rubi.ru.ac.za/	[54]
SuperDrug	A database of 3D-structures of active ingredients of essential marketed drugs	http://bioinf.charite.de/superdrug/	[55]
SYFPEITHI	A database of major histocompatibility complex (MHC) ligands and peptide motifs	http://www.syfpeithi.de/	[56]
TIPdb	A database of anticancer, antiplatelet, and antituberculosis phytochemicals from indigenous plants in Taiwan	http://cwtung.kmu.edu.tw/tipdb/	[57]
p-ANAPL	A collection of samples of natural products from African sources	http://www.african-compounds.org/about/p-anapl/	[58]
PubChem	A public repository for information on chemical substances and their biological activities	https://pubchem.ncbi.nlm.nih.gov/	[59]
ZINC15	A free database of over 100 million purchasable compounds for virtual screening	http://zinc15.docking.org/	[60]

Table 1. Summary of some small molecule libraries currently available for anticancer drug discovery.

model based on known CXCR2 antagonists and used it to screen a database of 5 million commercially available compounds from different vendors. The authors were able to identify small molecule hits, which were further tested by *in vitro* screening in a cell-based CXCR2-mediated β-arrestin-2 recruitment assay, followed by several other cell-based assays. *In vivo* studies were conducted by lipopolysaccharide (LPS)-induced lung inflammation in mice. It was also shown that one of the compounds inhibits CXCR2 signaling through down regulation of surface CXCR2.

Moreover, the same compound was shown to inhibit CXCL8-mediated neutrophil migration and LPS-induced lung inflammation in mice significantly. The identified compounds were shown to also inhibit CXCR2/β-arrestin-2 association, cell migration and proliferation, and acute inflammation in mouse models. Another study combined structure-based docking and pharmacophores to design novel indole and chromene analogues, which were cyclin-dependent kinase 2 (CDK2) inhibitors. The identified compounds proved to be active against MCF-7 and HeLa cell lines. The study was conducted by exploiting stereo-specific information obtained from crystal structures of CDK2, substituting the pharmacophores on their moiety and docking the target protein to calculate the binding affinities [66]. Other recent successful cases, where docking, QSAR, machine learning, and pharmacophore-based screening were combined to search for small molecules targeting cancer, are abundant in the literature [67–70].

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3. Case study: structure-based virtual screening and QSAR studies on PRK1 inhibitors

3.1. Structure-based design studies (X-ray target structures and cross docking)

Four crystal structures of PRK1 are available in the protein data bank (PDB), **Table 2**. The PRK1 binding site is reported to exhibit either intrinsic or induced flexibility [17, 24]. Moreover, different bound inhibitors are known to induce different conformational changes in the binding site residues [71]. Furthermore, chemical characteristics of the co-crystallized ligands play an important role in the applicability of the X-ray structure for virtual screening. Therefore, a cross-docking procedure was carried out, as a retrospective study aimed at determining the appropriate structure for the virtual screening. The selected structure should be able to bind the nonnative ligands with low root mean square deviation (RMSD), with respect to the reference binding conformation. On the other hand, the performance of the docking power and scoring functions is varying for different targets [32]. The docking power measures the ability of docking algorithms to predict the correct conformation of the ligands [72].

The four X-ray structures of PRK1, including the apo-form, were used for a cross-docking study [24]. The co-crystallized ligands were docked toward the target binding sites, using the Glide cross-docking script, implemented in Schrödinger Suite (2014 version) with standard precision (SP) for flexible ligand docking. In order to optimize the docking solution, an option was selected to perform post-docking minimization and includes number of poses up to 5 per ligand. The structures were prepared using the default setting implemented in Protein Preparation Wizard (Schrödinger 2014). The co-crystallized water molecules were deleted. Hydrogen atoms and partial charges were assigned. Finally, the structure energy minimized applying the OPLS 2005 force field. The binding site was defined using Grid Generation of Schrödinger suite 2014 and sets the co-crystallized ligand as a center of the binding pocket. In the case of apo-structure 4OTD, the binding site was defined by applying centroid of selected residues Leu650 and Lys753 with box size 14 Å. A hydrogen bond constraint was defined at PRK1 hinge region reside Ser704. In addition, all ligands were prepared using LigPrep implemented in Schrödinger utility 2014 involving generation of ionization and tautomeric states at pH 7.4 with less than 10 low-energy ring conformations. The ligands were energy minimized using MMFFs force field.

Ligand	4OTD		4OTG		4OTH		4OTI	
	RMSD	SP	RMSD	SP	RMSD	SP	RMSD	SP
Lestaurtinib-4OTG	6.56	−4.29	0.24	−11.67	4.65	−7.82	7.56	−4.29
Ro-318220-4OTH	6.79	−7.01	2.98	−11.4	0.64	−11.76	6.47	−6.55
Tofacitinib-4OTI	3.14	−4.54	6.11	−8.09	2.7	−8.18	0.55	−8.8
Average RMSD	5.5		3.11		2.67		4.86	

Table 2. RMSD values (Å) for the top-ranked docking solutions (using Glide SP as scoring function).

The RMSD values were calculated, with respect to the respective reference ligand conformations. **Table 2** shows the RMSD values and Glide SP scores for the top-ranked poses in the corresponding structures. It was observed that none of the available structures had a binding site conformation, which allowed all the three different ligands to be docked with RMSD < 2.0 Å. Furthermore, the calculated average RMSD values for each structure were high. However, the applied docking method performed well by reproducing the binding mode of the co-crystallized inhibitor in self-docking. Moreover, the docking method correctly scored the co-crystallized inhibitors at the top of the list (**Table 2**), e.g., the X-ray structure of the ligand lestaurtinib was re-docked with an RMSD = 0.24 Å into its co-crystallized structure (PDB: 4OTG), being also ranked as the top scoring pose (Glide SP = −11.67 kcal/mol). Similar results were observed with the other inhibitors when X-ray structures were docked toward their respective co-crystallized target structures. Meanwhile, the binding pocket conformation for the apo-form of PRK1 was not found to be suitable for docking when using any of the current inhibitor structures. RMSD values were high and the docking scores were low when compared with those obtained in the rest PRK1 structures (**Table 2**). Therefore, an ensemble of PRK1 structures was proposed for the further virtual screening study.

3.2. Docking of active inhibitors

The data set DS1 includes 28 active (**Figures 1** and **2** [18]) and 300 inactive compounds. First, the active compounds were docked toward the ensemble of the three PRK1 structures using the previously described docking methods. The docking solutions were first inspected, with the aim of comparing them with the experimental conformations (the co-crystallized ligands in other kinases). The active compounds could be divided into four subsets, the first set being staurosporine derivatives (10 actives, including the co-crystallized inhibitors lestaurtinib and Ro-318220). The second subset is made of the tofacitinib or pyrrolopyrimidine family (nine actives, in addition to the inhibitor tofacitinib in PDB ID: 4OTI). The third set was made of two isoquinoline derivatives, forming a group of five actives. The last subset contains the remaining inhibitors, with diverse scaffolds. The binding mode for each compound was analyzed and compared with the experimental data. As previously seen in the cross-docking for the co-crystallized inhibitors (**Table 2**), the binding modes for lestaurtinib and Ro-318220 were correctly reproduced. The binding modes for the further staurosporine derivatives were compared with these two analogues. Since the ensemble docking procedure was applied to dock the actives, eight compounds were docked toward the 4OTG structure target site, while the others (Ro-318220 and BIM1) were docked toward the 4OTH site. It was interesting to notice that the top-ranked active poses had a quite conserved binding mode, which shares two H bonds, interacting with the hinge region of PRK1. The obtained binding modes for all staurosporine derivatives were identical to the published structure (in the PRK1 X-ray structure 4OTG). Since a part of the C-terminal regulation region (C-tail) is not resolved, resulting in a more open and accessible ATP binding pocket, most of the staurosporine derivatives could be docked into it. Furthermore, all staurosporine derivatives were docked into both PRK1 structures, which are co-crystallized with one of staurosporine derivatives (4OTG or 4OTH). For pyrrolopyrimidine and tofacitinib derivatives, the inhibitors were docked into three X-ray structures of PRK1. Tofacitinib was co-crystallized in the structure 4OTI. The RMSD value of re-docking tofacitinib was 0.55 Å. The binding modes

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for the other analogues were compared with the experimental data (4OTI). Additionally, several other analogues were co-crystallized with the Akt1 kinase (PDB IDs: 3MV5, 3MVJ, and 3OCB).

Each active compound forms 2 H bonds with the hinge region, besides additional H bonds with the surrounding residues in the binding pocket. There are also some isoquinoline derivatives that were co-crystallized with other kinases, e.g., cAMP-dependent kinase (PDB IDs: 1YDS and 1YDR) and Rho kinase (PDB IDs: 2GNI). This subset of actives interacts with the PRK1 hinge region by mediating 1 H bond. A former virtual screening campaign had identified several actives, including CB-6046000 and CB-5743914, which were docked using the same previously described docking method. The binding mode showed two H bonds between the dihydroindol-2-one and the backbone of the hinge region, mainly Ser704 and Glu702. Furthermore, the binding mode of PD-0166285 matched the experimental data observed for the LCK kinase (PDB ID: 3KMM).

3.3. Scoring power

The rescoring for the derived docking poses was performed by calculating BFEs and using different solvation models implemented in the AMBER12 package. IC_{50} values had been previously determined for 26 of the active compounds (**Table 3**), with only percentage inhibitions available for two others) [19]. Thus, the correlation coefficient (R^2) between the experimental pIC_{50} and the calculated enthalpy changes (ΔH) for protein-ligand binding was calculated (**Table 3** and **Figure 3**). The BFE calculation was carried out using one snapshot after two consecutive minimizations steps and applying the Generalized Born solvation model [73, 74]. First, the minimization was carried out for water and counterion molecules without the ligand-protein complex, which was restrained to their initial coordinates with a force constant of 500 kcal/mol/Å^2. In this step, there were 2000 iterations (beginning with 1000 steepest descent and followed by 1000 conjugate gradient). The second minimization step was applied for the whole system through 10,000 iterations (first 5000 steepest descent and then 5000 conjugate gradients). A significant correlation was found when using the Nguyen and Simmerling (igb = 8) version of the Generalized Born solvation model [73, 74] and applying the two minimizations steps. The cross-validated R^2 was found to be 0.60, with a root mean square error (RMSE) of 0.89. To understand the effect of the chemical modification on the main scaffold, the binding mode of the actives and the interactions in the binding pocket was analyzed. As mentioned previously, PRK1 actives can be divided into four subsets (**Table 3**). The docking score (Glide SP) and the MM/GBSA BFE values were employed to explore the differences in the inhibitory activity. However, there was a weak correlation between the pIC_{50} and Glide SP scores (R^2 of 0.43, see **Table 4**).

It was observed, from docking, that the isoquinoline derivatives are able to form an H bond between the N atom of the isoquinoline motif and the NH backbone of Ser704 located in the hinge region. The isoquinoline motif occupied the adenine binding site; meanwhile, the substituents were located in the sugar-binding site, which is surrounded by several hydrophilic residues (e.g., Asp708, Asp750). Rescoring, using MMGBSA, showed that H-7 (IC_{50} = 658 μM and ΔH = −34.51 kcal/mol) had a higher score than HA-1077 (IC_{50} = 1.95 μM and ΔH= −29.42 kcal/mol), **Table 3**. The more favorable value resulted from the additional

Compound	IC_{50} (nM)	Exp. pIC_{50}	Target structure	Glide SP	MM/GBSA**	PEOE_PC-	Pred. pIC_{50}*
Ro-318220	78.3	7.11	4OTH	−11.36	−45.97	−2.71	7.16
BIM1	579	6.24	4OTH	−9.82	−46.10	−2.29	6.58
PKC-412	14.2	7.85	4OTG	−10.98	−55.52	−3.26	8.26
Lestaurtinib	8.6	8.07	4OTG	−11.43	−46.92	−2.77	7.30
K252a	3.2	8.49	4OTG	−11.38	−51.40	−2.95	7.80
Staurosporine	0.81	9.10	4OTG	−11.83	−60.86	−2.72	8.59
Calbiochem681640	1441	5.84	4OTG	−10.61	−47.90	−1.96	6.71
BIM	154.2	6.81	4OTG	−9.16	−38.70	−1.96	5.58
K252c			4OTG	−10.53	−40.12		
Quercetin			4OTG	−8.43	−48.23		
CP-690550 **(tofacitinib)**	129	6.89	4OTI	−8.81	−38.88	−2.01	5.55
Z1139203558	55730	4.25	4OTG	−8.52	−27.91	−1.84	4.41
Z1129905037	53380	4.27	4OTI	−9.61	−35.25	−1.82	5.29
CB-38374289	42560	4.37	4OTI	−10.49	−33.36	−2.11	5.49
CB-85384930	34020	4.47	4OTH	−9.04	−35.48	−1.48	4.98
Z1139202903	31470	4.50	4OTI	−9.37	−37.77	−2.09	5.62
Z991906728	26400	4.58	4OTG	−9.18	−28.92	−2.02	4.75
CB-76536004	13090	4.88	4OTG	−9.43	−33.02	−1.45	4.83
Z1039084878	6210	5.21	4OTG	−8.76	−29.13	−2.10	4.73
PZ0151	1060	5.97	4OTI	−8.54	−43.19	−2.16	5.97
H-7	658.5	6.18	4OTH	−8.68	−34.51	−1.43	4.78
HA-1077	1945	5.71	4OTI	−8.71	−29.42	−1.39	4.31
F2457-0067	70300	4.15	4OTH	−8.49	−36.31	−1.76	5.10
F2458-0011	53730	4.27	4OTI	−8.07	−29.18	−1.70	4.34
Ambnee93542761	29330	4.53	4OTH	−9.12	−30.33	−1.68	4.66
CB-6046000	5350	5.27	4OTG	−7.39	−47.01	−1.57	5.70
CB-5743914	2940	5.53	4OTG	−9.89	−41.07	−1.72	5.81
PD-0166285	5517	5.26	4OTG	−7.48	−39.17	−2.41	5.53

Comparison of experimental pIC50 versus predicted pIC50 values calculated by QSAR_3 for the 26 training set molecules [18].

The BFE values were calculated according to the docked structure (target structure), which are supplied with the PDB ID.

Table 3. Biological activity, calculated BFE and docking scores of the active compounds.

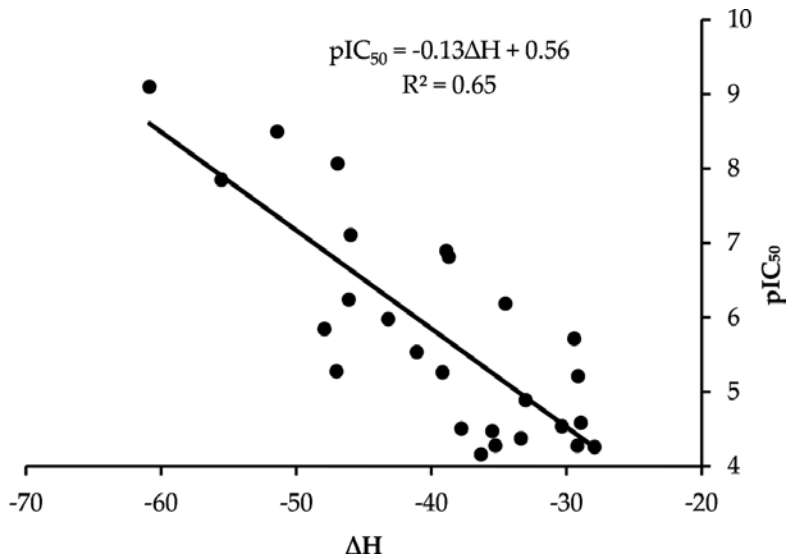

Figure 3. Correlation curve of pIC50 versus Δ*H* scores of actives using MM/GBSA.

hydrophobic interactions between the front hydrophobic pocket residues and Phe910 (from the C-tail) and the methyl substituent on the piperazine ring. Meanwhile, the van der Waals interaction became weaker in the case of HA-1077. This could be explained by the shifting of the position of the substituent, since its seven-membered ring is bulkier. Both derivatives contain a positively charged ring nitrogen, which consequently forms an additional H bond in the ATP-sugar binding pocket (**Figure 4**). Meanwhile, the interaction between Asp708 and the piperazine ring enables the methyl group to get closer to Phe910 and its surrounding residues. Moreover, the sulfonyl moiety of both compounds is located under the P-loop, where it forms hydrophilic and hydrophobic interactions.

	GLIDE		***ΔH***
	SP	**XP**	**GB8**
R^2	0.43	0.27	**0.65**
EF1%	**100**	**100**	25
EF3%	**30.3**	**33.33**	9.1
AUC*	**0.90**	**0.88**	0.66

*AUC = area under the ROC curve.

Table 4. Enrichment study results using an ensemble of PRK1 structures, where the available scoring functions in Schrödinger and BFE methods were considered. Where **SP**: Standard Precision; **XP**: Extra Precision and **ΔH**: enthalpy score; GB8: Generalized Born theory model.

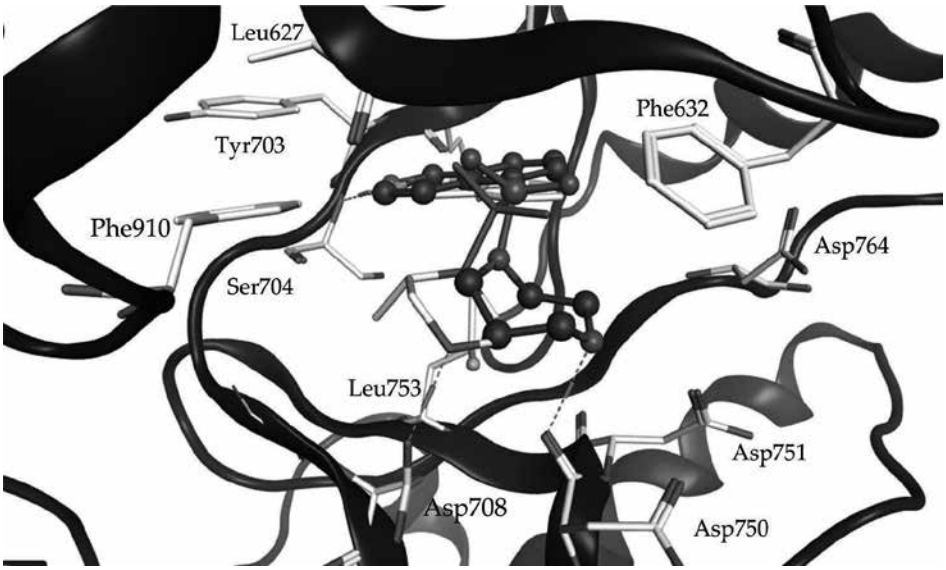

Figure 4. Comparison between the docking poses of two isoquinoline derivatives H-7 (magenta) and HA-1077 shown as green sticks (PRK1 PDB code: 4OTH). The figure was designed using MOE.

The remaining compounds share common interactions (as previously mentioned), but the biological activities of these compounds could not be predicted using the derived BFE model. However, the lower activity of compound ambnee93542761 could be explained by the loss of the interaction between of the sulfonyl moiety and weak hydrophobic interactions with Phe910. Nonetheless, it forms a hydrogen bond with the catalytic Asp764 (IC_{50} = 29.33 μM and ΔH = −30.32 kcal/mol). The results of this subset show the importance of the hydrophobic interactions with the Phe910 of the C-tail and its surrounding residues. However, the BFE values and docking scores failed to predict the affinity of F2457-0067, which shows a favorable BFE value but a low IC_{50} value (**Table 3**). This could probably result from the long linker between the isoquinoline motif and the positively charged morpholine ring. The generated conformations show additional van der Waals interactions. On the other hand, the morpholine ring shows more polar properties compared to the other compounds.

The second subset of actives is made of staurosporine derivatives. Most of these compounds form two H bonds between the pyrrole ring and the hinge backbone (NH of Ser704 and CO of Glu702). The docking results for staurosporine derivatives were compared with two PRK1 crystal structures (4OTG and 4OTH). The favorable BFE values of staurosporine derivatives could be attributed to the strong hydrophobic interactions with residues from the P-loop, e.g., Val635 and Leu627, in addition to the interactions with Leu753. The only observed deviation was with parts of the docked ligand interacting with the sugar pocket, e.g., staurosporine (the most active compound) showed a BFE value of ΔH = −60.85 kcal/mol and an IC_{50} = 0.0008 μM (**Table 3**). The favorable BFE value and activity of staurosporine could be estimated through the targeting of both hydrophobic pocket residues (behind the gatekeeper Met701 and near Phe910); on the other hand, several polar groups occupied the sugar polar

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pocket. By comparison with lestaurtinib (IC_{50} = 0.0086 μM and ΔH= −46.92 kcal/mol), the differences in the activity and BFE values could be attributed to the smaller ring/shorter linker in lestaurtinib for targeting Asp708 and Asp750 (**Figure 5**).

The third subset of PRK1 inhibitors is made of tofacitinib analogues. Ten compounds belong to this subset (**Table 3**). All derivatives interact with the hinge region by forming two H bonds through the pyrrolopyrimidine scaffold (**Figure 6**). The major substituents, which influence notably the BFE values and the biological activity of tofacitinib, are the N-methyl of the pyridine ring. Any chemical modification changing these interactions will consequently change the BFE value and the activity. A comparison of the calculated BFE values of tofacitinib with those of the other derivatives could clarify the effects of these interactions. As previously mentioned, the loss of the interactions made with the P-loop residues clearly influences the calculated BFE values and, therefore, affects the biological activities. Consequently, Z1129905037 (IC_{50} = 55.73 μM and ΔH = −27.92 kcal/mol) possesses a BFE value lower than tofacitinib (IC_{50} = 0.129 μM and ΔH = −38.88 kcal/mol) (**Table 3**). Furthermore, contrary to tofacitinib, the binding mode of Z1129905037 does not show hydrophobic interactions with the residue located at the bottom of the binding pocket. The ATP-sugar binding site is occupied by non-polar substituents in the case of Z1129905037, which is unfavorable and consequently reduces the activity. The other tofacitinib analogues, which do not contain any tertiary amine as the linker, show higher BFE values and obviously lower biological activities. One exception is PZ0151 (IC_{50} = 1.06 μM and ΔH= −43.19 kcal/mol) which displays the same binding mode with tofacitinib. The pyrrolidine

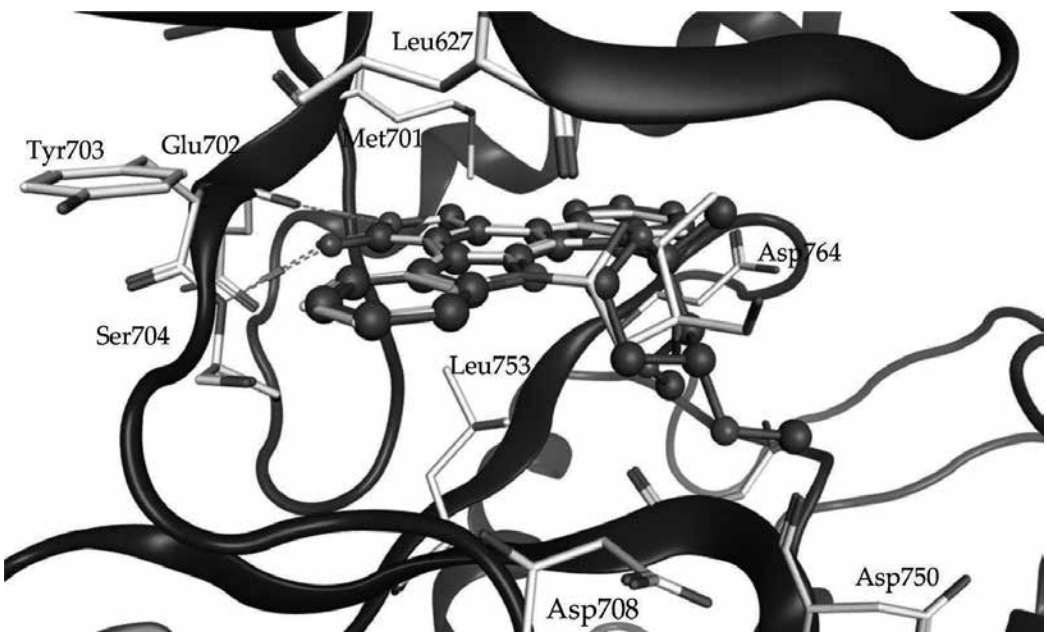

Figure 5. Comparison between the docking pose of staurosporine (seen in cyan sticks) and the binding mode of lestaurtinib (shown in white brown sticks) at PRK1 structure 4OTG. The figure was designed using MOE.

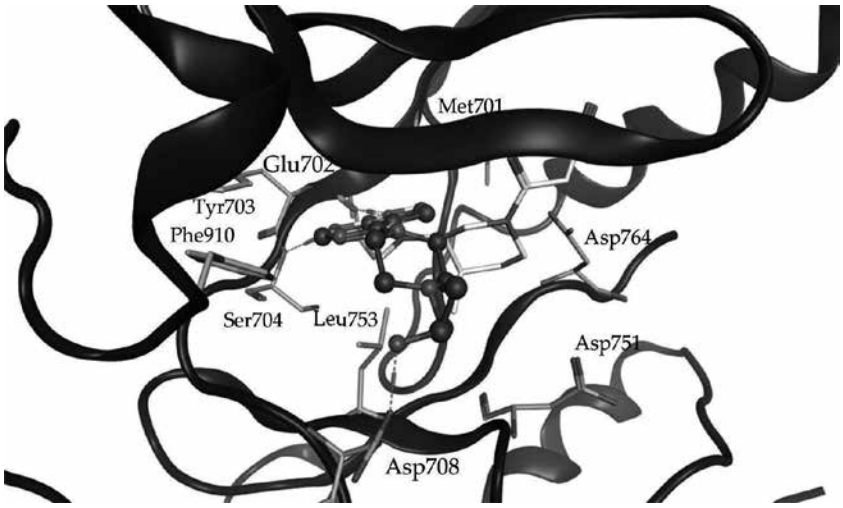

Figure 6. Comparison of the docking pose of Z1129905037 (shown as cyan sticks) and the binding mode of tofacitinib seen in magenta, at the binding site of PRK1 structure 4OTI. The figure was designed using MOE.

ring in PZ0151 takes the position of the CN group of tofacitinib but rather shows a lower activity. This could be as a result of the small size of the binding site under the P-loop residues, which is not suitable for large substituents. In general, it is difficult to explain the changes of the BFE values caused by chemical modification of all compounds under study. The observed correlation coefficient ($R^2 = 0.65$) could only be used to explain major differences in biological activities. However, it could be used as an indicator to identify the interactions, which play major roles in the determination of biological activity.

3.4. Ranking power

An enrichment study was performed in order to measure the ability to identify known binders or actives in large databases of inactives. The previously mentioned data set (named DS1), containing 28 actives and 300 inactives, was used to perform the enrichment study. The ligands were docked using the same ensemble and rescored depending on the previous findings. The results are presented in a BOX-PLOT and receiver operating characteristic (ROC) curves (**Figures 7** and **8**). **Table 4** shows enrichment study results, using an ensemble of PRK1 structures, where the available scoring functions in Schrödinger and BFE methods were considered (ΔH, Glide SP and Glide XP). As seen in **Figure 7**, Glide SP was able to discriminate between actives and inactives better than the BFE scoring (ΔH).

The median values of the pair active/inactive were −9.17/7.23 and −38.79/34.02 for Glide SP and ΔH, respectively. The area under the curve (AUC) and enrichment factors (EF) at two different percentages were calculated. **Table 4** shows the enrichment study results. It is interesting to note that at EF1%, all screened hits were actives when using both Schrödinger's scoring functions (Glide SP and XP). Interesting, it was also observed that for the EF3% (for Glide SP and XP), the rate of true positives was 100% (**Table 4**). The main factor to measure the ranking

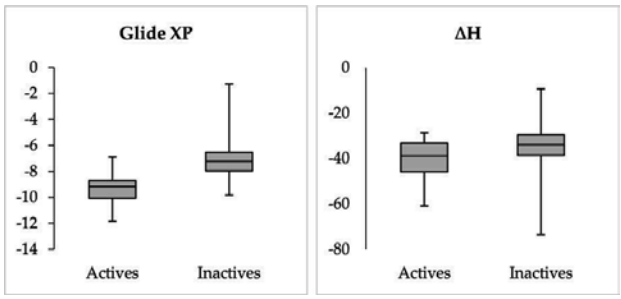

Figure 7. Box plots for the active and inactive compounds using the respective score values.

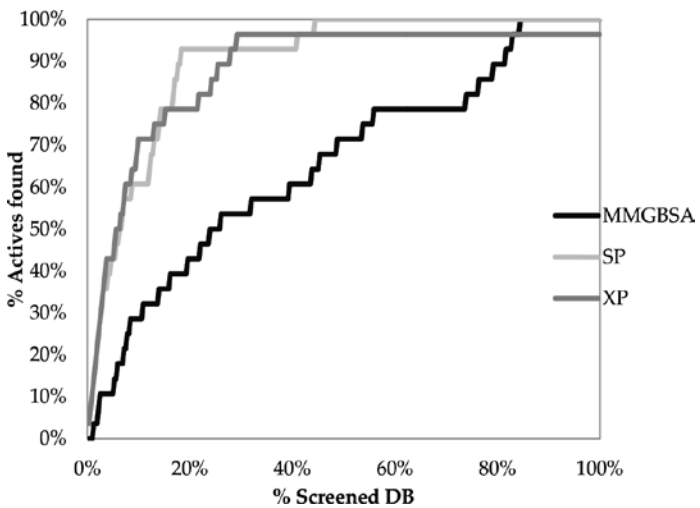

Figure 8. Enrichment plot showing comparison of the % actives found at a given percentage of the ranked database for DS1, using GLIDE SP, XP, and Δ*H* (MMGBSA).

power of the applied docking methods was the AUC value (**Table 4**). It is clear that the Glide scoring functions performed better to discriminate between the actives of inactives, having AUC = 0.90 and 0.88 for SP and XP, respectively.

3.5. QSAR model generation

Several QSAR models were generated to identify a possible correlation between experimentally obtained pIC_{50} values and calculated BFE or descriptor values (for the compounds on **Figures 1** and **2**). Calculated BFE (obtained by the MM/GBSA approach) from the previous study and 2D molecular descriptors, e.g., different descriptors referring to the partial charge and number of rotatable bonds, were considered. Since the Glide SP score showed a significant discrimination power between the active and inactive compounds, further scoring functions were also tested to optimize the QSAR models.

The best correlation coefficient in the previous study was found by applying Nguyen and Simmerling (igb = 8) Generalized Born solvation model [73, 74], after a minimization step (R^2 = 0.65, RMSE = 0.89 and cross-validated q^2 = 0.60, n = 26). In order to improve the correlation, partial charges of the ligands were calculated to compute several molecular descriptors for generating PLS models [75]. The inclusion of the PEOE_VSA-0 descriptor improved the correlation coefficient value only slightly (model QSAR_, R^2 = 0.68, RMSE = 0.8, and q^2 = 0.61, n = 26). This descriptor indicates the partial equalization of electronegativities with approximated accessible van der Waals surface area (in Å^2) of the molecule [76]. Furthermore, the inclusion of another descriptor based on ligand partial charges (PEOE_PC-) [76], which indicates to the total negative partial charge, was used instead of PEOE_VSA-0 to generate QSAR_2 (R^2 = 0.70, RMSE = 0.78 and q^2 = 0.62, n = 26; Eq. (1)). The model QSAR_3, containing three descriptors, showed a slightly better correlation coefficient (R^2 = 0.71, RMSE = 0.76 and q^2 = 0.63, n = 26; Eq. (2)). The relative importance of the individual descriptors was MM-GBSA: 1, GLIDE_SP: 0.417, and PEOE_PC-: 0.371. The other tested descriptors were not helpful and were not considered for the final models.

Further internal validation of the generated QSAR models was carried out by means of bootstrapping, in which the samples are randomly selected from the used inhibitors to form training and test sets [77, 78]. The respective models are generated, and their statistical parameters (R^2 and q^2) are calculated and compared with those of the original QSAR models (which had been generated from the whole data set). The random selection of the samples was performed within each cluster generated by hierarchical clustering, depending on the structural similarity or within activity distribution clusters of PRK1 inhibitors (low, moderate, and high active inhibitors) [77]. PRK1 inhibitors can be divided into four subsets depending on the chemical scaffolds (**Figures 1** and **2**). In the next step, hierarchical clustering using maximum common substructure (MACCS) keys and calculation of Tanimoto coefficients were carried out using the molecular operator environment (MOE) software tool (chemical computing group, Montreal, Canada, version 2014). Then, several samples within each cluster were taken, considering their activities. From 26 compounds, the sample subset contains only 20 inhibitors. The statistic parameters of the obtained QSAR models are presented in **Table 5**. The goal of the bootstrapping is to perturb the training set while not considering the statistical parameters of the test set. Since the obtained QSAR models were built from subset of the total data set, the values of R^2_{BT} and q^2_{BT} satisfy the minimum acceptable statistical parameters when

	Training set (20 inhibitors)			**Test set (6 inhibitors)**		
	R^2	**RMSE**	q^2	R^2_{BT}	$\mathbf{RMSE}_{\mathbf{pred}}$	q^2_{pred}
QSAR_BT_1	0.71	0.73	0.65	0.43	1.11	0.06
QSAR_BT_2	0.72	0.72	0.63	0.58	0.95	0.19
QSAR_BT_3	0.72	0.71	0.60	0.67	0.84	0.28

Table 5. Statistical parameters of obtained QSAR models in bootstrap validation.

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compared with the three original QSAR models. These values also revolve around the values of the original QSAR models, generated from 26 PRK1 inhibitors (**Table 5**). It was observed that the obtained QSAR models possess acceptable statistic parameters ($q^2 > 0.5$) and were comparable with those found in the original QSAR models. However, the QSAR_BT_3 model showed poor correlation when compared with the results found in the whole data set (q^2 = 0.60, **Table 5**). The remaining six inhibitors were used as the external test set to verify the ability of the obtained QSAR models (QSAR_BTs) to predict the biological activities of the test set compounds. The values of R^2_{pred} between the calculated and measured pIC_{50} are 0.43, 0.58, and 0.67 for QSAR_BT_1, QSAR_BT_2, and QSAR_BT_3, respectively (**Table 5**). These results may confirm the ability of the model QSAR_3 and its derivatives (QSAR_BT_3) to predict the biological activities of novel compounds.

Interestingly, QSAR_3 have predicted pIC_{50} values higher or close to 7 for the most potent PRK1 inhibitors (IC_{50} < 100 nM). Moreover, QSAR_3 possessed the ability to distinguish between weak, moderate, and highly potent compounds (**Table 3**). Thus, the model could be used as a filter for the prioritization of specific compounds (hits) for synthesis and testing. **Figure 9** displays the graph of the correlation between the observed and predicted pIC_{50} values using QSAR_3 model.

3.6. Validation of the generated QSAR models and GSK databases screening

It has previously been demonstrated that considering only the statistic parameters (R^2, RMSE, and q^2) to validate QSAR models is insufficient and sometimes misleading [78–82]. Therefore, it is important to validate QSAR models by testing the ability to accurately predict the biological activities of ligands not used for QSAR model generation. In the current study, two data

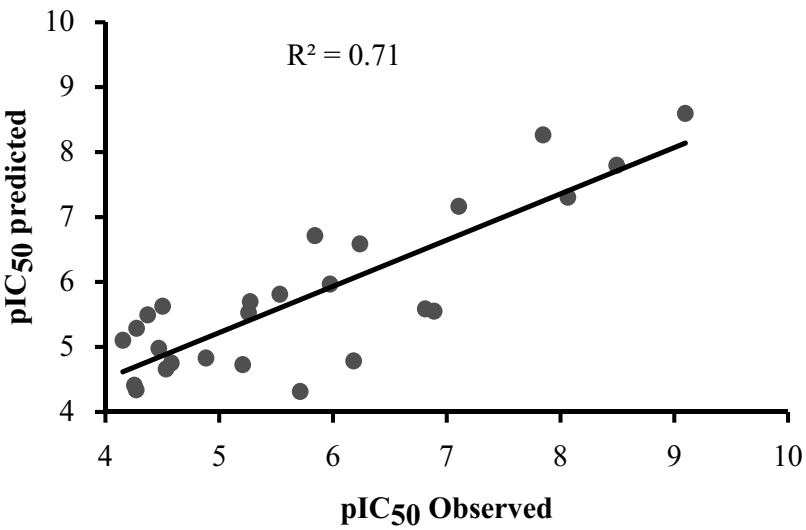

Figure 9. Correlation plot of the observed versus predicted pIC_{50} values of the training set (n = 26) using QSAR_3 model.

sets of compounds were available: the first set containing 26 PRK1 inhibitors with IC_{50} values was used as a training set for QSAR model generation (**Figures 1** and **2**). The second set was used as an external test set to validate the QSAR model. These compounds were identified from an *in vitro* screening (GSK PKIS1) and contain 35 compounds including 14 active compounds and 21 inactive compounds (**Figures 10** and **11**) [18]. Seven of the active compounds, for which IC_{50} values were measured (0.04–4.9 μM, **Table 6**), were taken as external test set to further evaluate the predictive power of the QSAR models.

Table 5 provides a comparison of the calculated pIC_{50} versus experimental pIC_{50} values. The calculation of pIC_{50} values was performed by using the QSAR_1, 2, and 3 models. An important statistic parameter to test the predictive power of the QSAR models is the correlation coefficient R^2_{pred} between the predicted and observed pIC_{50} values for the test set [82]. The correlation coefficients between the experimental and predicted pIC_{50} values for the seven test set compounds were quite weak (R^2_{pred} = 0.49, n = 7) when using QSAR_3 model. The predictive power of the QSAR_1 and 2 models was investigated. The correlations of both models were within a similar range (R^2_{pred} = 0.58 and 0.37, n = 7). A further analysis was performed to detect the outliers depending on Z-score values. The Z-score values were calculated using MOE, for QSAR_3. Golbraikh et al. mentioned that at least five compounds are required for a test set to validate a QSAR model [80]. After the analysis of the correlation and Z-scores, compound SB-750140 found to negatively affect the correlation coefficients. The correlation using QSAR_3 shows an improvement (R^2_{pred} = 0.95, RMSD = 0.17 and q^2 = 0.89, n = 6). A similar observation was made for the two other QSAR models (QSAR_1: R^2_{pred} = 0.93, RMSD = 0.21 and q^2 = 0.82, n = 6; QSAR_2:R^2_{pred} = 0.98, RMSD = 0.12 and q^2 = 0.95, n = 6). The outcomes confirm that the generated

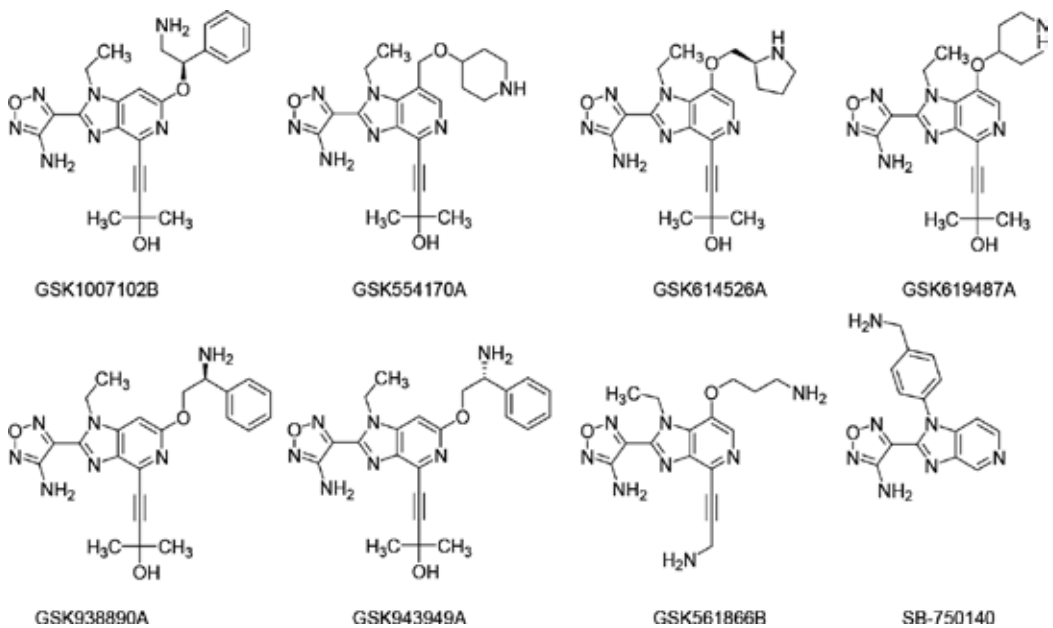

Figure 10. Chemical structures of PRK1 14 actives identified from screening the GSK PKIS1 [18].

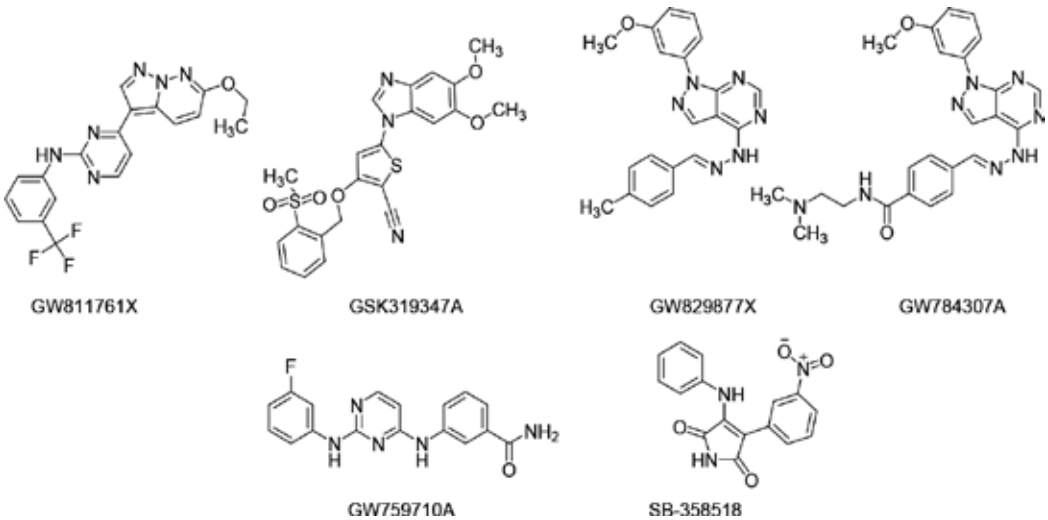

Figure 11. Chemical structures of PRK1 14 actives identified from screening the GSK PKIS1 (continued) [18].

Compound	Binding 1 μM	IC_{50} nM	pIC_{50}	Pred. pIC_{50} QSAR_3	Pred.pIC_{50} QSAR_1	Pred. pIC_{50} QSAR_2
GSK943949A	Yes	40	7.40	7.33	7.79	7.99
GSK614526A	Yes	68.1	7.17	7.09	7.68	7.69
SB-750140	Yes	120	6.92	5.70	6.06	5.88
GSK619487A	Yes	181.3	6.74	7.11	7.62	7.65
GSK554170A	Yes	710	6.15	6.34	6.76	7.02
GSK938890A	Yes	3600	5.44	5.67	5.77	6.49
SB-358518	Yes	4900	5.31	5.84	6.16	6.24
GW811761X	Yes	No IC_{50}		5.72	5.97	6.05
GW784307A	Yes	No IC_{50}		6.86	7.03	7.36
GSK1007102B	Yes	No IC_{50}		6.34	6.72	7.21
GSK561866B	Yes	No IC_{50}		5.82	6.10	6.45
GSK319347A	Weak	No IC_{50}		6.77	6.26	6.96
GW759710A	Weak	No IC_{50}		4.99	5.26	5.41
GW829877X	Weak	No IC_{50}		5.26	5.52	5.53
GSK1030059A	No			7.66	8.08	7.98
GSK625137A	No			4.16	4.43	4.47
GW290597X	No			6.52	5.67	6.53
GW513184X	No			5.27	5.25	5.55

Compound	Binding 1 μM	IC_{50} nM	pIC_{50}	Pred. pIC_{50} QSAR_3	Pred.pIC_{50} QSAR_1	Pred. pIC_{50} QSAR_2
GW643971X	No			5.27	5.19	5.60
GW794607X	No			5.44	5.66	5.71
SB-409514	No			5.13	5.56	5.48
GSK994854A	No			5.93	5.90	6.50
GW874091X	No			5.26	5.65	5.56
GW693481X	No			3.25	3.80	3.92
GSK1000163A	No			6.21	7.02	6.91
GSK1220512A	No			6.03	5.51	6.58
GSK317314A	No			5.94	6.64	6.48
GSK579289A	No			5.14	4.89	5.60
GSK949675A	No			7.20	7.99	7.89
GW580496A	No			6.18	5.37	6.77
SB-476429-A	No			4.68	4.67	4.92
SB-744941	No			4.73	5.52	5.22
GSK571989A	No			6.26	6.42	6.75
GW779439X	No			4.83	4.76	5.35
GSK978744A	No			7.76	7.75	8.10

Table 6. Comparison of the experimental pIC_{50} versus the calculated pIC_{50}, using the generated models QSAR_1, 2 and 3 [18].

QSAR models show satisfactory ability to predict the biological activities of the test set compounds. Thus, they could be accepted having an R^2_{pred} value > 0.5 [82]. Moreover, taking the last consideration of the threshold for the predicted $pIC_{50} > 7$ found in case of actives to refer to potent compounds was clearly in the test set, since the most potent inhibitors in the test set ($IC_{50} < 100$) were identified with predicted $pIC_{50} > 7$ (**Table 6**). Meanwhile, the biological activities of the moderately active compounds were predictive with calculated pIC_{50} in the range 5.5–7 (**Table 6**). Among the inactive compounds in the test set, three compounds were predicted to be highly potent compounds, but these compounds were inactive in the assay. The visualization of the false positives could not clarify the absence of activity of both compounds. However, GSK978744A and GSK1030059A, which mainly target the PLK kinase, possess the same chemical scaffold, and their binding modes were investigated and compared with the binding modes of analogues co-crystallized with other kinases (CDK2; PDB ID: 2I40, NEK2; PDB ID: 2XNN). However, it is not clear, from structural interactions, why this compound is not active. Additionally, it might possible that the exclusion of entropy changes or solvation of the binding pocket could play a role in the wrong estimation of the biological activity.

Since the QSAR_2 model shows high value of R^2_{pred} in the test set (QSAR_2: $R^2_{pred} = 0.98$, RMSD = 0.12 and $q^2 = 0.95$, $n = 6$), the discrimination performance was therefore discussed. Similar to QSAR_3, the most potent inhibitors in the test set ($IC_{50} < 100$) were identified with predicted $pIC_{50} > 7$

(**Table 6**). Furthermore, between the remaining active compounds, two of them were predicted to be high potent inhibitors $pIC_{50} > 7$ (GW784307A: predicted $pIC_{50} = 7.36$; GSK1007102B: predicted $pIC_{50} = 7.21$). However, the IC_{50} values of these two compounds have not been measured yet, but it reported to be active since it binds to PRK1 at 1 μM. Thus, the consideration of the threshold for the predicted $pIC_{50} > 7$ could also be applicable in the QSAR_2 model. The weak active compounds GSK319347A, GW759710A, and GW829877X (shown in **Table 6**) also predicted as moderate or low active compounds where predicted pIC_{50} values were less than 7. Among the inactive compounds, three compounds (the same compounds as in the case of the QSAR_3 model) were predicted to be highly potent compounds, but these compounds were inactive in the assay.

$$\begin{aligned} &\text{QSAR\_2}: pIC_{50} = 0.15509 - 0.09778 \\ &* \text{PEOE\_PC} - -0.09778 * \text{MM-GBSA} \\ &\left(R^2 = 0.70, \text{RMSE} = 0.78\, q^2 = 0.62,\ n = 26\right) \end{aligned} \tag{1}$$

$$\begin{aligned} &\text{QSAR\_3}: pIC_{50} = -1.37129 - 0.56938 * \text{PEOE\_PC} - \\ &-0.08688 * \text{MM-GBSA} - 0.26312 * \text{GLIDE\_SP} \\ &\left(R^2 = 0.71,\ \text{RMSE} = 0.76 \text{ and } q^2 = 0.63, n = 26\right) \end{aligned} \tag{2}$$

By comparing the three generated QSAR models (QSAR_1, 2, and 3) depending on the predicted pIC_{50} values among them to the measured ones, it was found the predicted pIC_{50} values when using QSAR_3 model were closer to the experimental values (**Table 6**), but QSAR_2 model showed the highest value of R^2_{pred} in the test set. Furthermore, in bootstrap validation, it was found that the QSAR_3 model had the ability to predict the biological activity of the test set compounds more accurately than the models QSAR_1 and 2 (**Table 6**). Thus, the QSAR_3 model could be used to predict the biological activities of the highly potent compounds, which should exhibit biological activities power in the nanomolar range and differentiate between the active compounds, according to their activity (low, moderate, and high).

3.7. Virtual screening using the p-ANAPL library

The above protocol was further used to virtually screen for possible PRK1 inhibitors from the p-ANAPL library [58]. As previously applied, ensemble docking was performed to dock the compounds to the X-ray structures of PRK1 stored in the PDB. Both data sets were prepared using Schrödinger 2014.U2, including the generation of several conformations per compound. The docking solutions were visualized using MOE to explore the conserved interactions with residues at the PRK1 hinge region. The selection of the hits was based first on the displayed binding mode, followed by their scoring, then predicting the pIC_{50} values using the generated QSAR models. Depending only on docking scores was insignificant for the selection of promising hit compounds. The docking solutions for the entire data set were visualized and several hits compounds were selected. In the next step, their predicted pIC_{50} values were calculated using QSAR_1, 2, and 3. Depending on the set threshold for the predicted pIC_{50}, several top scoring compounds could be selected as promising hits for the biological assays, e.g., those in **Figure 12**. **Figure 13** displays the binding modes of the selected hits and the interaction with residues at the binding pocket of the corresponding X-ray structure of PRK1.

One of the promising hits, DBT-6b, is Bartericin B (MW = 408.49; PubChem CID: 12136210) from *Dorstenia barteri* [83]. The binding mode of DBT-6b is shown in **Figure 13A**. The compound is predicted to interact with hinge region residue Ser704 with one H bond. The phenol ring forms an additional H bond with the backbone of Asp764 from the DFG-motif. The interaction with the front polar pocket and the ribose binding pocket is mediated by two H bonds between Asp708 and propanol substituent in DBT-6b. The calculated pIC_{50} values were 7.85, 7.64, and 7.45 using the generated QSAR models QSAR_1, 2, and 3, respectively. The second hit (P87), vitexin or apigenin-8-C glucoside (MW = 432.38; PubChem CID: 5280441), is a flavonoid glycoside from diverse sources, e.g., *Hyparrhenia hirta* [84]. The binding mode of P87 (**Figure 13B**) shows that the compound might form one H bond with the hinge region residue Ser704. Additionally, the substituents on the

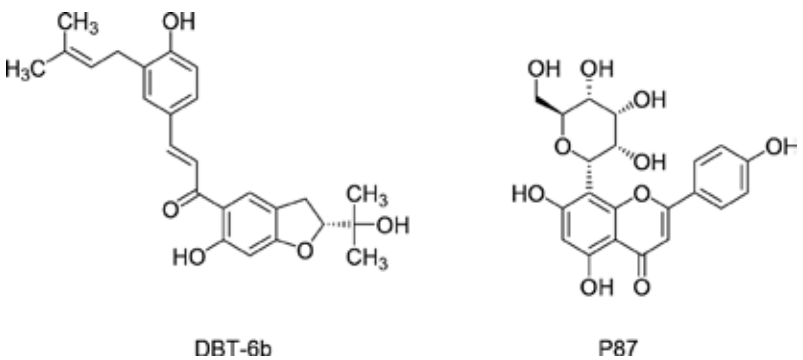

Figure 12. Chemical structures of the selected hits.

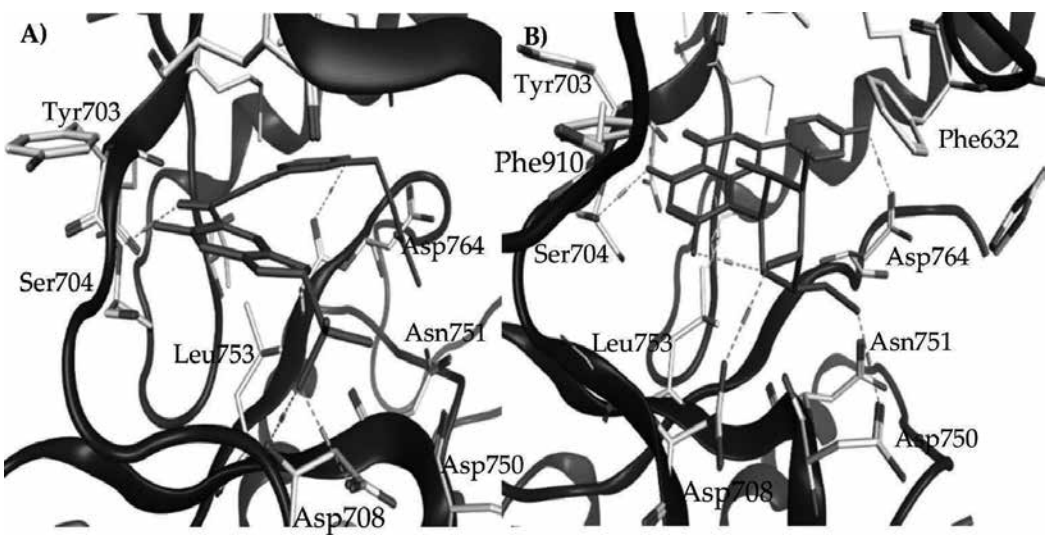

Figure 13. Docking poses of the selected hits in PRK1 binding pocket. (A) Docking poses of DBT-6b in the binding pocket of PRK1 structure 4OTG. (B) Docking pose of P87 in PRK1 structure 4OTH. The figure was designed using MOE.

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oxane ring are targeting the front polar pocket and forming two H bonds with Asp708 and Asp750. The phenyl ring interacts with the back hydrophobic pocket and further forms an H bond with Asp764 in the DFG motif. P87 was predicted to be a highly potent compound, with a predicted pIC_{50} value greater than 7, using QSAR models (pred_pIC_{50}= 8.02, 8.6, and 9, respectively). Bartericin B and vitexin are the only two examples of promising compounds from natural products which could target PRK1. Among the screened databases, other hits were selected as promising compounds, which could inhibit PRK1 activity in the nanomolar range. In further investigations, the selected compounds can be submitted for biological assay to figure out their ability to bind into PRK1.

4. Conclusions

This chapter provides a brief summary of database resources for anticancer drug discovery and some recent inputs and success stories for the identification novel inhibitors of selected anticancer drug targets by the use of computer modeling. While the experimental validation of some of the natural product hits identified in the case study is ongoing, the question about the applicability domains of the derived QSAR models reported is still to be answered. Among the identified hits, 50 closely similar compounds to Bartericin B (with cut-off Tanimoto coefficient of 0.7), having reported biological activities in PubChem [59] and ChEMBL [44], with 239 biological activities and 152 published patent data. A similarity search of vitexin in PubChem gives 112 similar compounds, corresponding to 524 biological activities and 208 patents. Bartericin B is known to exhibit antimicrobial activities against *Trichomonas gallinarum*, with minimum lethal concentrations (MLCs) of 0.244 and 0.121 μg/mL after 24 H and 48 H, respectively [83], while its analogue (Bartericin A from *Dorstenia angusticornis*) is known to be very active against some bacteria and yeasts associated with human pathologies [85]. Both chalcones are known to also have potential antiprotozoal activities [86], e.g., against *Plasmodium falciparum* [87]. Vitexin, on the other hand, is known for its antioxidative and [88] spasmolytic effects [89]. The compound is known to be abundant in plants of the genus *Vitex* (Verbenaceae), which is known to exert insect antifeeding activities, among others [90]. These compounds could now be tested in biological assays for potential PRK1 inhibition.

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Competing interests

The authors declare that they have no competing interests.

List of abbreviations

AfroCancer	African Anticancer Natural Products Database
BFE	Binding free energy
EC_{50}	Half maximal effective concentration, i.e., the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after a specified exposure time
ED_{50}	The median effective dose, a dose that produces the desired effect in 50% of a population
GI_{50}	The growth inhibition of 50%, drug concentration resulting in a 50% reduction in the net protein increase
IC_{50}	The drug concentration causing 50% inhibition of the desired activity
NANPDB	Northern African Natural Products Database
NP	Natural product
NPACT	Naturally Occurring Plant-based Anti-cancer Compound Activity-Target Database
p-ANAPL	Pan-African natural products library
PDB	Protein databank
PLS	Partial least squares
PRK1	protein kinase C–related kinase
QSAR	Quantitative structure-activity relationship
RMSD	Root mean square deviation
RMSE	Root mean square error
SM	Secondary metabolite
VS	Virtual screening

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Chapter 3

Annexin Proteins: Novel Promising Targets for Anticancer Drug Development

Filiz Bakar

Additional information is available at the end of the chapter

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Abstract

Intracellular Ca^{2+} signaling and Ca^{2+} homeostasis have long been an important subject area of cell biology. Several intracellular Ca^{2+} binding proteins have been demonstrated until now, and among these, annexins are characterized by their ability to interact with membrane phospholipids and they form an evolutionary conserved multigene family with the members being expressed throughout animal and plant kingdoms. Annexin proteins are defined by different structural and biochemical criteria, and this multigene family has several biological features. In certain clinical conditions, the alterations on the localization or expression levels of annexin proteins are considered as the causes of pathological results and/or sequelae of disease. So, annexin proteins are indirectly linked to severe human diseases such as cardiovascular disease and cancer. Since annexin proteins are known to play roles in cancer, the researches are focused on defining the clinical significance of certain annexin proteins in cancer development and by the way anticancer treatments in the last decades. This chapter presents detailed information about annexin proteins and the studies on anticancer drug development targeting certain annexins. The studies denominate that targeting of certain annexin proteins reduces tumorigenesis and therapeutic resistance. So, annexin proteins have growing importance for anticancer drug development.

Keywords: annexin, cancer, anticancer, drug development, treatment

1. Introduction

Annexins are commonly known to be a large multigene family of Ca^{2+}-dependent phospholipid-binding proteins.They were discovered in the late 1970s and before the name "annexin", they were first introduced in diverse names which in Greek means "hold together" [1].

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Over a hundred annexin proteins have been discovered in various species. Among these, 12 proteins are found in humans referred as A1–A13 (leaving A2 unassigned) [2], each having a differently positioned calcium/membrane-binding site within the core domain and a different N-terminal domain [3].

Annexins have a unique structure that allows them to locate onto membranes reversibly. They contain a conserved calcium and membrane-binding unit, which constitutes the core domain. It consists of four annexin repeats of about 70–80 amino acids. Its alfa-helical shape forms a slightly curved disc. The convex surface of it carries the calcium and membrane-binding sites as well as binding sites for phospholipids, heparin, and F-actin. The concave side on the other hand is responsible for other interactions. Ahead of the core domain comes the N-terminal region which differs in length and in sequence. It mediates regulatory interactions with protein ligands and annexin-membrane association [2]. It has been recently demonstrated that a part of N-terminal region integrates into the folded core, allowing the N-terminal region to be exposed for additional interactions upon calcium binding [4].

2. Functions of annexins

Annexins are responsible for calcium-regulated endocytotic and exocytotic events along with stabilizing organelle membranes and the plasma membrane [3]. One of the major roles of annexins is acting as scaffold proteins through calcium-regulated binding to phospholipids on the membranes. This allows the cytoplasm and the cytoplasmic side of the cell membrane to interact accordingly [5]. Mobilization of intracellular calcium triggers annexins to be recruited by cell membranes. However, some annexins can bind to membranes in the absence of calcium as well, such as annexins A9 and A10 [2].

Some annexin members specifically engage with certain sites of actin assembly at cellular membranes. For instance, the organization of raft and non-raft microdomains of smooth muscle cell membranes is regulated by annexins A2 and A6 through mediating interactions with the cytoskeleton [6].

2.1. Intracellular activities of annexins

Annexins are able to engage with cytoskeleton components reversibly. However, under certain circumstances, some annexins (A2 and A11) are found to be working together in the nucleus in the cell cycle [7]. Especially, annexin A11 plays an essential role in the terminal phase of cytokinesis. Without it, cells cannot form a midbody and hence end up in apoptosis [8]. Additionally, some annexins can be present on the cell surface. For instance, when cells are exposed to glucocorticoids, annexin A1 is found to be translocating from the cytosol to the cell surface [9]. It has also been demonstrated that annexin A2 functions as a co-receptor for plasminogen in several cell types including tumor cells, macrophages, and endothelial cells. There is also evidence that annexin A2 might be taking part in preserving vascular patency [10].

2.2. Extracellular activities of annexins

Annexins are typically known to be cytosolic proteins. However, some annexins can be found in extracellular fluids as well. There are binding sites on the outer side of cell membranes for these annexins, and they take part in several extracellular functions such as the role of annexin A5 as an anticoagulant protein, annexin A2 as an endothelial cell surface receptor for plasminogen, and the role of annexin A1 with anti-inflammatory activities on leukocytes [11]. As it is mentioned, annexin A2 functions as a receptor for plasminogen through its activities in fibrinolytic cascade as a positive modulator [12]. As a result, overexpression of annexin A2 on the surface of acute promyelocytic leukemia cells could lead to occurrence of bleeding [13].

Annexin A1 is the first member of the annexin family known to be present extracellularly. There are several findings about the extracellular activity of annexin A1. It can be found in human serum, particularly in inflammatory events such as colitis and myocard infarctus [14]. Even though annexins A1 and A4 are both present in ductal prostate epithelium cells, only annexin A1 is present extracellularly [15]. Several studies have shown that annexin A1 strongly inhibits the transendothelial migration of leukocytes, hence limiting the extent of inflammation [16].

3. Association of annexin proteins with diseases

The absence of annexin proteins can cause several abnormalities in the body. Altered expression of annexin A1 has led to a change in the inflammatory response of glucocorticoids and an increase in leukocyte migration. Additionally, it has been demonstrated that expression of other annexin proteins was affected by the loss of annexin A1 as well [17].

Recent studies have revealed that there are single-nucleotide polymorphisms (SNPs) in the genome of annexin proteins. According to studies, annexin A2 gene SNP exists in a higher level in sickle cell patients compared to control groups and is associated with osteonecrosis [18], and annexin A5 gene polymorphism has a role in recurrent pregnancy loss [19].

Annexins also take part in autoimmune diseases such as rheumatoid arthritis and type 1 diabetes. High levels of annexin V cause annexin V autoantibodies to be produced more than necessary, which may play a role in pathogenesis of these diseases [20, 21]. On the other hand, annexin A11 gene polymorphism is found to be associated with sarcoidosis, which is another autoimmune disease characterized by accumulation of epithelioid granulomas in many organs such as kidney and lungs [22].

4. Role of annexins in cancer

Annexin proteins generally exhibit diverse functions in coagulation, inflammation, signal transduction, cell proliferation, apoptosis, tumor development, angiogenesis, invasion/metastasis, and drug resistance. Several studies have revealed that annexins might be playing an important role in the process of tumor differentiation and tumor development through various mechanisms.

4.1. Annexin A1 and cancer

Annexin A1 also known as lipocortin is a member of annexin family [23], expressed in many cell types such as prostate, brain, epithelial cells, and phagocytes. It participates in various intracellular events such as cell growth, migration, cell differentiation, and mediating anti-inflammatory effects of glucocorticoids [24].

Up-regulation of annexin A1 functions as a tumor progression marker in hepatic, pancreatic, breast, and stomach carcinomas [25]. In contrast, it is down-regulated in head and neck cancers, prostate cancer, and esophageal cancers [26]. Increased annexin A1 levels have been correlated with various multidrug-resistant tumor cells as well. Annexin A1 regulates the expression of metastatic matrix metalloproteinase-9 (MMP-9) and its activity and induces the activation of NF-kB as well as promoting migration and invasion in MDA-MD-231 cells [27]. The studies have reported a significant correlation between annexin A1 levels and pathological differentiation of oral squamous cell carcinoma (OSCC) tissues [28]. According to data, the presence of annexin A1 also promotes small cell lung cancer (SCLC) cells adherence to brain endothelium leading to transendothelial migration [29]. These findings suggest that annexin A1 plays an important role in the regulation of tumor cell behavior and can be used as a potential target in breast cancer therapy.

4.2. Annexin A2 and cancer

Annexin A2, also known as Calpactin I or Lipocortin II, is a 36 kDa member of annexin family expressed by various cell types such as endothelial cells, tumor cells, and macrophages [30]. The N-terminal region of annexin A2 contains tissue plasminogen activator (tPA) [31] as well as S100A10 protein binding site [32]. On the other hand, the C-terminal region contains heparin [33], F-actin [3], and plasminogen binding sites [34].

Like the other members of annexin family, intracellular annexin A2 participates in endocytotic and exocytotic events. The down-regulation of annexin A2 inhibits cell proliferation and cell division [35], and degradation of this protein has been linked with apoptosis promoted by p53-induced pathways [36]. Annexin A2 can also function as an antioxidant. Down-regulation of annexin A2 leads tumor cells to apoptosis through pro-apoptotic p38MAPK/JNK/Akt signaling pathways upon hydrogen peroxide exposure [37].

Annexin A2 interacts with tPA which transforms plasminogen into plasmin, hence leading to extracellular matrix degradation and cell invasion. However, blocking off the surface of annexin A2 can prevent tumor cell growth and metastasis [38]. Overexpression of annexin A2 is observed in a wide range of cancer cells such as acute lymphoblastic leukemia (ALL), breast cancer, colorectal cancer (CRC), lung cancer, and many others.

In acute lymphoblastic leukemia (ALL) cells, annexin A2 has been linked with drug resistance. Experiments revealed that phosphorylated annexin A2 expression (and not annexin A2) is higher in prednisolone-resistant cells than in drug-sensitive cell lines, suggesting that preventing annexin A2 phosphorylation can bring therapeutic benefit to the treatment of drug-resistant ALL cells [39].

In pancreatic tumors, annexin A2 levels were observed to be 2- to 8-folds higher than in normal pancreas cells [40]. On the other hand, higher annexin A2 immunoreactivity is observed in lung and squamous cell carcinoma compared to control group [41]. The studies also suggest that annexin A2-dependent plasmin in human breast cancer cells may participate in angiogenesis and metastasis through ubiquitination in breast cancer tissue [42]. Recent studies have demonstrated that annexin A2 is a receptor for gastrin and progastric peptides, which are associated with growth-stimulatory effects on intestinal epithelial and colon cancer cells [43]. Annexin A2 expression is strongly correlated with disease recurrence. Hence, it could be regarded as a potential biomarker for CRC patients.

4.3. Annexin A3 and cancer

The absence of annexin A3 is believed to play an important role in drug resistance and tumor development. According to available data, there is a correlation between up-regulation of annexin A3 and increased drug resistance in ovarian carcinoma. It also increases the metastasis of lung adenocarcinoma and hepatocarcinoma. On the other hand, development of prostatic and renal carcinoma was observed with the down-regulation of annexin A3 [44].

Among digestive tract cancers, colorectal cancer (CRC) is seen very commonly. Since it bears no clinical symptoms at early stages, discovering a biomarker that will aid in diagnosis has become necessary. Annexin A3 is considered to be a potential biomarker for colorectal cancer. The higher level of annexin A3 expression has been determined in blood samples of patients with CRC, indicating the importance of annexin A3 as a biomarker in CRC [45].

4.4. Annexin A4 and cancer

Annexin A4 is a member of annexin family, also known as lipocortin IV with a size of 35.9 kDa [46]. It consists of four annexin repeats, and each region includes 5 alfa-helixes with a calcium-binding motif [47].

Annexin A4 plays an important role in membrane repair, promoting vesicle aggregation and regulation of passive membrane permeability [48]. It also takes part in calcium signaling, anticoagulation, and resistance to apoptosis [49]. Accumulated data show that annexin A4 also involves in tumor progression, invasion, metastasis, and drug resistance in various cancer types [50].

Experiments revealed that there is a positive correlation between annexin A4 and colorectal cancer progression [51]. Moreover, annexin A4 was found to be directly binding to HPA (one of the markers of CRC metastasis), which indicates that it can be considered an important marker for CRC progression [52]. Annexin A4 is also overexpressed in *Helicobacter pylori*-infected gastric cancer tissues compared to not infected ones [53]. The suggested mechanism is that *H. pylori* infection promotes gastric cancer progression through increasing annexin A4 levels in order to induce the expression of IL-8. Hence, annexin A4 can be regarded as a potential marker in gastric cancer development. The studies also showed the relation of annexin A4 with malignant mesothelioma [54], breast, laryngeal, and hepatocellular carcinoma [55, 56].

4.5. Annexin A5 and cancer

Annexin A5, also known as Endonexin II, Lipocortin V, or thromboplastin inhibitor V, plays an important role in cell membrane repair during anti-inflammatory, profibrinolytic, and anti-thrombotic activities. Intracellular annexin A5 participates in calcium channel activity on plasma membrane interacting with actin in platelets during the coagulation process [57]. On the other hand, extracellular annexin A5 plays an important role in apoptosis and phagocytosis [58].

As the most studied member of annexin family, annexin A5 also plays important role in cancer development and progression.

Experiments on tumor samples obtained from patients with hepatocellular carcinoma revealed that annexin A5 was up-regulated by 134% [59]. Hence, it could be a novel biomarker for portal vein tumor thrombus formation. Annexin A5 was also correlated with hepatocarcinoma lymphatic metastasis. Half of tumor metastasis occurs through lymphatic system leading to poor prognosis. Studies showed that in metastatic hepatocarcinoma, annexin A5 was increased by 216%, which indicates that annexin A5 levels could be used in diagnosing lymphatic metastasis of tumors [60]. Annexin A5 has been found overexpressed in human cutaneous SCC cell lines. Experiments showed that annexin A5 is mainly present in growing tumor areas [61], suggesting that annexin A5 may involve in cell proliferation and metastasis. On the other hand, knockdown of annexin A5 by siRNA decreased the invasion capability of human oral carcinoma cells while up-regulating a metastasis suppressor gene KISS-1 [62].

Annexin A5 is significantly up-regulated in pancreatic cancer cells under hypoxia condition, indicating that it may be a significant reference value in pancreatic ductal adenocarcinoma [63]. Results obtained from studies suggest that annexin A5 is involved in breast cancer since up-regulation of this protein suppressed Raf-1, MEK1/2, and ERK1/2 phosphorylation of breast cancer cells [64].

Additionally, the studies revealed that annexin A5 is also involved in cervical, colorectal, bladder carcinomas, and inflammation-associated carcinogenesis of fibrosarcoma by different mechanisms.

4.6. Annexin A7 and cancer

Annexin A7 (also known as synexin) is a member of annexin family. On human chromosome, it is located where several tumor-suppressor genes are present [65]. Although it can be found in the nucleus, it is mostly found in membranes [66].

Available data indicate that annexin A7 might function as a tumor-suppressor gene in prostate cancer, melanoma, and glioblastoma; however, it might act as a tumor promoter in gastric cancer, liver cancer, colorectal cancer, and breast cancer. Additionally, down-regulation of annexin A7 could participate in tumor invasion and metastasis [65].

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5. Annexin-targeted studies

The certain members of annexin family have important functions in the development and prognosis of several carcinomas mentioned above. Thus, the studies are focused on targeting these proteins to prevent or treat the disease. The recent findings on annexin-targeted treatments are summarized hereafter.

Prostate cancer is the most common malignant cancer diagnosed in men. It accounts for 10% of all male cancers and is difficult to detect at early stages. Therefore, it is necessary to discover a novel biomarker that will aid in early diagnosis [67]. To investigate the effect of Simvastatin and annexin A10 in human PC-3 prostate cancer cells, a nude mouse tumor xenograft model was used. Simvastatin was administered with 5 and 50 mg/kg doses. According to results obtained, Simvastatin up-regulated the expression of annexin A10 which led to a significant decrease in cell proliferation, invasion, and migration as well as a reduction in tumor size. In contrast, down-regulation of annexin A10 by siRNA increased the cell proliferation, invasion, and migration in PC-3 cells. Taken together, targeting annexin A10 with statins could be used in preventing or treating prostate cancer [68].

S100 proteins are known to regulate cell functions through interacting with other proteins, particularly with annexins [69]. The interaction between annexin A2 and S100A10 plays an important role in tumor metastasis and neo-angiogenesis [70]. Therefore, inhibiting this interaction could bring therapeutic benefits in cancer treatment. Several inhibitors have been identified using biochemical screening and receptor-guided random docking techniques based on '1,2,4-triazole' structure. One of these compounds was found to be a potent inhibitor: 2-[(5-{[(4,6-dimethylpyrimidin-2-yl)sulfanyl]methyl}-4-(furan-2-ylmethyl)-4H-1,2,4-triazol-3-yl)sulfanyl]-N-[4-(propan-2-yl)phenyl]acetamide [71].

Various chemicals can cause DNA damage and mutagenesis such as As^{3+} or reactive oxygen species, and mutagenesis has an important role in cancer initiation and progression [72]. Annexin A1 is known to participate in signal transduction of growth factors and cell proliferation or differentiation. Nevertheless, in certain types of cancers, the expression of annexin A1 can be reduced such as squamous cell carcinoma, whereas it can be increased in other cancers such as bladder cancer [73]. Moreover, in some cancer cells, the expression of annexin A1 is found higher in nucleus than in cytosol, which indicates that the nuclear presence of annexin A1 could correlate with progression of certain cancers [74]. Annexin A1 requires calcium signaling and tyrosine phosphorylation in order to translocate into the nucleus. This process is triggered by DNA-damaging agents and oxidative stress [75]. Signals of damage in DNA form a mono-ubiquitinated annexin A1, which stimulates translesion DNA synthesis by heavy metals [76]. Since annexin A1 is thought to involve in responses of DNA damage and mutagenesis, the inhibition of binding activity of annexin A1 by several substances including flavonoids has been researched. Results have revealed that Quercetin, Silibinin, and Genistein inhibited the binding activity of annexin A1 in a concentration-dependent manner. Moreover, they inhibited thymidine kinase gene mutation induced by As^{3+} in lymphoma cells through

suppressing the translesion DNA synthesis which was mediated by mono-ubiquitinated annexin A1 in the nucleus [77]. These findings indicate that annexin A1 could be a novel target protein in preventing DNA damage induced by gene mutation.

Hepatocarcinoma is one of the most common malignancies with a high mortality rate and no effective treatment. A study has shown that in a mouse hepatocarcinoma cell line (Hca-P), down-regulating the expression of annexin A7 decreases the proliferation and induces apoptosis [78]. To investigate the role of it further, an experiment targeting annexin A7 has been performed. In order to down-regulate the expression of annexin A7, an RNA interference technique (RNAi) was used to demonstrate the changes in cell viability where annexin A7 levels are altered after Cisplatin treatment. According to data obtained, following the down-regulation of annexin A7, treatment with Cisplatin reduced the proliferation of Hca-P cells significantly and induced apoptosis. Additionally, altering the expression of annexin A7 decreased the expression of Bcl2 and increased the expression of caspase-3 and cytochrome-C, which indicates that presence of annexin A7 inhibits apoptosis through the mitochondrial pathway [79] (see **Figure 1**).

Annexin A1 is known to participate in the process of inflammation along with a wide range of cellular activities [2]. It has been revealed that annexin A1 plays a role in the process of apoptosis in inflammatory cells as well [80]. Experiments have shown that elevated annexin A1 levels in U937 cells and bronchoalveolar epithelial cells induce apoptosis through caspase-3 activation [81]. Moreover, it has been shown that in thyroid cancer cells, apoptosis induced by TRAIL is also mediated through annexin A1 expression [82]. Additionally, in

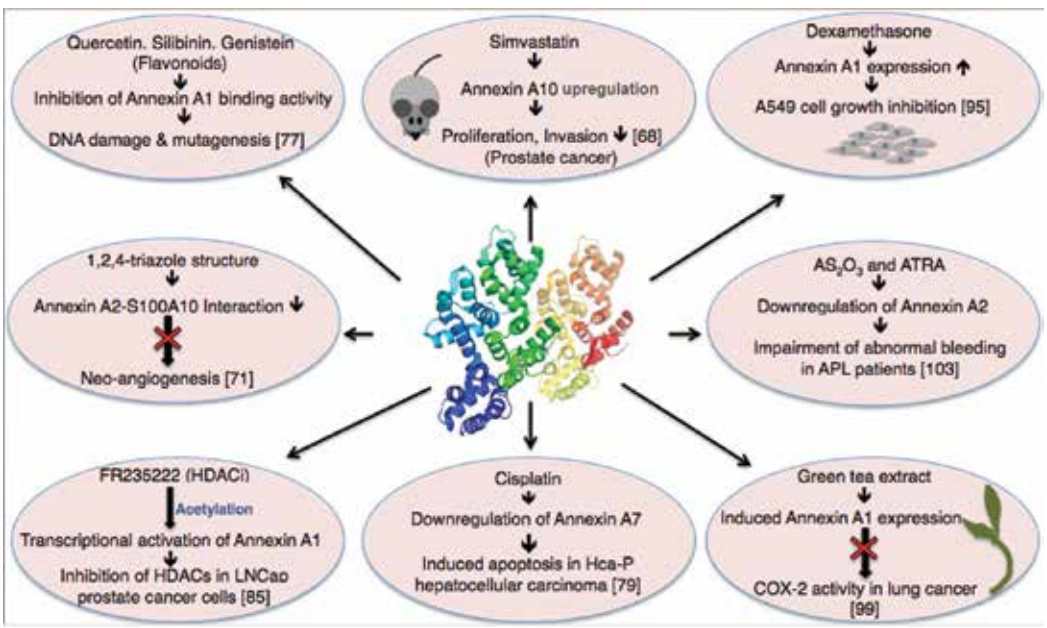

Figure 1. Schematic representation of annexin-targeted novel studies (the centered protein figure was prepared by Pymol Educational Program using annexin IV protein (PDB ID: 2ZOC) from Protein Data Bank).

prostate cancer cells, down-regulation of annexin A1 has been suggested to contribute in cancer initiation and progression [83]. On the other hand, up-regulation of annexin A1 has decreased the cell viability and induced apoptosis through caspase activity [84], which indicates that annexin A1 could be taken as a tumor-suppressor protein in prostate cancer cell line (LNCaP).

Experiments have shown that the expression of annexin A1 decreases in prostate cancer cells. Therefore, the mechanism of this reduction has been investigated. The fact that annexin A1 levels only decrease and are not completely eliminated brings the possibility that dysregulation of annexin A1 occurs at the level of gene transcription [85]. It has been proposed that deacetylation of histone proteins leads to altered gene expressions [86]. The turnover of histone acetylation is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes can induce and inhibit transcription [87], and they show dysregulated activities in human cancers leading to neoplastic transformation of tumor cells [88]. Hence, the balance of HAT/HDAC has been a target in cancer therapy. Various compounds have shown antitumor effects through inhibiting HDACs such as valproic acid and some cyclic peptides (FK228) [89].

Recently, a novel compound, FR235222, with inhibitory effect on histone deacetylases has been isolated from a fungus [90]. Experiments have revealed that FR235222 induces apoptosis and regulates annexin A1 expression in leukemia cell lines. The possible mechanism suggested was that reduced levels of annexin A1 could be mediated by deacetylation of histone proteins [85]. To confirm this hypothesis, the effect of FR235222 on apoptosis and annexin A1 expression has been studied in prostate cancer cell lines (LNCaP). Western blotting results have shown that FR235222 induces the expression of annexin A1 in a time-dependent manner with a peak at 48 h. Also, experiments with actinomycin D indicated that the increase of annexin A1 was at transcription level. In contrast, when annexin A1 expression was down-regulated by siRNA transfection protocol, a partial decrease in FR235222-induced apoptosis has been observed by 26% and in caspase-3 activity by 22% in LNCaP cells [91]. These findings suggest that transcriptional activation of annexin A1 is induced by FR235222 through acetylation of histone proteins and inhibition of HDACs in LNCaP cells, and the increased levels of annexin A1 lead to apoptosis through caspase activity.

Lung cancer is one of the most common cancer types with a high rate of mortality [92]. Studies have shown that inflammation participates in the development of lung cancer. One of the components of inflammatory pathways is the COX-2/PGE_2 pathway. Increased expression of COX-2 is often seen in human non-small cell lung cancer (NSCLC). This leads to overexpression of PGE_2 which involves in various cancer-related activities such as resistance to apoptosis, angiogenesis, invasion, and metastasis [93]. Annexin A1 acts as a phospholipase A2 inhibitor and is associated with several functions such as cell differentiation, cell growth arrest, and anti-inflammation [94]. The effect of annexin A1 in human NSCLC cell line (A549) has been investigated. Studies have concluded that Dexamethasone increased the expression of annexin A1 in A549 cells which inhibited cell growth [95]. In contrast, gene deletion of annexin A1 led to an excessive inflammatory stimuli characterized by increased leukocyte migration and IL-1B generation [17].

Green tea (*Camellia sinensis* leave extract) is known to contain polyphenols which are natural antioxidants. Accumulated data have shown that green tea exhibits a protective role against various cancers including lung cancer [96]. It has been observed that green tea extract (GTE) induced annexin A1 in human urothelial cells and in lung cancer (A549) cells in a dose-dependent manner [97]. Moreover, GTE-induced annexin A1 also mediated cytoskeletal actin remodeling, which led to an increase in cell adhesion and decrease in cell motility. Additionally, inhibition of COX-2 and PGE2 was observed in NSCLC cell lines following GTE-induced annexin A1 expression. In contrast, silencing annexin A1 expression overturned the inhibitory effect of GTE on COX-2. These results suggest that GTE shows its effect through inducing annexin A1 expression, therefore targeting annexin A1 could be a promising mechanism in preventing lung cancer [98].

Annexin A2 is present in various cell types including endothelial cells, neuronal cells, and cancer cells. It acts as a co-receptor for plasminogen and tissue plasminogen activator (tPA) [99]. In acute promyelocytic leukemia (APL) cells, annexin A2 is found to be overexpressed. This causes plasmin to be highly produced leading to hyperfibrinolysis and then abnormal bleeding in patients [100]. A study has been performed to investigate the regulation of annexin A2 expression in APL cells as well as the effect of arsenic trioxide (As_2O_3) and all-trans retinoic acid (ATRA). Results have shown that annexin A2 is expressed abnormally on the surface of APL cells. Additionally, it has been observed that annexin A2 exhibits a unique activity of binding the tPA substrate plasminogen leading to enhanced plasminogen activity in APL cells [101]. Following the administration of As_2O_3 and ATRA in patients with APL, the expression of annexin A2 was significantly down-regulated on the surface of APL cells compared to the control group. Bleeding started to disappear a week after the treatment with ATRA and As_2O_3 as well as parameters of fibrinolysis [102]. These findings suggest that targeting annexin A2 could help treat the abnormal bleeding in patients with APL.

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Chapter 4

Immunotherapy in Pediatric Acute Leukemia: A Novel Magic Bullet or an Illusory Hope?

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Additional information is available at the end of the chapter

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Abstract

The last decade became the renaissance for investigating and exploring the potential role of immunotherapy in pediatric acute leukemia (AL). It is beyond question that there is an interaction between innate immune system and hematological malignancy. Leukemia cells inhibit the host immune response according to multiple mechanisms, but exploiting the innate immune system mechanisms can overcome the resistance to the conventional treatment. What is the role of immunotherapy in pediatric AL treatment? Does it have the potential to substitute or combine the standard chemotherapy? What is the best possible timing to take advantage of immune interventions? This review is considered to follow through the possible treatment options including their foundation, strong and weak points, but also information about possible implementation into the clinical practice.

Keywords: immunotherapy, acute lymphoblastic leukemia, acute myeloblastic leukemia, children

1. Introduction

Acute leukemia (lymphoblastic and myeloblastic) is the most common malignancy diagnosed in children with an incidence of about 4.2 and 4.9 per 100,000 in the age groups of 0–19 and 0–14, respectively. In the population of children aged 0–19, acute lymphoblastic leukemia (ALL) accounts for approximately 75% while acute myeloblastic leukemia (AML) accounts for 20% of pediatric leukemia cases. Contemporary therapy allows achieving complete remission in approximately 90% of patients with ALL and 70% with AML [1, 2]. It is worth mentioning that 50 years ago acute leukemia was almost universally incurable [3]. The breakthrough has been achieved through standardized and optimized multi-agent therapeutic regimens and through therapy individualization according to the risk stratification. However, even though

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great progress in therapy is reported, refractory or relapsed leukemia remains one of the major causes of cancer-related mortality. Failure to respond to chemotherapy is almost universally connected with poor prognosis. Survival rates for patients with relapsed or refractory AML receiving a second treatment attempt was estimated between 25 and 30% [4]. In 15% of ALL patients who experience relapse of the disease, long-term survival rates vary from 40 to 50%, even though the remission is achieved in over 70% of patients [5, 6]. What is more, current chemotherapy regimens are consisted of very intensive blocks of treatment that are responsible for multiple acute and long-term sequelae, especially in the pediatric population. According to multiple research, 60% of children after an anticancer treatment present at least one organ late effect [7]. New approaches that redirect treatment toxicity accurately to the neoplastic cells, sparing the normal cells and hematopoietic counterparts, will significantly reduce the possible complications and improve the survivor's quality of life.

1.1. Contemporary treatment strategy for acute leukemia in children

The therapy for ALL and AML in children is based on standardized protocols and is composed of four major phases: remission induction, followed by consolidation, reinduction (intensification), and maintenance. In order to provide the most effective and harmless treatment for every patient, children are classified into three groups based on the risk of treatment failure (standard, intermediate, or high). This way, less toxic regimens can be administered to patients with more favorable prognosis, whereas those children with features showing higher risk of relapse are receiving more aggressive blocks of chemotherapy. Protocols that are currently used in treatment of acute leukemia in Polish children are ALL IC-BFM 2009 and AML-BFM 2012 [8, 9]. Allogeneic hematopoietic stem cell transplantation (allo-HSCT), which is a form of immunotherapy, is generally not recommended in the first remission of pediatric AML patients except for those at high risk. Comparably, only the children with high-risk ALL and additional particular unfavorable prognostic factors, like T-cell ALL, high initial leukocytosis, hypodiploidy, and genetic impairments, like t(9;22) or t(4;11), benefit from allo-HSCT in the first complete remission [10, 11]. Radiotherapy is considered as a treatment option in case of extramedullary organ (central nervous system, testicular) involvement, but also as a prevention of central nervous system relapse in every patient with AML and, in strictly defined circumstances, children with the high-risk ALL. This approach is reserved only for selected group of patients according to an increased risk and severity of ionization-related late sequelae in the pediatric population [12].

Chemotherapy regimens used in acute leukemia in children are distinguished as extremely intensive, especially the treatment in patients with AML. This causes a long period of bone marrow aplasia that causes vulnerability to numerous infectious complications. Notably, 5% of treatment failures using previous versions of chemotherapy regimens were the result of treatment-related deaths in the first complete remission. According to the significant improvement in supportive care and therapy individualization, the treatment-related mortality has decreased gradually over the last decade [13]. However, there are still patients with drug-resistant or recurrent leukemia who require further efforts to identify effective treatment strategies based on the advances in our knowledge, understanding of leukemic cell biology, and interactions between them and the innate immune system. Without searching

for new approaches and confining ourselves only to chemotherapy regimens, their prognosis remains unfavorable.

1.2. Rationale for immunotherapy in acute leukemia

The evidence supporting the idea of interactions between immune system and malignant cells is based on multiple observations of leukemia course depending on immune system function. For example, shift reconstitution of the immune system after induction regimens correlates with improved survival, and absolute leukocyte count is an independent prognostic factor for survival in patients with acute leukemia [14]. What is more, it is proven that malignant cells use multiple pathways to interfere the host immune system promoting the number and function of regulatory T cells (T regs) and subsequently reducing the ability of cytotoxic T cells to target leukemia [17].

The most popular and the only undisputed and thoroughly investigated form of immunotherapy, which has been applied in clinical practice for a few decades, is **allogeneic HSCT**. This form of treatment is considered in a subgroup of high-risk patients in the first remission or in refractory and relapsed hematological malignancies. The **graft-versus-leukemia effect (GvL)**, which occurred to be an additional immunological benefit to this approach, is mediated by donor T cells and natural killer (NK) cells against residual leukemia blasts. This phenomenon was discovered according to the observations of a decreased risk of relapse in allogeneic graft recipients compared to patients after syngeneic HSCT or those who received T-depleted grafts to reduce the risk of graft-versus-host-disease (GvHD) [15].

Understanding the impact on immune response against malignant cells was a trigger to further investigations that enabled a better understanding of mechanisms of how leukemia cells manage to evade immune surveillance. This study has laid the foundation for novel approaches using immune interventions. Immunotherapy approaches are mostly investigated in the context of HSCT. However, possible strategies are feasible also in settings which are not related to transplantation. The next chapter indicates the immunotherapeutic approaches that can be potentially implemented into the treatment regimens of acute leukemia in children (**Table 1**).

To boost the immunity	
Inhibiting excessive function of regulatory T cells	CTLA-4 inhibition: Iipilimumab
	PD-1 inhibition: Nivolumab
To enhance the cytotoxic effect	
Using T cells, NK cells, and dendritic cells	Allogeneic HSCT
	Donor lymphocyte infusion (DLI)
	CAR-T cell therapy
	Transfer of allogeneic NK cells
	CAR-engineered NK cell therapy
	Dendritic cell (DC) vaccines

To bridge the tumor cell to the killer	
Monoclonal antibodies	Anti-CD20: Rituximab, Ofatumumab
	Anti-CD22: Epratuzumab
	anti-CD52: Alemtuzumab
	anti-CD33: Lintuzumab
Monoclonal antibodies conjugated to cytotoxic compounds	Anti-CD22 linked to calicheamicin: Inotuzumab ozogamicin
	Anti-CD33 linked to calicheamicin: Gemtuzumab ozogamicin
Bispecific T-cell engagers (BiTEs)	Blinatumomab, anti-CD3, and -CD19

Table 1. Immunotherapeutic strategies in acute leukemia.

1.3. To boost the immunity: potential therapies inhibiting excessive function of regulatory T cells

Regulatory T cells' (T reg, CD4+, CD25+) major role is to control immune tolerance. They are crucial to maintain unresponsiveness against self-antigens, but also to prevent autoimmune diseases and allogeneic graft rejection. In terms of their role in hematological malignancies, they may suppress the anticancer effect mediated by activated T cells. As a consequence of tumor activity, their immunosuppressive effect on T cells may be aggravated compared to healthy individuals (**Figure 1**). Studies show that high plasma and tissue T regs level at the moment of diagnosis correlate with a worse response to chemotherapy and prognosis [16].

One of the mechanisms that leukemia cells tend to interfere T regs function is **overexpression of the FOXP3 gene** and high levels of Foxp3 mRNA, which is considered to be essential for their inhibitory effect. This phenomenon was described in particular subtypes of AML [17], but there are a few reports of Foxp3 overexpression and T regs activity in ALL. However, it

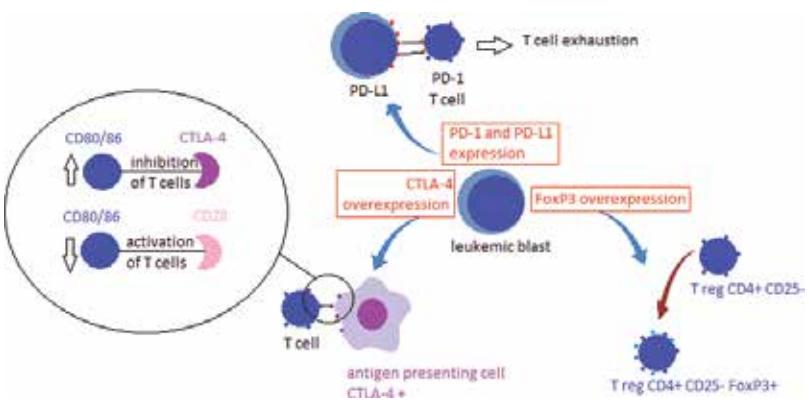

Figure 1. Immune surveillance evasion of leukemic blasts by promoting T regs–inhibitory function.

has been described that B-ALL patients' T regs presented higher immunosuppression than T reg cells from normal healthy individuals. What is more, chemotherapy corresponded to the reduction of Foxp3 and interleukin-10 expression which is also a mediator of cytotoxic T cells suppression [18, 19].

Another way to support inhibitory T regs function mediated by leukemic blasts is the expression of **cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4)** on T cells and the surface of leukemia cells. CTLA-4 binds the ligands which are essential for early T cell activation (CD80 and CD86) and as a result it inhibits T cell activation and increases inhibitory cytokine production by T regs. It has been proved that the higher levels of soluble CTLA-4 and CD86 in B-ALL patients worsen the prognosis and should be considered as potential high-risk factors [20, 21]. Inhibition of CTLA-4 by specific antibody ipilimumab was not yet investigated in acute leukemia in children, but there are ongoing clinical trials assessing its potential in small groups of adults with acute myeloid leukemia, relapsed after allo-HSCT showing promising regression of malignancy, but also immune-related adverse events connected with drug infusion [22–24].

Programmed cell death protein 1 (PD-1) high expression on activated immune system cells and **Programmed cell death ligand 1 (PD-L1)** on blasts due to the tumor influence are mechanisms for leukemia evasion from an immune attack. This molecule induces T cell tolerance by direct inhibition of activated T cells and enhancement of T regs–inhibitory function in myeloid malignancies. Exhausted T cells are no longer capable to produce the cytokines: interleukin 2 (IL-2), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), which impair their cytotoxic effect. This is also the signal to induce the apoptosis of activated T cells. Overexpression of PD-1 is associated with leukemia relapse after hematopoietic stem cell transplantation [25]. Using PD-1 is being investigated as its inhibition (nivolumab) may have the potential to break immune tolerance to AML cells. It may also enhance the cytotoxic effect of donor-derived cytotoxic T cells [26–28].

1.4. To enhance the cytotoxic effect: potential therapies are promoting innate or using adoptive T cells, NK cells, and dendritic cells

Donor lymphocyte infusion is the basic method of the relapse treatment and prevention after allogeneic HSCT mediated through the GvL effect. Its major complication was the high risk of graft-versus-host-disease, which is associated with a donor lymphocyte reaction against host antigens [29]. Its efficacy is nonetheless assessed as disappointing. A major obstacle is tumor-mediated evasion from the immune surveillance by downregulating surface antigens and costimulatory molecules (CD80 and CD86). As a result, T cells are not appropriately activated in vivo to induce an antitumor response. To improve the efficacy of DLI, multiple methods have been used: costimulation with CD3/CD28 and activation ex vivo [30], enrichment of donor T cells with leukemia-specific antigens (WT1) [31] or tumor-specific and host-restricted minor histocompatibility complex antigens [29, 32].

The DLI and GvL effect were the foundation to search for modified approaches to avoid side effects and use the potential T cells against leukemia. The next step was using **genetically modified and activated autologous T cells** to target tumor-specific antigens. The major advantage of this approach is eliminating the risk of GvHD.

At first, genetic modifying was based on **transferring α/β heterodimer of T-cell receptors (TCRs)** to the autologous T cells, but the limitation was the fact that the TCR receptor was only able to recognize antigens presented by human leukocyte antigen (HLA) molecules, which can be downregulated on the tumor cells avoiding immune surveillance. The next idea was to **transfer chimeric antigen receptors (CARs)** instead that are composed of a single-chain-variable fragment (scFv) antibody, which is specific for tumor antigens. CARs have an ability to recognize and fight the cells presenting any specific antigen without HLA molecules. The engineered cells express antigen receptors against tumor-associated surface antigens, thus redirecting the effector cells and enhancing tumor-specific immunosurveillance [33].

CAR-T cell therapy is now being actively investigated in refractory or relapsed ALL in adults and children. At the moment, majority of patients benefit from this approach having achieved remission when the disease appears to be incurable in terms of using standard chemotherapy. Side effects are mostly immune-related and reversible. The studies were carried out on small groups of patients, and the results are now to be confirmed in the larger multicenter trials [34, 35].

The potential limitation that make a CAR-T therapy ineffective in some groups of patients is the lack of the antigens that would be specific only for leukemia cells and their ability to downregulate the antigens by the neoplastic cells, but also unsatisfactory persistence of CAR-T cells after an adoptive transfer and predominance of an immunosuppressive microenvironment, which is a result of leukemia and the host immune system interactions [36].

One of the major challenges in terms of defining the ideal CAR-T target antigen is identifying a leukemia-specific molecule, expressed primarily, if not exclusively on the neoplastic cells, absent on their normal hematopoietic counterpart. This is an important field of research in terms of immunotherapy efficacy improvement [37]. The antigen that is commonly used as a target against B-linear blasts is CD19; however, this molecule is not a specific one. Another target that is being currently evaluated in a context of CAR-T therapy in ALL is **CD22**. Targeting CD22 turned out to be effective in vitro and is currently investigated in vivo, but its expression is still not limited to leukemia cells as this antigen is naturally presented by HLA class I on dendritic cells (DCs) and macrophages [38, 39]. In AML, **CD33** is one of the most popular among various antigens that are being investigated. However, it is highly expressed on both leukemic cells and their normal hematopoietic counterpart which explains the severe toxicity of immunotherapy targeting CD33 established in the clinical trials [40]. **CD123** molecule has emerged as more specific for AML blasts as it is expressed at low levels by normal progenitor cells, which makes it more applicable [41]. There is no defined target that could be addressed in the treatment of T-linear ALL, which has a worse prognosis in the pediatric population compared to the B-linear analog.

Further investigations led to multiple improvements of the method, like producing NK CAR cells as an alternative to T cells [42, 43], enhancing cytotoxicity of CAR-T cells by the addition of costimulatory molecules (second and third generation) or by adding chemokine receptors to enable the effective infiltration to the tumor site. For example, the expression of CD40 ligand by genetically modified T cells leads to increased proliferation and secretion of proinflammatory TH1 cytokines, but also enhances the immunogenicity of tumor cells by upregulation of costimulatory molecules (CD80 and CD86), adhesion molecules (CD54, CD58, and CD70),

and human leukocyte antigen molecules on their surface. Improved survival was confirmed on a model with mice [44]. In terms of managing cytokine release syndrome, the researchers work on the antibody-based switches to mediate the interaction between the CAR-T cell and target cells to improve the safety of therapy [45].

In terms of using NK cells in the treatment of leukemia, a possible strategy is using **adoptive transfer of allogeneic NK cells** or **genetically modified autologous NK cells** (CAR-engineered NK cells). Supremacy of NK cells over T cells is connected with its lower potential to cause GvHD while being donor-derived [46, 47]. The limitation in using autologous NK cells is the overexpression of killer immunoglobulin-like receptors (KIRs) on target cells which counteract the activation of the NK cells and tumor lysis. To overcome this problem, it is more accurate to use allogeneic NK cells, but it is also important to examine the donor and recipient in terms of incompatibility of the KIR ligand (which is presented with HLA-Bw4 and HLA-C) [48]. Only mismatched donor's NK cells would be effective in the treatment of residual disease. The efficacy of using adoptive immunotherapy with NK cells is being examined in AML patients who are not eligible for stem cell transplantation. The results indicate that this approach can help to sustain the remission in patients with AML, but its efficacy is limited in active disease and it was only examined in a small group of elderly patients [46]. There are no reports in applying the therapy in patients with ALL.

Eliciting the T cells immunity can also be performed by using **dendritic cell vaccines**, which are modified to present antigens that are characteristic for leukemia blasts. This way, cytotoxic T cells are activated to kill tumor cells overcoming the mechanisms of evading immune surveillance, like downregulating of surface antigens and then T regs function enhancement are present. DCs can be autologous or allogeneic, but the HLA restriction is essential for the second option. The specific antigen that has been used so far is **Wilms' Tumor-1 (WT1)**, which is a characteristic for myeloblasts, especially in relapse, but it is also detectable in some patients with ALL and different solid tumors. This approach was assessed as effective in several patients with posttransplant-relapsed AML or ALL. The GvHD was assessed as mild and no serious adverse events were reported [49–51]. Ongoing clinical trials are developed to assess WT1 dendritic cell vaccines in larger groups of patients.

1.5. To bridge the tumor cell to the killer

Antigens expressed on leukemic blasts can also be utilized as a target for specific antibodies. Hematological malignancies express surface molecules that are accessible in the circulation. Epitopes presented exclusively on leukemic cells would be preferential for the antibody therapy. However, identifying unique ones, characteristic only for the neoplastic cells and not for their normal hematopoietic counterparts, is challenging. There are several mechanisms that can be used to eliminate blasts including internalization of toxins or drugs that are conjugated to the antibodies, antibody-dependent cellular toxicity (ADCC), complement-dependent cytotoxicity (CDC), induction of apoptosis, and direct-engaging endogenous T cells at the leukemic cells surroundings. Antigens that are candidates for antibody therapy in ALL are mostly characteristic for B-linear differentiation, like CD19, CD20, and CD22, but it is necessary to look for the targets not only presented on B-linear blasts, like CD52. Epitope that is targeted in AML is CD33 [52].

Monoclonal antibodies (MoAbs) are capable of eliminating blasts not only by promoting cytotoxic or complement-dependent cell lysis but also by blocking the effects that are advantageous for neoplastic cells, mediated by growth signals and various agonists. They are selective to the targeted molecules so that the treatment-related toxicity can be reduced. Also, the treatment response can be improved by using monoclonal antibodies as they sensitize leukemic blasts to the conventional chemotherapy. Their efficacy is generally limited when employed as a single agent, but in combination with the standard regimens they improve the overall survival even in chemoresistant or posttransplantation-relapsed patients [53, 54].

CD20 was the first epitope that was successfully applied in the therapy of hematological malignancies. **Rituximab** is a chimeric monoclonal antibody, approved in 1997 by the Food and Drug Administration (FDA) in the treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukemia, but its efficacy is also being assessed in combination with chemotherapy in adults with B-cell acute lymphoblastic leukemia. In several studies, targeting CD20 was related to obtaining a prominent improvement of chemotherapy results in the Philadelphia chromosome-negative BCP-ALL [55–57]. **Ofatumumab** is also developed to target CD20; however, its binding site is closer to the cell membrane and with greater avidity than rituximab. This **second-generation anti-CD20** monoclonal antibody is considered to be more effective, even in patients who did not benefit from rituximab. Unconjugated monoclonal antibody that targets **CD22** is called **epratuzumab**. Treatment with epratuzumab was assessed in combination with conventional chemotherapy showing its feasibility in children with relapsed CD22-positive ALL. In several clinical trials, majority of patients achieved early responses [58, 59].

Alemtuzumab is a humanized monoclonal antibody against **CD52**. CD52 is expressed in about 50% of leukemia blasts, including B- and notably in T-ALL and AML. It was assessed in small groups of patients in combination with granulocyte-colony-stimulating factor (G-CSF) to boost antibody-dependent cell cytotoxicity mediated by neutrophils showing transient good responses [60]. One of the promising monoclonal antibodies that can be potentially used in AML is anti-**CD33, lintuzumab** [61, 62]. Clinical trials revealed high efficacy in the reduction of leukemic blasts, but remissions were only reported after effective cytoreduction, not in patients with high tumor burden [63].

To improve leukemic-targeted toxicity, we can also take one step further. If a target is known to internalize on binding, it is effective to use **monoclonal antibodies conjugated to cytotoxic compounds**, producing an additional mechanism for cytotoxicity. For example, CD22 is proven to be internalized on antibody binding. **Inotuzumab ozogamicin** is the antibody targeting **CD22 linked to calicheamicin** that showed improvement over chemotherapy including complete hematologic remission, longer progression-free, and overall survival [64, 65]. The analog that could have been potentially used in AML is **gemtuzumab ozogamicin**, targeting **CD33.** However, according to its toxicity and increased risk of veno-occlusive disease (VOD), it has been withdrawn in 2010. Another approach using antibody-dependent mechanisms of tumor cell lysis is using **immunotoxins**, which are recombinant anti-CD22 or anti-CD19 conjugated with Pseudomonas or Diphtheria endotoxins. **Radioimmunoconjugates** are monoclonal antibodies linked to radioactive isotopes that can be beneficial as the part of hematopoietic stem cell transplantation regimens, but they are non-preferential to be used in children.

Bispecific T-cell engagers (BiTEs) are designed to redirect and activate cytotoxic T cells precisely at the site of a tumor. The idea is to create antibody-based constructs that temporarily bridge T-cells and cancer cells. The most popular and widely investigated bispecific antibody, **Blinatumomab, targets CD3 and CD19.** Based on multiple clinical studies that have shown an achievement of durable complete remission and acceptable safety profile, the FDA granted accelerated approval for blinatumomab for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia in 2014 [66–68]. AML treatment requires using BiTEs that are compatible to antigens on myeloblasts. **AMG 330** is designed to target **CD33 and CD3**. Clinical studies indicate that this approach is efficient in relapsed AML, especially when combined with blockade of the PD-1/PD-L1 [26].

2. Conclusions

In the era of discovering new approaches to improve survival in childhood hematological malignancies, immunotherapy has a strong position. Potential benefits that can be achieved by implementation of highly active targeted therapies in pediatric acute leukemia are numerous. Improved overall survival and event-free survival is the major advantage, but the possibility to reduce treatment-related toxicity is also extremely important for improving the convalescent's quality of life. Treatment strategies are now actively investigated in multiple clinical trials, mostly in adults, but also in the pediatric population and the results are promising. However, they can be evaluated only in situations when there are no longer better treatment strategies present in refractory or relapsed leukemia, which means that their efficacy is being assessed in desperate settings with high blasts burden and more aggressive neoplastic cells mutated according to the previous treatment [69]. It would be important to evaluate the role of immunotherapy combined with frontline regimens, whether this approach optimizes the treatment efficacy. For example, remission induction is the phase of impaired T regs number and function, which indicates that it is potentially beneficial to combine the T regs depletion with cytoreduction. What is more, it has been proven that the combination of different immunotherapeutic strategies has the synergistic effect. T regs depletion with CAR-T or bispecific antibodies straightens the efficacy of T cells cytotoxicity [26, 62].

There are still many challenges and difficulties to overcome to make the treatment of childhood acute leukemia more effective and safe. Apart from numerous studies that provide a better understanding of the biology and genetics of leukemia, the impact of immunological processes that influence the treatment response was underestimated for a couple of years. Significant breakthroughs achieved in immunotherapy that improved survival in patients with the most resistant disease triggered a renewed interest in this field of treatment. Immunotherapeutic strategies are being constantly improved using the advances of engineering techniques and a better understanding of immunological mechanisms that play a role in tumor surveillance. The assortment is impressive at the moment and is getting even wider, but it appears that in everyday clinical practice the opportunities are not adequately utilized.

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Chapter 5

Lupan-Skeleton Pentacyclic Triterpenes with Activity against Skin Cancer: Preclinical Trials Evolution

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Additional information is available at the end of the chapter

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Abstract

Skin cancer is an increasingly frequent pathology, with a dangerous high percentage of malignant melanoma. The use of synthetic chemotherapy raises the problem of severe adverse effects and the development of resistance to treatment. Therefore, the use of natural therapies became the focus of numerous research groups due to their high efficacy and lower systemic adverse effects. Among natural products evaluated as therapeutical agents against skin cancer, betulinic acid was emphasized as a highly selective anti-melanoma agent and is currently undergoing phase II clinical trials as topical application. Several other pentacyclic triterpenes exhibit antiproliferative activities. This chapter aims to present the latest main discoveries in the class of pentacyclic triterenes with antitumor effect and the evolution of their preclinical trials. Furthermore, it includes reports on plant sources containing pentacyclic triterpenes, as well as the main possibilities of their water solubilization and cancer cell targeting. A review on recent data regarding mechanisms of action at cellular and molecular levels complements information on the outstanding medicinal potential of these compounds.

Keywords: pentacyclic triterpenes, betulinic acid, lupane, preclinic, mechanism of action

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1. Introduction

Skin cancer represents one of the most frequent cancers with an increasing incidence over the past decades [1]. Malignant melanoma, squamous cell carcinoma and basal cell carcinoma represent 98% of all skin cancers [2]. Malignant melanoma determines a higher mortality compared to nonmelanoma skin cancers, being responsible of 75% of skin cancer deaths [2, 3]. Sun exposure is one of the major risk factors, but it influences differently the types of skin cancer. Squamous cell carcinoma is more often related to chronic sun exposure, while malignant melanoma is caused by intermittent sun exposure and overexposure in childhood [4].

Nonmelanoma skin cancer is considered to have the highest incidence of all cancers and occurs more frequently in people with white skin [5]. However, 232,000 new cases of malignant melanoma were diagnosed in 2012, with the highest incidence in Australia. The number of deaths due to this type of skin cancer was 55,000 worldwide in the same year [6]. Malignant melanoma cases tripled in the last 30 years in the United States and Europe [1]. According to World Health Organization [7], each year occur 132,000 cases of melanoma skin cancers worldwide. The increasing incidence is associated with an increase of treatment costs. This aspect underscores the important role of prevention and early detection efforts for this type of cancer [8].

Even though numerous efforts were made for finding effective treatments in melanoma, prognosis for these patients remains unsatisfactory. The standard treatment in early stages is represented by surgical excision, followed by an adjuvant therapy or enrollment in a clinical trial [9]. An early detection of melanoma and a proper treatment increase the chances for cure. Surgery, chemotherapy, immunotherapy or radiation therapy can be used for the treatment [10]. Interferon-α (IFN-α) is used as an adjuvant therapy in patients with high-risk cutaneous melanoma, improving mainly disease-free survival but also overall survival, though not without side effects [11]. An improvement of survival in patients with stage III melanoma has been noticed for ipilimumab therapy. This monoclonal antibody increases the immune response and is approved for treatment in advanced melanoma, but also causes gastrointestinal, endocrine and hepatic adverse effects [12]. New drugs have also been used in the treatment of patients with inoperable or stage IV cutaneous malignant melanoma. The BRAF inhibitors vemurafenib and dabrafenib, the anti-PD1 (programmed death 1) antibodies pembrolizumab and nivolumab or the MEK (mitogen-activated protein kinase) inhibitors trametinib and cobimetinib are new agents proposed in melanoma therapy [13]. Associations of BRAF inhibitors (dabrafenib) and MEK inhibitors (trametinib) have also been evaluated in order to improve overall survival and to delay the appearance of drug resistance in patients with metastatic melanoma with BRAF mutations [14].

In nonmelanoma skin cancer, the therapy is different depending on the severity of the tumor. Standard excision or Mohs micrographic surgery (MMS), radiotherapy, photodynamic therapy (PDT), cryosurgery and topical treatment with imiquimod or 5-fluorouracil are employed in the management of this type of skin cancer [15].

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Despite the numerous studies and the advances in targeted therapy and immunotherapy, the treatment options in melanoma are limited [9]. The main inconveniences of chemotherapeutic agents currently used in skin cancer are the severe side effects and the multi-drug resistance [16].

Due to these disadvantages of conventional therapies, new alternatives have been investigated in order to find compounds that can serve for the synthesis of new drugs [16]. Plant-derived compounds are intensively studied as anticancer agents, many studies being performed to evaluate their properties in different types of cancer, including skin cancer [17].

2. Plant sources of pentacyclic triterpenes with lupane scaffold

Plants have gained over the time an important place in the prevention and treatment of various medical conditions. Extracts from plants were obtained since ancient times following simple procedures and used as teas, potions and ointments in an attempt to alleviate pain and to cure diseases. Natural sources of drugs remain an important branch in pharmaceutical drug discovery and therapeutic implementation. Combinatorial chemistry as an initial source of information was unable to offer the expected amount of final products, but is considered a tool for preliminary analysis of new drugs even on cancer treatment. Several groups of researchers provided routes to refine and improve the skeleton of natural compound and to prepare novel active agents [18]. Links between natural sources, synthetic chemistry and knowledge about genetic analysis of microbes are new trends in preclinical evaluations [18, 19]. Drugs derived from nature may be fall in one of the following categories: natural product botanical, derived from natural product or made by total synthesis but with the specification that the pharmacophore is in relation with a natural product [18].

During the last decades, natural remedies are engaged in an unprecedented evolution aimed at an increased efficacy. The development of sophisticated technologies in the fields of phytochemistry, drug formulation and pharmacology, as well as the focus on the mechanism of action on a cellular and molecular level, enables the obtainment of highly efficient drugs from plants.

One of the numerous categories of plant phytochemicals are triterpenes (**Figures 1**–**3**). So far, over 20,000 triterpenes have been isolated from the plant kingdom. They include a variety of structural subtypes: squalene, lanostane, dammarane, tetranortriterpenoids, lupane, oleanane, ursane, hopane and other [20, 21]. Pentacyclic triterpenes, their natural sources and biological effects are presented in **Table 1**.

Among plant sources containing lupan-skeleton pentacyclic triterpenes (**Figure 2**), birch bark has received particular attention due to its high content in these substances, its well-proven application and uses over the time [41]. Currently, it is acknowledged that the outer birch bark is a rich source of pentacyclic triterpenes, which include: betulin (B lup-20 (29)-ene-3β, 28-diol), betulinic acid (BA, 3β acid, hydroxy-lup-20 (29)-en-28-oic) and lupeol

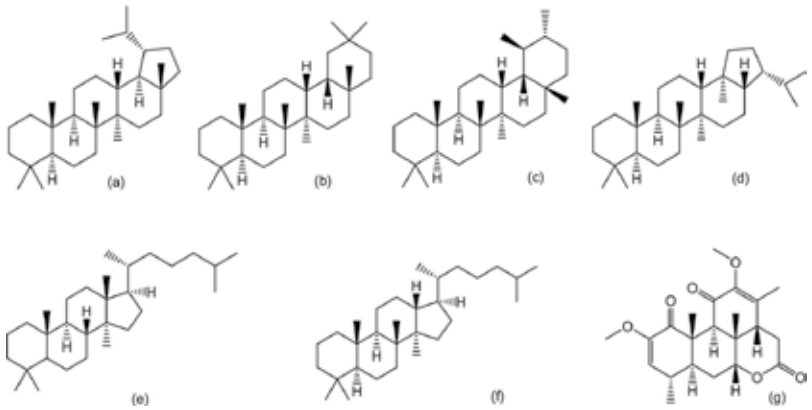

Figure 1. Triterpene structures (a) lupane; (b) oleanane; (c) ursane; (d) hopane; (e) lanostane; (f) dammarane and (g) quassin.

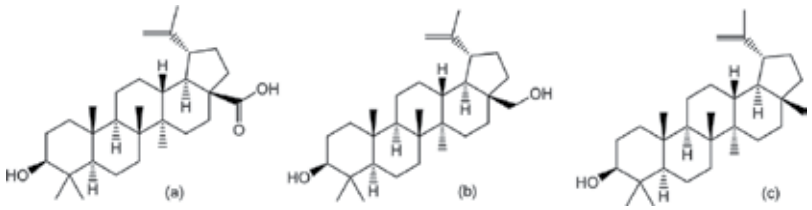

Figure 2. Lupan skeleton triterpenes (a) betulinic acid; (b) betulin and (c) lupeol.

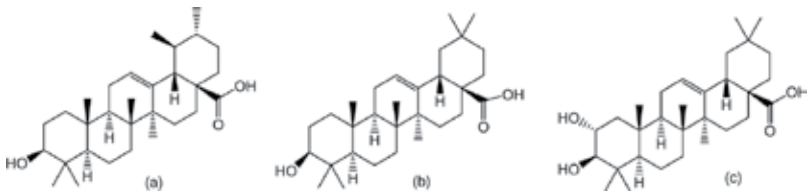

Figure 3. Other triterpenes (a) ursolic acid; (b) oleanolic acid and (c) maslinic acid.

(L, Lup-20 (29)-en-3-ol). The development of birch bark extracts, their applications and bioactivity has comprehensively been reviewed [42]. By analyzing birch bark extract, it was shown that betulin is present in the highest amount, while betulinic acid content is lower. However, it is possible that plants from different geographical regions present a variable content in pentacyclic triterpene, which requires a rigorous analysis of the content [43]. Differences between barks of birch species regard the content in: betulin, betulinic acid, betulinic aldehyde, lupeol, oleanolic acid, oleanolic acid 3-acetate, betulin 3-caffeate, erythrodiol and other.

Substance	Plant (family)	Plant part	Study/effect	Reference
BA	*Ziziphus mauritiana* Lam. (Rhamnaceae)	Stem bark	*In vitro*—inhibitory effect on (MEL-1, -2, -3, -4) cells; apoptotic effect on MEL-2 cells *In vivo*—antitumor effect on athymic mice injected with MEL-2 cells	[22]
BE, LU	*Betula x caerulea* Blanch., *Betula cordifolia* Regel, *Betula papyrifera* Marsh., *Betula populvolia* Marsh. (Betulaceae)	Bark	N/A	[23]
BA, LU, BE, UA, OA	*Syzygium formosanum* Hay. Mori (Myrtaceae)	Leaves	N/A	[24]
BE, BA, UA	*Diospyros leucomelas* Poir. (Ebenaceae)	Leaves	*In vivo*—anti-inflammatory activity on Swiss mice, for induced ear edema and induced paw edema	[25]
OA	*Rosa canina* L. (Rosaceae)	Rose hip, powder	*In vitro*—immunomodulatory activity on Mono Mac 6, obtained when a mixture of OA, BA and UA was used	[26]
BA	*Rosmarinus officinalis* L. (Labiatae)	Stems and leaves	*In vivo*—antidepressant-like effect in the TST, for Swiss mice; anti-immobility effect	[27]
BE, BA	*Betula pendula* Roth, syn. *Betula verrucosa* (Betulaceae)	Bark	*In vitro*—cytotoxic effect in EPG85-257 and EPP85-181 cells line	[28]
A, BA, BE, LU, UA, OA	*Ligustrum pricei* Hayata, *Ligustrum sinense* Lour., *Ligustrum lucidum* W.T.Aiton (Oleaceae)	Leaves	*In vivo*—analgesic and anti-inflammatory effect on Sprague Dawley rats	[29]
OA, BA	*Viscum album* L. (Santalaceae)—harvested from *Malus domestica* Borkh.	Sprout	*In vitro*—cytotoxic and apoptotic effect on B16.F10 cells	[30]
OA, BA	*Viscum album* L. (Santalaceae)—harvested from *Malus domestica* Borkh.	Sprout	*In vivo*—antiapoptotic, antiproliferative effect on C57BL/6NCrL mice injected with B16.F10	[31]
BE, UA	*Myrica cerifera* L. (Myricaceae)	Bark	*In vitro*—cytotoxic activity against HL60, A549 and SK-BR-3 cell lines	[32]
LU	*Taraxacum* sp. Dandelion (Asteraceae)	Root	*In vitro*—cytostatic, not cytotoxic effect on B16 2F2 cells; inhibition of cells proliferation by differentiation	[33]
LU	*Lactuca indica* L. (Asteraceae)	N/A	*In vivo*—prevents local tumor progression, distant metastasis in dogs with COMM Dogs: two miniature Dachshunds, two Beagles, two miniature Schnauzers, one Golden Retriever, one Labrador Retriever, one American Cocker Spaniel, one Cavalier King Charles Spaniel and 1 mixed-breed dog	[34]
LU	*Lactuca indica* L. (Asteraceae)	N/A	*In vivo*—tumor growth suppression and induced cell cycle arrest in C57BL/6 mice injected with B16 2F2 cells	[35]

Substance	Plant (family)	Plant part	Study/effect	Reference
LU	*Bombax ceiba* L. (Malvaceae)	Stem Bark	*In vitro*—antiangiogenic effect on SK-MEL-2, A549 and B16-F10 cell lines	[36]
BA, OA	*Paeonia rockii* ssp. rockii T.Hong & J.J.Li (Paeoniaceae)	Root	*In vitro*—antiapoptotic effect induced selectively in the M-14 cell line	[37]
UA	*Salvia officinalis* L. (Lamiaceae)	N/A	*In vivo*—antiprotease and antimetastatic effects on C57BL/6N mice injected with B16 cells	[38]
BA	*Avicennia officinalis* L. (Acanthaceae)	Leaves	*In vivo*—anti-inflammatory effect on rats	[39]
BA, UA, MA	*Bridelia cambodiana* Gagnep. (Phyllanthaceae)	Whole plant	*In vitro*—cytotoxic effect against HL60 and LCC cell lines	[40]

BA (betulinic acid); BE (betulin); LU (lupeol); UA (ursolic acid); OA (oleanolic acid); MA (maslinic acid); N/A (not applicable); MEL-1, -2, -3, -4 (human melanoma cell line); Mono Mac 6 (human monocytic cell line); TST (tail suspension test); EPG85-257 (human gastric carcinoma cell line); EPP85-181 (human pancreatic carcinoma cell line); B16.F10 (murine melanoma cell line); HL60 (human promyelocytic leukemia cell line); A549 (human lung carcinoma cell line); SK-BR-3 (human breast cancer cell line); B16 2F2 (mouse melanoma derived subclone with high differentiation capability); COMM (canine oral malignant melanoma); SK-MEL-2 (human melanoma cell line); M-14 (human melanoma cell line); B16 (mouse melanoma cell line derived from spontaneous skin tumor); LLC (mouse lewis lung carcinoma).

Table 1. Bioactivity of various plant products containing pentacyclic triterpenes.

3. Obtainment of lupane-skeleton triterpenes with efficacy in skin cancer

Pentacyclic triterpenes from plants are secondary metabolites with high lipophilicity. Therefore, they are mainly located in hydrophobic histological structures. In the cork of trees, which represents the outer tissue of the secondary bark, triterpenes are associated with suberin; a well-known example is birch bark [44]. Triterpenes are as well components of cuticular and epicuticular waxes covering leaves [45] and fruits [46].

Betulin was isolated for the first time in the 1788 by Lowitz [47] from birch cork. The elucidation of its structure was performed by only in 1953 by Guider et al. [48]. Additional plant sources for betulin include hornbeam (*Carpinus betulus* L) and hazel (*Corylus avellana* L.), plants which are phylogenetically closely related to birch [49]. Betulinic acid, a triterpene of major therapeutic relevance, was isolated under the name of "graciolon" from *Gratiola officinalis* [50] and recognized as such only 40 years later [51]. "Platanolic acid," isolated from *Platanus acerifolia* bark [52], proved later to be betulinic acid as well [53]. Furthermore, betulinic acid could be obtained from an alcoholic extract of *Cornus florida* L. bark [54]. Ko and co-workers [55] used mistletoe (*Viscum album*) to obtain an ethanol extract enriched in triterpenes, including betulinic acid and botulin.

The obtainment of triterpenes from the plant matrices employs as a first step extraction with organic solvents such as methanol or ethanol [56]. Other solvents are chloroform, dichloromethane, ethyl acetate, petroleum ether or various mixtures thereof, in accordance with the low polarity of these phytocompounds. Recovery procedures may include Soxhlet extraction,

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maceration and ultrasound-assisted processes [57]. In order to progressively enrich/isolate triterpenes, the usual phytochemical approaches are employed: partition among solvents of increasing polarity, column chromatography on silica gel, countercurrent chromatography and preparative chromatography. Triterpene acids are extracted after alkalinization with sodium hydroxide [57] or calcium hydroxyde [58]. Pure betulin was prepared from a crude mixture using a chromatographic column with silica gel as a stationary phase and a mixture of hexane and ethyl acetate as eluent, followed by recrystallization from 75% ethyl alcohol [59]. An effective preparation of crystalline betulin (99% purity) from birch bark is clearly described in a recent work, following the steps to remove betulinic acid and lupeol. Additionally, the authors demonstrate the obvious relationship between the cytotoxic activity of betulin and its purity [58]. The analytic determination of triterpenes in samples is performed by reverse-phase HPTLC and gas chromatography, coupled with the detection using mass spectrometry detection, is a widely used method for analysis of betulin and other triterpenes in samples. High-performance thin-layer chromatography (HPTLC) is a valuable straightforward tool for the visualization of impurities [60].

Betulinic acid received high attention due to its properties to inhibit the growth of cancer cell lines, without being cytotoxic to normal cells. In plant materials used as sources of triterpenes such as birch, the content in betulinic acid is much lower than that in betulin. For this reason, various attempts have been made to obtain betulinic acid, using betulin as a starting point. In the study of Melnikova and co-workers [61], the most intense catalytic activity was noticed for aluminum salts, which also have a selective activity. The reaction proceeds via the intermediate betulonic acid, purified by recrystallization. Betulonic acid is reduced with $NaBH_4$ (THF or isopropanol) at room temperature to obtain a mixture of 3α-betulinic acid and betulinic acid-3β [61].

Enzymatic transformations, privileged for their simplicity, eco-friendliness and safety, are currently a mainstay in the obtainment of drugs. In an enzymatic approach, the fungus *Armillaria luteo-virens* Sacc ZJUQH100-6 was employed in the biotransformation of betulin into betulinic acid [62]. Optimization of the obtainment was monitored by variation of parameters like pH, glucose, betulin content, addition of tween 80 and stage of inoculation; the presence of the surfactant had a significant impact on the yield of the biotransformation.

While betulin is readily available from birch and other plants, its anticancer activity is only moderate. Being an accessible starting point for derivatizations, betulin has been the subject of many researches, aiming to obtain compounds with enhanced anticancer activities [63]. The acetylated derivates were tested for antiproliferative effect on several cell lines: colorectal adenocarcinoma, leukemia and breast cancer. By esterificating betulin with propionic acid in dichloromethane solution in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine, derivatives: 28-O-propynoylbetulin and 3.28-A, IB, dipropynoylbetulin were obtained. Column chromatography was employed in order to obtain the pure components with a yield of 60% in the case of the first derivative and 12% in the second. The reaction of betulin with propargyl chloroformate and 3-butyl-1-yl chloroformate in benzene, in the presence of pyridine, resulted in the formation of a mixture of 28-O-propargyloxycarbonylbetulin monoesters and 28-O- (3-butynyloxycarbonyl) toxin and di-3,28-A sheep-di (pro-pargyloxycarbonyl)

3.28-A toxin and sheep-di (3-butyn-yloxycarbonyl) toxin. The resulting mixture was separated by column chromatography; thus, pure components were obtained in a 64–69% yield for monoesters and 23–27% in the case of diesters [64].

4. Advanced formulation of lupane triterpenes

The most challenging aspect of the biomedical use of lupane triterpenes is their low water solubility which subsequently causes poor bioavailability [65]; so far, several delivery systems have been developed in order to achieve superior pharmacokinetic outcomes. The current subchapter aims to review the most recent and promising delivery options for betulin, betulinic acid and lupeol.

The first step in the attempt to modulate the aqueous solubility of an insoluble compound relies in its convenient derivatization with water-soluble partners; such an attempt was conducted by Drag-Zalesinska and co-workers [66], who prepared mono- and diesters of betulin and betulinic acid with amino acids. All esters revealed higher water solubilities and significant cytotoxic activity via apoptosis induction; the type of ester as well as the type of the amino acid side chain strongly influences the biological effect of the respective compounds [66]. C(2)-propargyl-substituted pentacyclic triterpenoids conjugated with 1,2,3-triazole glucopyranosides were synthesized via "click" chemistry in order to achieve optimized water solubility as well as pharmacokinetic and pharmacological properties [67].

Cyclodextrin (CD) complexation represents an attractive solution to increase the aqueous solubility of numerous compounds; through their hydrophobic interior and hydrophilic surface, cyclodextrins are able to accommodate various lipophilic guest molecules which can thus be water-solubilized. According to molecular studies [68], the bulky structures of betulinic acid and betulin fit best inside the cavity of γ-CD and its semisynthetic derivatives, such as hydroxypropyl-γ-CD (HPGCD). As a result of HPGCD complexation, a 14-fold increased water solubility was reported for BA accompanied by superior biological activity [69], *i.e.*, strong antiangiogenic and antitumor effect [70, 71]. Similar outcomes were achieved in terms of anti-melanoma activity tested on B16 cell line (murine melanoma) [72]. Fontanay et al. conducted a study on the inclusion of hydroxy pentacyclic triterpenoid acids, including BA, inside native γ-CD [73]; the physicochemical analysis revealed the formation of a 1:1 complex with a significantly improved aqueous solubility.

β-CD derivatives also served as complexation partners for BA, and its inclusion in the cyclodextrin hydrophilic matrix led to significantly improved dissolution rate and, subsequently, antiproliferative *in vitro* activity against MCF7 (breast cancer) cell line [74]. The same tumor cell line was involved in the study of the biological activity of betulinic acid accommodated inside native β-CD [75]; a dose-dependent antiproliferative activity was reported, through mitochondria-mediated apoptosis induction and G2/M cell cycle arrest.

An important parameter in the cyclodextrin inclusion process is the stability constant of the final complex, its value giving the measure of the potential use of the complex as biologically active agent; significantly high stability constants were achieved for both betulin and

betulinic acid in complex with newly synthesized hydrophilic γ-CD derivatives [76]. Such a high stability constant characterizes a strong interaction between the host- and the guest molecule, thus enabling the delivery of the active drug at the target site in the absence of systemic adverse effects. Both complexes, with betulin and betulinic acid, respectively, were *in vitro* and *in vivo* tested, revealing moderate *in vitro* antiproliferative activity; however, *in vivo* results on murine models showed a significant decline in tumor size and volume [77, 78].

The inclusion of betulin inside HPGCD led to optimized outcomes in terms of bioavailability and antiproliferative activity [79, 80]; similar results were reported for betulin complexation with hydrophilic β-CD derivatives that caused stronger inhibitory activity against MCF7 (breast cancer) cell line than pure betulin [74].

Lupeol was also subjected to inclusion inside γ-CD by kneading in a 1:2 molar ratio; the complex revealed optimized antiproliferative and antiangiogenic activities compared to the pure drug [81].

The use of triterpenes as mixtures such as total extract of birch outer bark may trigger simultaneously various mechanisms of apoptosis induction and therefore result in an additive or synergistic effect. Hertrampf et al. [82] used HPBCD as solubilizer for birch total extract; a series of dilutions were prepared using the main ingredient, betulin, as a reference to calculate concentrations. The study reported the multivalent cytotoxic activity of the birch bark at lower concentrations than previously used presumably due to a higher bioavailability of triterpenes provided by cyclodextrin solubilization; moreover, a synergistic effect was suggested. Triterpene-rich mistletoe extract (6.9% BA) was solubilized by Strüh et al. [30] by using HPBCD and tested against B16F10 melanoma cell line; a dose-dependent reduction of cellular ATP was reported accompanied by high cytotoxicity due to DNA fragmentation. The research was continued by in vivo studies on C57BL/6 mice bearing B16F10 subcutaneous melanoma, revealing an increased antitumor effect and a prolonged mice survival [31].

An alternative research direction was the preparation of cyclodextrin conjugates instead of inclusion complexes; "click chemistry" was involved in the synthesis of triazole-bridged conjugates between β-CD and pentacyclic triterpenes [83]. All bioconjugates showed higher hydrophilicity than the parent compound, and several conjugates displayed significant cytotoxicity on various cancer cell lines; in addition, the cyclodextrin conjugation led to the disappearance of haemolytic toxicity. The authors continued their research by synthesizing α-CD conjugates with several pentacyclic triterpenes including BA [84]; all conjugates exhibited lower hydrophobicity than the parent molecules accompanied by significant anti-HCV (hepatitis C virus) entry activity.

An excellent review was published in 2016 by Lima et al. [85], describing the main attempts to use cyclodextrins as nanocarriers for various terpenes; the authors concluded that cyclodextrins are feasible tools in improving the pharmacological profile of terpenes, limited mainly by the scarce pharmacokinetic and clinical studies.

Liposomes are small vesicles displaying one or more phospholipidic layers and an aqueous core [86] that may incorporate both lipophilic and hydrophilic compounds [87, 88]. Betulinic acid was trapped inside large liposomes by Mullauer et al. [89] and administered to mice

bearing experimental models of colon (SW480) and lung (A549) cancer; no systemic adverse effects were reported following parenteral (i.v.) and oral administration. Similar studies reported liposomal and proliposomal formulations with BA with 95% yield of the incorporation process [90]. Phospholipidic nanosomes prepared by means of supercritical fluids were used to entrap BA in order to increase its efficacy as antiviral agent [91, 92]. Several betulin derivatives such as 28-acetylenic derivatives [93] and pyrazoles and 1,2,3-triazole derivatives [94] were synthesized and formulated as liposomes; the nanoformulations exhibited strong apoptotic activity due to both higher biological effect of the active compound and optimized delivery. PEG-ylated BA liposomes were obtained by Liu et al. in 2016, entrapping BA in the lipid bilayer of the liposomes by the ethanol injection technique [95]; the hydrophilic outer PEG layer ensured improved sustained release and antitumor effect compared to free BA or BA liposomes.

Another attractive option in drug delivery is the use of micro- and nanoemulsions; a nanoemulsion containing BA was prepared using flax-seed oil as lipophilic phase and the high-pressure homogenization method [96]; the *in vivo* testing on the chorioallantoic membrane (CAM assay) revealed a significant antiangiogenic activity. The same procedure was applied for betulin nanoemulsion, followed by *in vivo* testing by CAM assay and experimental murine cancer model; the study reported strong anti-inflammatory, antiproliferative and antiangiogenic activities of the incorporated betulin as well as its potential benefits in inhibiting metastasis [97]. An oil-in-water nanoemulsion with BA was prepared through the use of phospholipase-catalyzed modified phosphatidylcholine as emulsifier in an ultrasound device; various factors such as composition, ultrasound amplitude, temperature and pH significantly influenced nanoparticle size and stability [98].

A different approach consists in the administration of betulin via the nasal route; in order to avoid mouth sedimentation of betulin particles, the solvent exchange method was used to limit particle sizes to nanoscale, thus leading to higher bioavailability of betulin in the lower respiratory tract [99].

Water solubility may be increased through grinding with hydrophilic polymers (i.e., polyvinylpyrrolidone, polyethylene glycol, arabinogalactan) [100, 101]; solid dispersions of BA with various hydrophilic polymers (*i.e.*, Soluplus, HPMCAS-HF, Kollidon VA64, Kollidon K90, Eudragit RLPO) in 1:4 (w/w) ratio were prepared and analyzed by Yu et al. [102] in 2014, revealing a great potential to increase BA water solubility. Moreover, hydrophilic bioconjugates can be synthesized between active drugs and hydrophilic polymers. BA-monomethoxy polyethylene glycol (mPEG) conjugate was synthesized by covalent bonding of the carboxyl moiety of BA and the amine groups of mPEG [103]; the conjugate exhibited cytotoxicity through cell apoptosis on hepatic cancer cells (Hep3B, Huh7) as well as *in vivo* antitumor efficacy in Ehrlich ascites tumor (EAT) model while lacking any sign of biochemical and histological toxicity. A step further was represented by the use of multiarm-PEGs as conjugation partner which offer a high density of functional groups; through the formation of an ester bond, BA was linked to eight-arm PEG (8arm-PEG) and then to a targeting molecule (folate) followed by the self-assembly into nanoparticles [104]. A second anticancer drug, hydroxycamptothecin, was added by nanoprecipitation; the ensemble achieved a dramatically increased cytotoxicity, prolonged blood circulation, enhanced tumor targeting and lower systemic toxicity than the

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free drugs; in addition, a synergistic antitumor efficacy was reported [104]. BA also shows the ability to self-assemble into nano- and microfibers with antileukemic efficacy and cytoprotective activity as well [105].

Biodegradable polymeric nanospheres based on poly(lactide-co-glycolide)-poly(ethylene glycol) (PLGA-PEG) were prepared by nanoprecipitation to incorporate 40% BA [106]; the study reported an increased cytotoxicity and lower IC50 value compared to the pure drug. PLGA was used as building material by interfacial deposition for nanocapsules that efficiently entrapped lupeol [107]. Polymer matrixes can be involved in regional chemotherapy, an approach that avoids systemic adverse effects [108] and allows the controlled release of the pure drug; betulin was incorporated as model compound in such a matrix (poly(3,4-ethylenedioxythiophene), its release being conducted by passive or active mode. The novel formulation exhibited efficient cytotoxic activity against KB and MCF7 cancer cell lines.

BA conjugates with carboxyl-functionalized single-walled carbon nanotubes were synthesized via π–π stacking interaction, leading to a 20% loading of the active drug [109]; following physicochemical and biological analysis, the authors reported the controlled, prolonged release of the drug, with no sign of toxicity on normal fibroblasts and significant cytotoxicity against A549 (lung cancer) cell line. The research continued by coating the nanotubes with four biopolymers: tween 20, tween 80, polyethylene glycol and chitosan in order to further improve biocompatibility [110]; the procedure induced sustained and prolonged release compared to the uncoated nanotubes, while cytotoxicity depended on the chosen biopolymer.

Metallic nanoparticles were also used as nanocarriers for pentacyclic triterpenes; as an example, magnetic nanoparticles coated with chitosan were loaded with BA and exhibited a pseudo-second-order kinetic model release of the active drug [111]; the nanoparticles were cytotoxic on MCF7 cells in a dose-dependent manner while lacking toxicity against normal mouse fibroblast cells. Silver nanoparticles coated with BA were involved in the in vitro testing on a panel of cancer cell lines, including A375 (murine melanoma) [112]; the new formulation revealed strong antiproliferative and antimigratory activity, in particular against melanoma cells.

5. Innovative approaches in preclinical evaluations of pentacyclic triterpenes of the lupane series in skin cancer

Preclinical trials are important in the initial evaluation of new drugs, formulations or specific new design of pathology. They are complex processes with an uncertain ending, as just a reduced percent of evaluations lead to a market product. It is estimated that around 90% of tested drugs are not launched to the market [113]. Despite intense of basic research, the actual delivery of accepted drugs is scarce.

The classical route of a tested compound in preclinical trials includes *in vitro* tests followed by *in vivo* tests on animals [113]. These tests may be conceived in a variety of ways and are

constantly improved and diversified. The mainstay of preclinical tests regarding a potential efficacy against skin cancer types is *in vitro* tests using different types of cell lines. In this regard, lupane triterpenes were tested on human melanoma (G361, SK-MEL-28, MEL-2, SK-MEL2, A375), mouse melanoma (B16-F1, B16 2F2) and human skin epidermoid carcinoma A431. In case of betulin, the latter showed a particularly high sensitivity (with a IC50 value below 10μM), while various types of human melanoma cell lines may display a high variation range of the sensitivity to betulin, with differences in IC50 values of one order of magnitude, from 12.4 to over 250 μM [114]. For betulinic acid, IC50 was 154 μM when tested on A375 melanoma [115], and 70 μM when tested on B16-F10 murine melanoma [116]. Information of particular relevance for the actual clinical utilization as anticancer agent comes from comparative data on the cytotoxicity against normal cells and cancer cell lines. Betulin, for example, is more cytotoxic against cancerous cells than nontumoral ones [114]. Further steps in preclinical evaluation are the investigation of the mechanism of action; lupeol, betulin, betulinic acid and their semi-synthetic derivatives have so far shown significant effects on apoptosis and cell cycle regulation [117, 118]. As inflammation is an important player in the pathogenesis of cancer, the anti-inflammatory effects and mechanisms are as well explored to give a correct picture of the antitumoral potential [117]. Furthermore, it is important to explore the antimigratory potential of natural products, as it has a seminal importance for malignant melanoma—a cancer type with a high invasiveness [119]. A global approach to relevant preclinical tests regarding triterpenes should thus be multi-component. In this regard, our workgroup has established an efficient battery of tests for aimed at establishing the potential of natural triterpenes with anticancer/anti-inflammatory activity as agents against skin cancer. The module of preclinical evaluations includes as follows:

- **Step 1**: *in vitro* tests on normal cells (e.g., HaCat) comparing with specific pathological tests; evaluation of cytotoxic activity;
- **Step 2:** *in vitro* evaluations concerning the impact on apoptosis, and observations of specific markers via DAPI/HOPI staining, and evaluation of Annexin V, caspases and other cellular markers [120];
- **Step 3:** *in vivo* embryonated egg membrane assay for toxicological evaluation (HET CAM assay) and investigations of the potential to affect angiogenesis; future aspects include cultivation of cancer cells on embryonated eggs and direct research of the therapeutic potential;
- **Step 4:** *in vivo* tests including a large number of experimental protocols: photochemical model, inoculation of murine cells, xenograft of human pathological cells on adequate mouse hosts and correlations with therapeutical surveillance. Furthermore, histopathological evaluations and immuno-histochemical assays are correlated with innovative approaches like RAMAN skin evaluation, noninvasive methods for skin quality and surface damage characterization.

Additional determinations could require selection of cells from a primary experimental tumor, cultivation of cells and evaluation of compounds, PET animal observations and other methods applied for a detailed pathologic surveillance of drugs.

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6. Pentacyclic triterpenes: mechanism of action at cellular and molecular level

Apoptosis is a programmed cell death consisting in morphological changes including cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, plasma membrane blebbing and caspase activation [121]. In this regard, apoptosis is considered a crucial physiological process in tumor clearance, being a major target for anticancer drugs [122]. The molecular mechanisms of apoptosis can include extrinsic and intrinsic pathways. The extrinsic pathway of apoptosis is initiated by external signals, which can activate TNF/Fas-receptor, which in turn activates procaspase-8 [123]. The activated caspase-8 is involved into the caspase-3, -6 and -7 cascade activation [124]. Caspases are important cellular enzymes synthesized as inactive zymogens, which can be activated into their active tetrameric forms by various apoptotic signals [124]. The activation of caspase-3, -6 and -7 leads to cell death not only by breaking down the cytoskeleton, but also the nucleus.

The intrinsic pathway of apoptosis, known as the mitochondrial pathway, is initiated by internal stimuli which can activate the proapoptotic genes from the outer membrane of mitochondria. Bcl-2 family proteins (Bax, Bak, Bcl-xs) are important proapoptotic genes involved in permeabilization of the mitochondrial membrane in order to release cytochrome c in cytosol, where it binds to the caspase-activating protein apoptotic protease activating factor-1 (Apaf-1) and with the procaspase-9, transforming into an apoptosome [124]. The apoptosome releases the activated form of caspase-9, which is also involved into the caspase-3, -6 and -7 activations, which lead to cell death [123].

Previous evidences showed that the pentacyclic triterpens, especially betulin, betulinic acid, lupeol and ursolic acid, have induced apoptosis in different types of cancer cells via activation of the mitochondrial pathway and not to the death receptor pathway (extrinsic way) [125, 126]. These data have been supported by Drag-Zalesinska et al. [118] study in which betulin and betulinic acid proved to induce apoptosis in human metastatic melanoma cells (Me-45) by releasing cytochrome c or the apoptosis inducing factor (AIF) through the mitochondrial membrane. Liu et al. [122] have also demonstrated that betulinic acid, as well as betulin, could kill CNE2 cells through the mitochondrial pathway. Betulinic acid induced the DNA fragmentation, caspase activation and cytochrome c release but independent of Bax proteins [115, 122]. Moreover, betulinic acid has been also involved in activation of nuclear factor kappa B (NF-κB) responsible for apoptosis in various types of cancer cells [127].

The increased production of reactive oxygen species (ROS) caused by betulin and betulinic acid stimulation [128, 129] has been considered a stress factor involved in the depolarization of mitochondrial membrane [130]. Furthermore, both calcium overload and ATP depletion were additional stress factors responsible for increasing the permeability of the inner mitochondrial membrane through formation of nonspecific pores [116]. For instance, the dimethylaminopyridine triterpenoid derivatives have also caused the depolarization of the mitochondrial membrane in situ, in order to increase the permeability transition pore [131].

Unlike the previous data, the study of Şoica et al. [77] on B164A5 murine melanoma cells and on a mouse melanoma model showed that BE and its derivatives had no effect on caspase-2 regulation, the apoptotic mechanism of betulin being suggested to be probably through the transformation of BE into betulinic acid inside the cells.

According to the study of Muceniece et al. [132] *in vitro*, betulin had a mimetic effect on melanocortin (MC) receptor, especially on MC-1 subtype. This observation has been also supported by Şoica et al. [77] study in which botulin had revealed strong inhibitory effects on B64A5 murine melanoma cells, by binding to the melanocortin receptors. Betulin has been not involved by itself in stimulation of cAMP generation, but it acted as a weak antagonist on alpha-melanocyte-stimulating hormone (alpha-MSH)-induced cAMP accumulation in B16-F1 mouse melanoma cells [132].

In vitro and *in vivo* studies have also revealed that the birch bark extract and betulin have significantly increased the expression of PARP-1 in melanoma cells [118], exhibiting interferon-inducing activity [133].

According to Zhang et al. [126] study, betulinic acid induced apoptosis by suppressing the cyclic AMP-dependent transcription factor ATF-3 and NF-κB pathways and decreasing the expression of topoisomerase I, p53 and lamin B1. On one hand, earlier studies indicated that betulinic acid had induced apoptosis of cells due to the p53 pathways [134]. This conclusion has been also supported by Tiwari et al. [135] study, in which BA proved a dose-dependent apoptotic effect on both p53 mutant and wild-type cells probably because of its involvement in p53-independent apoptotic pathway. On the other hand, a recent study has shown that the apoptotic effect of betulinic acid in human metastatic melanoma cells (Me-45) had been independent of p53-apoptotic pathway [118]. The presumable mechanisms of action of betulinic acid and betulin in skin cancer are depicted in **Figure 4**.

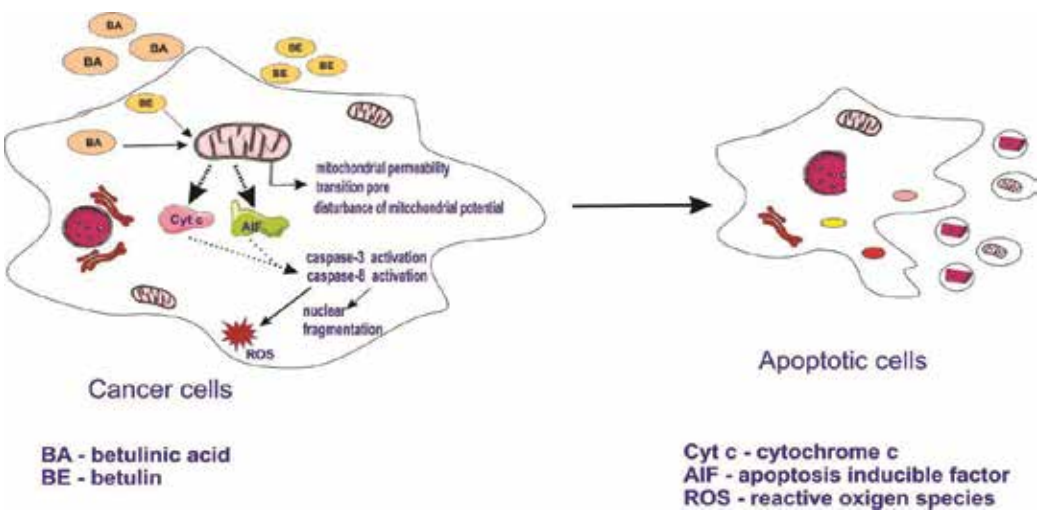

Figure 4. The mechanism of action of betulinic acid and betulin in skin cancer.

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Lupeol is a complex multitarget phytochemical, being involved in controlling IL-1 receptor-associated kinase-mediated toll-like receptor 4 (IRAK-TLR4), Bcl-2 family, nuclear factor kappa B (NF-κB), phosphatidylinositol-3-kinase (PI3-K)/Akt and Wnt/β-catenin signaling pathways [136]. According to the Tarapore et al. study, the anticarcinogenic effect of lupeol has been related to the Wnt/β-catenin signaling pathway. That study has revealed that lupeol caused a dose-dependent decrease in Wnt target genes in Mel 1011 cells. Moreover, there has been also observed a decrease of nuclear β-catenin expression, associated with an enhancement of plasmatic β-catenin expression in melanoma cells (Mel 928 and Mel 1241). Consequently, lupeol has been involved in blocking the movement of β-catenin between cytoplasm and nucleus [137].

An *in vivo* study on Swiss Albino mice showed that lupeol exerted apoptotic effects through the enhancement of bax and caspase-3 genes expression and downregulation of bcl-2 anti-apoptotic genes [138].

Unlike botulin and betulinic acid, lupeol has also induced apoptosis via extrinsic pathway by enhancing the expression of FADD protein and Fas receptors [127].

Ursolic acid has strongly increased the IR-induced apoptotic effect in various types of cancer cells, likely DU145, CT26 and B16F10, playing a major role in DNA fragmentation, mitochondrial dysfunction and apoptotic marker modulation [139]. Moreover, ursolic acid has induced apoptosis in M4Beu cells human melanoma through intrinsic pathway by enhancing the caspase-3 activity in a dose-dependent manner, correlated with a low caspase-9 activity [140]. Ursolic acid has also proved to act as an inhibitor of the endogenous reverse transcriptase (RT) activity in the following tumor cells: melanoma (A375), glioblastoma (U87) and thyroid anaplastic carcinoma (ARO), as well as on nontransformed human fibroblast cell line (WI-38), exhibiting strong antiproliferative effects [141].

The mechanism of apoptosis induced by pentacyclic triterpens is not fully understood, although, according to the previous studies, we can conclude that these triterpens exhibited strong apoptotic effects, especially via intrinsic pathway, being involved in increasing the permeability of inner mitochondrial membrane, activation of caspase-9 and 3, as well as cell death.

7. Conclusion

Pentacyclic triterpenes represent an important issue in the field of antiskin cancer formulations; nowadays, the researches focus on the development of nanoformulations that provide multiple advantages over the classical pharmaceutical formulations, including the possibility of being decorated with targeting moieties that significantly improve the antiproliferative activity of the loaded active drug. Different mechanisms of action have been identified so far at cellular and molecular level, in particular for betulinic acid; however, future studies are needed in order to fully comprehend the intimate details of the anticancer treatment with pentacyclic triterpenes and formulations thereof.

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Chapter 6

Recent Progress on the Molecular Mechanisms of Anti-invasive and Metastatic Chinese Medicines for Cancer Therapy

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Additional information is available at the end of the chapter

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Abstract

Despite of the recent advances in diagnostic and therapeutic approaches, cancer remains as the leading cause of death worldly with diverse causal factors regarding genes and environment. Invasion and metastasis, as one of the most important hallmarks for cancer, have restrained the successful clinical therapy and are the primary causes of death among cancer patients. So far, most chemotherapeutic drugs are not effective for metastatic cancer due to drug resistance and serious side effects. Therefore, it is urgently essential to develop more effective therapeutic methods. Owing to their diverse biological activities and low toxicity, naturally active compounds derived from Chinese medicines, as a complementary and alternative approach, are reported to promote the therapeutic index and provoked as an excellent source for candidates of anti-metastatic drugs. With the rapid development of molecular biology techniques, the molecular mechanisms of the effects of potential anti-invasive and metastatic Chinese medicines are gradually elucidated. This chapter reviews the potential anti-invasive and metastatic mechanisms of naturally active compounds from Chinese medicines, including suppression of EMT, proteases and cancer-induced angiogenesis, anoikis regulation of circulating tumor cells and regulation of miRNA-mediated gene expression, providing scientific evidence for clinically using Chinese medicines in the field of cancer therapy.

Keywords: Chinese medicines, anti-invasion and metastasis, molecular mechanisms, cancer therapy

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1. Introduction

Despite of all the recent advances in diagnostic and therapeutic approaches, cancer remains the leading cause of death and primary public health hazard all over the world [1, 2]. With diverse causal factors (genetic and environmental, physical, psychological and biochemical factors), cancer has a various disease spectrum to more than a hundred different kinds of malignancies, such as lung cancer, breast cancer, renal carcinoma, hepatocellular carcinoma, and so on [3]. It is a progressive disease with multiple pathological processes covering cancer initiation, development, and metastasis. Cancer is characterized by several key hallmarks [4–6], namely uncontrolled replication ability of abnormal cells, resistance to programmed cell death, invasion into the surrounding extracellular matrix (ECM), sustained capability of angiogenesis, and metastatic spread to other sites.

As one of the most important hallmarks for cancer, metastasis is an intricate process concerning the following six steps (as shown in **Figure 1**): (i) detachment of cancer cells through degrading ECM, (ii) local migration and invasion into the surrounding tissues, (iii) intravasation into blood and/or lymphatic vessel systems, (iv) survival and circulation in the circulatory system, (v) extravasation into the targeted secondary organ site, and (vi) multiplication and formation of a secondary tumor [7–9]. During these steps, the metastatic cancer cells should have special properties to overcome the obstacles, such as the capability of invasion, resistance to anoikis, and angiogenesis. Basically, these steps are regulated by multiple factors, including but not limited to changes of expression of related genes, cytoskeleton remodeling, proteolysis degradation of ECM, and so on [10]. Metastasis is a nonrandom process, and different metastatic cancer types possess their corresponding preferred sites of metastasis. For instance, the preferred sites of breast cancer cells are lung, liver, and bone [11]. Since invasion and metastasis restrain the successful clinical therapy and are the primary causes of death among cancer patients, it has been widely accepted that invasion and metastasis become a highlighted topic of research interests, and active efforts are still needed to understand the underlying molecular mechanisms and develop effective anti-metastatic therapies.

At the present day, there are three conventional therapeutic approaches which are used to treat metastatic cancers, namely surgical resection, chemotherapy, and radiotherapy. Though remain as the main treatment approach for metastatic cancer patients, most chemotherapeutic drugs are not effective for metastatic cancer due to drug resistance and serious side effects. Most chemotherapeutic drugs fail to selectively kill cancer cells without destroying normal cells at the sites of metastasis [12] and thus cause severe toxicity, such as appetite loss, weight loss, insomnia, fatigue, even life threat etc [13, 14]. Although chemotherapeutics significantly leads to regression of the primary tumor, some investigations even report that it may also promote and enhance metastatic formation of a secondary tumor [15, 16]. Besides, metastatic cancers are demonstrated to be largely resistant against chemotherapeutics. Despite that various approaches have been applied to treat metastatic cancers, the clinical outcomes of metastatic cancer treatment are still not at a satisfactory level. Therefore, it is urgently essential to develop more effective therapeutic methods with minimal adverse effects for metastatic cancer treatment.

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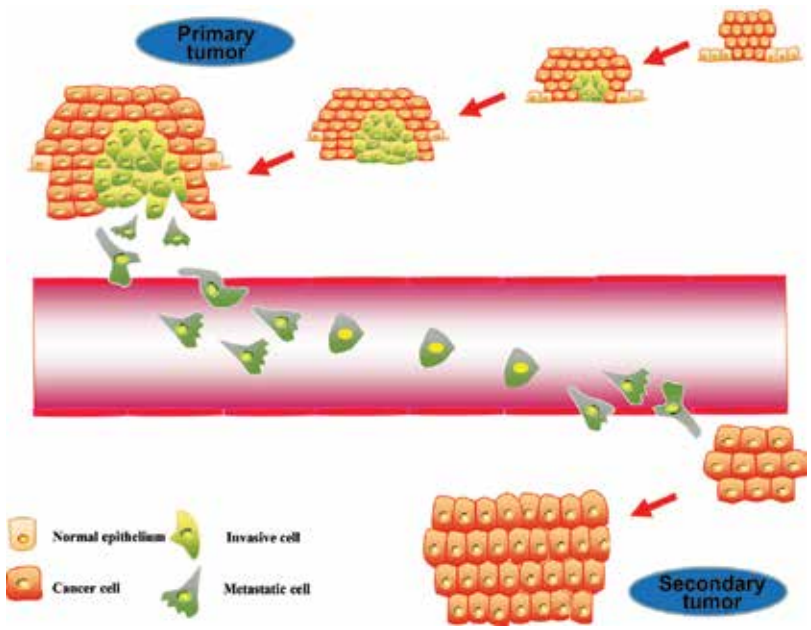

Figure 1. The process involving in cancer metastasis.

Traditional medicine, such as Chinese medicine, has been shown to exhibit various pharmacological activities and used in treatment of various diseases in Asian countries and regions for a long time [17]. The numerous natural compounds obtained from Chinese medicines chemically range from flavonoids and polyphenols to mineral salts, which have been reported to be an excellent source for anti-cancer agents [18]. Owing to their long-lasting efficacy, diversity in biological activities, and low toxicity, natural active products from Chinese medicines, including single compounds and various extracts, are being developed for treatment of metastatic cancer [19, 20]. In line with such a concept, several natural active products from Chinese medicines have been currently investigated as a complementary and alternative approach, and their anti-metastatic properties have been focused to find newly discovered mechanisms with the hope to promote the therapeutic index of metastatic cancer.

With the rapid development of molecular biology techniques, the molecular mechanisms underlying the effects of potential anti-invasive and metastatic Chinese medicines are gradually elucidated. Understanding of the underlying molecular mechanisms may in turn lead to the discovery of novel anticancer drugs. In summary, this chapter reviews the anti-invasive and metastatic effect of natural active compounds from Chinese medicines and their molecular mechanisms. **Tables 1** and **2** respectively summarized the potential underlying molecular mechanisms of single pure compounds and various extracts from Chinese medicines to suppress cancer invasion and metastasis.

Single pure compound	Cancer type	Study type	Mechanism of actions	Ref. (PMID)
Arctigenin	Breast cancer	In vitro MCF-7 and MDA-MB-231 cells	Suppress MMP-9 and uPA	28035371
	Colorectal cancer	In vitro CT26, MC38, CCD-18Co and SW620 cells and in vivo BALB/c female mice	Induce anoikis via MAPKs signaling, inhibit EMT through increasing E-cadherin and decreasing N-cadherin, vimentin, β-catenin, and Snail and downregulate MMP-2/9	27618887
Astragaloside IV	Breast cancer	In vitro MDA-MB-231 cells and in vivo athymic Balb/c nude mice	Downregulate Vav3 and MMP-2/9	27930970
Berberine	Hepatocellular carcinoma	In vitro MHCC-97L, Bel-7402, SMMC-7721 cells and in vivo nude mice	Downregulate uPA and suppress Id-1 via HIF-1α/VEGF pathway	27092498 25496992
	Nasopharyngeal carcinoma	In vitro HONE1 cells	Suppress Rho GTPases including RhoA, Cdc42, and Rac1	19513545
Notoginsenoside R1	Colorectal cancer	In vitro HCT-116 cells	Reduce MMP-9, integrin-1, E-selectin and ICAM-1 expressions	27840961
Matrine	Prostate cancer	In vitro DU145 and PC-3 and male Balb/c nude mice inoculated subcutaneously with cells	Downregulate MMP-2/9	28000853
Bibenzyl 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl	Lung cancer	In vitro H292 cells	Suppress EMT markers (vimentin and Snail) and increase the level of E-cadherin and induce anoikis by reduction of activated protein kinase B (p-AKT) and activated extracellular signal-regulated kinase (p-ERK)	24692728
Curcumin	Lung cancer	In vitro H460 cells	Sensitize anoikis by down-regulating Bcl-2	20127174
Imperatorin	Lung cancer	In vitro H23, H292 and A549 cells	Sensitize anoikis by down-regulating Mcl-1 protein and up-regulating Bax	23108812
Artonin E	Lung cancer	In vitro H460, A549 and H292 cells	Sensitize anoikis by down-regulating Mcl-1 protein	23225436

Single pure compound	Cancer type	Study type	Mechanism of actions	Ref. (PMID)
Ecteinascidin 770	Lung cancer	In vitro H23 and H460 cells	Sensitize anoikis by down-regulating Mcl-1 protein and up-regulating Bax	23393342
Renieramycin M	Lung cancer	In vitro H460 cells	Sensitize anoikis by down-regulating survival proteins p-ERK and p-AKT and anti-apoptotic proteins BCL2 and MCL1	27069144
Oroxylin A	Lung cancer	In vitro A549 cells and in vivo nude mice	Sensitize anoikis by inactivating the c-Src/AKT/HK II pathway	23500080
Geraniin	Lung cancer	In vitro A549 cells	Inhibit the TGF-β1-induced EMT	26169124
Genipin	Hepatocellular carcinoma	In vitro HepG2 and MHCC97L cells and in vivo male nude mice	Overexpress TIMP-1 and inhibit MMP-2	23029478
Kukoamine A	Glioblastoma	In vitro C6, U251 and WJ1 cells and in vivo nude mice (BALB/C-nu/nu)	Inhibit EMT and induce anoikis by downregulating expressions of C/EBPβ and 5-LOX	27824118
Gigantol	Lung cancer	In vitro H460 cells	Decrease EMT markers including N-cadherin, vimentin, and Slug	26733180
Moscatilin	Lung cancer	In vitro H460 cells	Inhibit EMT by suppressing mesenchymal cell markers (vimentin, Slug, and Snail) and induce anoikis by survival proteins (ERK and Akt) suppression and Cav-1 down-regulation	26384689
2,3,5- Trimethoxy-4-cresol	Lung cancer	In vitro A549 cells	Suppress Akt, MMP-2 and MMP-9 and increase E-cadherin and TIMP-1	25951809
Deoxyelephantopin	Lung cancer	In vitro A549 cells	Suppress MMP-2, MMP-9, uPA, and uPAR	25686703
Bufalin	Lung cancer	In vitro NCI-H460 cells	Suppress MMP-2, MMP-9, MAPKs, and NF-kB	26446205
Epicatechin-3-gallate	Lung cancer	In vitro A549 cells and in vivo BALB/c nude mice	Inhibit the TGF-β1-induced EMT by up-regulating epithelial marker (E-cadherin) and down-regulating mesenchymal markers (fibronectin and p-FAK)	27224248

Single pure compound	Cancer type	Study type	Mechanism of actions	Ref. (PMID)
Rocaglamide-A	Prostate cancer, breast cancer and cervical cancer	In vitro PC-3, MDA- MB-231, HCT116, HeLa, and 293T cells	Inhibit the activity of Rho GTPases RhoA, Rac1 and Cdc42	27340868
Chamaejasmenin B	Breast cancer	In vitro MDA-MB-231, ZR75-1 and 4T1 cells and in vivo BALB/c mice	Block TGF-beta induced EMT	27374079
Artesunate	Cervical cancer	In vitro CaSki and Hela cells	Inhibit HOTAIR and COX-2 expressions	27736969
Ginsenoside Rd	Breast cancer	In vitro 4T1 cells and in vivo BALB/c mice	Derepress miR-18a-mediated Smad2 expression	27641158
Quercetin	Colorectal cancer	In vitro CT26 and MC38 cells and in vivo BALB/c female mice	Induce apoptosis through the MAPKs pathway, regulate EMT markers including E-, N-cadherin, β-catenin, and snail and regulate MMPs and TIMPs	27823633
Sulforaphane	Lung cancer	In vitro H1299, 95C and 95D cells and in vivo male BALB/c nude mice	Inhibit EMT by silencing miR-616-5p	27890917
Tricetin	Osteosarcoma	In vitro U2OS and HOS cells	Repress MMP-9 via p38 and Akt pathways	27860196
Arsenic trioxide	Chondrosarcoma	In vitro HCS-2/8, OUMS-27, SW1353, and JJ012 cells	Inhibit EMT via the miR-125b/Stat3 axis	27576314
Cucurbitacin B	Breast cancer	In vitro MDA-MB-231 and 4T1 cells	Inhibit angiogenesis via downregulating VEGF/FAK/MMP-9 signaling	27210504
2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside	Colorectal cancer	In vitro HT-29 cells	Suppress MMP-2 and ICAM-1 via NF-κB pathway	27278328
7,7″-Dimethoxyagastisflavone	Melanoma	In vitro B16F10 cells and in vivo female C57BL/6JNarl mice	Down-regulate the polymerization of F-actin via Cdc42/Rac1 pathway and inhibit lamellipodia formation via suppressing CREB phosphorylation	27487150
Nobiletin	Osteosarcoma	In vitro U2OS and HOS cells	Block ERK and JNK-mediated MMPs expression	27144433

Table 1. Summary on the potential underlying molecular mechanisms of single pure compound from Chinese medicines to suppress cancer invasion and metastasis.

Various extracts	Cancer type	Study type	Mechanism of actions	Ref. (PMID)
Ethanol extract of baked Gardeniae Fructus	Melanoma	In vitro B16F10 and in vivo C57BL/6 mice	Inhibiting the release of pro-angiogenic factors from tumor cells	27779658
Mixture of flavonoids extracted from Korean *Citrus aurantium*	Lung cancer	In vitro A549 cells and in vivo NOD/SCID mice	Induce apoptosis through regulating the apoptosis related protein cleaved caspase-3 and p-p53	No
Bibenzyl compounds isolated from *Dendrobium pulchellum*	Lung cancer	In vitro	Induce anoikis	23472473
Aqueous extract of *Andrographis paniculata*	Esophageal cancer	In vitro EC-109 and KYSE-520 cells	Inhibit anoikis resistance	26885447
Methanol extracts of *Euphorbia humifusa* Willd	Breast cancer	In vitro MDA-MB-231 and in vivo Balb/c mice	Reduce TNFα-induced MMP-9 expression	27776550
Ethanol extract of Lophatheri Herba	Fibrosarcoma, breast cancer, prostate carcinoma and melanoma	In vitro HT1080, MDA-MB231, DU145, B16F10 cells and in vivo C57BL/6J mice and ICR mice	Suppress tumor-induced angiogenesis by decreasing the pro-angiogenic factors	27808120
Coptidis Rhizoma aqueous extract	Hepatocellular carcinoma	In vitro Hep G2 and MHCC97-L cells and in vivo nude mice	Suppress Rho/ROCK signaling pathway and inhibit VEGF secretion	21106616 24363282
Methanol extracts and butanol extracts of *Oldenlandia diffusa*	Breast cancer	In vitro MCF-7 cells	Inhibit PMA-induced MMP-9 and ICAM-1 expressions	27876502
Annona muricata leaf aqueous extract	Breast cancer	In vitro 4 T1 cells and in vivo female BALB/c mice	Induce the apoptosis	27558166
Ethanol extract of *Siegesbeckia orientalis*	Endometrial Cancer	In vitro RL95-2 and HEC-1A	Reverse the TGFβ1-induced EMT	27527140
Polyphenols of *Artemisia annua* L.	Breast cancer	In vitro MDA-MB-231 cells	Suppress EMT by inhibiting MMP-2/-9 and vascular cell adhesion molecule-1	27151203
Gegen Qinlian decoction	Renal carcinoma	In vitro ACHN and Caki-1 cells and in vivo male BALB/c nude mice	Suppress neoangiogenesis via MMP-2 inhibition	25228536

Table 2. Summary on the potential underlying molecular mechanisms of various extracts from Chinese medicines to suppress cancer invasion and metastasis.

2. Suppression of epithelial-mesenchymal transition

Recent studies clearly showed that epithelial-mesenchymal transition (EMT) plays an important role in the metastasis of cancers [21]. As the fundamental step during cancer metastasis, EMT is a complex process during which immotile epithelial cells undergo a morphological transformation into motile mesenchymal-appeared cells, triggering cancer cells to detach from the primary site via the loss of cell-to-cell junctions and thus promoting cell migration [22]. There are

three different subtypes of EMT, and the third subtype of EMT is associated with the invasion and metastasis of cancers [23]. EMT-phenotypic cells can decrease the level of epithelial marker E-cadherin, a junction protein for cell-cell contact. Besides, they can also increase the level of mesenchymal markers, such as N-cadherin, β-catenin, and vimentin, as well as promote transcription factors of EMT switch, such as Slug and Snail [24, 25]. As EMT has been significantly linked to the metastatic behaviors of cancer cells, natural products obtained from Chinese medicines with the ability to suppress EMT are attracting attention for the development of anti-metastasis therapies.

Among potential natural products, geraniin, a polyphenolic component derived from Phyllanthus amarus, has gained considerable attention over the past decade. Previous study has demonstrated that EMT can be induced by transforming growth factor-beta 1 (TGF-β1) and thus stimulates the migration and invasion of lung adenocarcinoma. Geraniin has been shown to inhibit TGF-β1-induced EMT of lung cancer A549 cells in vitro by inducing the epithelial marker E-cadherin and suppressing Snail and mesenchymal marker N-cadherin and vimentin [26]. A compound derived from *Dendrobium ellipsophyllum*, bibenzyl 4,5,4′ -trihydroxy-3,3′-dimethoxybibenzyl was shown to inhibit EMT of lung cancer cells via downregulating EMT markers (vimentin and Snail) and upregulating E-cadherin [27]. Such EMT suppression was also observed in lung cancer cells treated with other single compounds obtained from Chinese medicine, such as moscatilin [28], gigantol [29], and epicatechin-3-gallate [30]. A flavonoid obtained from *Stellera chamaejasme* L., namely chamaejasmenin B, was also reported to block the TGF-β-induced EMT in breast cancer [31]. 5-lipoxygenase (5-LOX) is an enzyme to convert arachidonic acid to leukotrienes [32] and abrogating its expression can inhibit the migration, invasion, and metastasis of cancer cells by suppressing EMT via inactivating E-cadherin and activating snail [33]. CCAAT/enhancer binding protein β (C/EBPβ) was also reported to be related to the migration, invasion, and metastasis of cancer cells by EMT regulation [34]. Kukoamine A, a spermine alkaloid extracted from Cortex lycii radicis, was demonstrated to suppress the migratory and invasive ability of human glioblastoma cell both in vitro and in vivo, and this action was mediated through EMT attenuation via decreasing the levels of 5-LOX and C/EBPβ [35]. Likewise, similar EMT inhibitory effects have also been observed in various extracts from Chinese medicines. *Siegesbeckia orientalis* Linne is a traditionally used Chinese medicinal herb that exhibits various pharmacological activities. Its ethanol extract (SOE) has been reported as a potential anti-metastatic agent by reversing the TGFβ1-induced EMT via ERK1/2, JNK1/2, and Akt pathways [36]. SOE can inhibit the migration and invasion of endometrial cancer RL95-2 and HEC-1A cells in a dose-dependent manner. *Artemisia annua* L. is a traditional medicine which has been applied for treating multiple diseases. The polyphenolic compounds from *Artemisia annua* L. (pKAL) were found to exhibit anti-metastatic property on highly metastatic breast cancer cells MDA-MB-231 [37]. This anti-metastatic property of pKAL was achieved through suppressing EMT by inhibiting MMP-2/-9 and vascular cell adhesion molecule-1 (VCAM-1).

3. Suppression of proteases expression

Matrix metalloproteinases (MMPs) is regarded as primary factors to trigger metastasis [38]. As extracellular zinc-dependent endopeptidases, they can degrade the basement membrane

and ECM and thus play an important role in the migration and invasion of cancers. There are 23 members in MMPs family, among which MMP-2 and MMP-9 are considered to be the key enzymes and play crucial roles in cancer metastasis [39, 40]. The activities of MMPs are finely mediated by tissue inhibitors of metalloproteinases (TIMPs) via their non-covalent binding to the active zinc-binding sites of MMPs [41]. In addition, as a serine-specific protease, urokinase-type plasminogen activator (uPA) can also degrade ECM via binding to uPA receptor (uPAR) and activating plasmin [42]. It is well-known that reorganization of the actin cytoskeleton plays an important role in the migration of cancer cell [43]. This process is mainly regulated by the Rho family GTPases, such as RhoA, Rac1, and Cdc42 via a shuttle between an inactive GDP-bound form and an active GTP-bound form [44, 45]. Since these proteases play an important role in cancer invasion and metastasis via proteolysis, natural products obtained from Chinese medicines with the ability to suppress these proteases are attracting attention for the development of anti-metastasis therapies.

As a phytoestrogen-botanical lignan derived from Arctium lappa, arctigenin was shown to exert its anti-metastatic property through suppressing MMP-9 and uPA of breast cancer cells via inhibiting the upstream signaling pathways including Akt, NF-κB, and MAPK (ERK 1/2 and JNK 1/2), which is dependent to the modulation on estrogen receptor (ER) expression [46]. Such protease regulation was also observed in breast cancer cells treated with astragaloside IV [47]. Notoginsenoside R1 (NGR1) is a primary compound in Panax notoginseng, and its anti-metastatic property has also been revealed [48]. NGR1 can inhibit the migration, invasion, and adhesion of cultured human colorectal cancer cells (HCT-116) via suppressing MMP-9, integrin-1, E-selectin, and ICAM-1 expressions. Such inhibition on colorectal cancer cells was also observed when treated with 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside [49]. As an alkaloid derived from Sophora flavescens, matrine can inhibit the invasion and migration of castration-resistant prostate cancer DU145 and PC3 cells by suppressing MMP-9 and MMP-2 expressions through NF-κB pathway [50]. The phenol derived from Taiwanese edible fungus *Antrodia cinnamomea*, 2,3,5-trimethoxy-4-cresol, was recently described as an effective anti-metastatic agent against lung cancer via suppressing Akt, MMP-2 and MMP-9, and increasing E-cadherin and TIMP-1[51]. Such protease regulation was also observed in lung cancer cells treated with other single compounds derived from Chinese medicine, such as deoxyelephantopin [52] and bufalin [53]. Genipin, a natural compound obtained from the fruit of Gardenia jasminoides, was reported to exhibit anti-metastatic effect on hepatocellular carcinoma both in cell and animal model. This effect may be related with TIMP-1 overexpression and MMP-2 inhibition of genipin [54]. As an isoquinoline alkaloid isolated from Coptidis rhizome and other medicinal plants, berberine has been shown to exhibit multiple pharmacological actions in treating human diseases, including cancers [55]. Recently, it was reported to inhibit nasopharyngeal carcinoma cell migration and invasion in vitro through suppressing Rho GTPases including RhoA, Rac1, and Cdc42 [56]. The anti-metastatic ability of Rocaglamide-A was also recently described via inhibiting the activity of Rho GTPases RhoA, Rac1, and Cdc42 [57]. As a dietary flavonoid in Eucalyptus honey and Myrtaceae pollen, tricetin was shown to attenuate osteosarcoma cell migration via suppressing MMP-9 via p38 and Akt pathways [58]. Such inhibition on osteosarcoma cells was also observed when treated with nobiletin [59]. The compound obtained from Taxus x media cv. Hicksii, 7,7″-Dimethoxyagastisflavone (DMGF) has been reported to inhibit the invasion and metastasis of melanoma cells in vivo and in vitro

[60]. The mechanism study provided evidence that DMGF can downregulate the polymerization of F-actin via Cdc42/Rac1 pathway and inhibit lamellipodia formation via suppressing cAMP response element-binding protein (CREB) phosphorylation. Likewise, similar protease inhibitory effects have also been observed in various extracts from Chinese medicines. The methanol extracts of *Euphorbia humifusa* Willd was reported to have anti-metastatic effects on breast cancer both in vitro and in vivo via reducing TNFα-induced MMP-9 expression [61]. In addition, the methanol extracts and butanol extracts of *Oldenlandia diffusa* were also shown to block the metastasis of breast cancer via inhibiting PMA-induced MMP-9 and ICAM-1 expressions [62].

4. Suppression of cancer-induced angiogenesis

Angiogenesis is a normal physiological process to sprout new vessels during the development of embryogenesis. To the contrary, pathological angiogenesis is associated with multiple diseases including cancers [63]. Highly malignant tumors can induce angiogenesis to provide sufficient oxygen and nutrients for themselves [64]. Additionally, angiogenesis also provides paths for cancer cells to metastasize distant tissues [65]. In tumor microenvironment, tumor and host cells release pro-angiogenic and anti-angiogenic factors. The pro-angiogenic factors include transforming growth factor (TGF), vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF), epidermal growth factor (EGF), and so on, and there is a fine balance between them. When the balance is skewed to the pro-angiogenic state, tumor shifts from a dormant state to a hyper-vascularized state [66]. Since angiogenesis plays an important role in the metastatic behaviors of cancer cells, natural products obtained from Chinese medicines targeting on tumor-induced angiogenesis have been regarded as promising agents to metastatic cancers.

Berberine has been shown to exhibit a significant inhibition on the migratory and invasive ability of hepatocellular carcinoma cells. Except for downregulation of uPA, berberine also inhibits angiogenesis through suppressing inhibitor of differentiation/DNA binding (Id-1) via HIF-1α/VEGF pathway [67, 68]. Cucurbitacin B (CuB), a plant triterpenoid, obtained from Cucurbitaceae family has been shown to inhibit the metastasis and angiogenesis of breast cancer MDA-MB-231 and 4T1 cells via downregulating VEGF/FAK/MMP-9 signaling [69]. Recently, a study of artesunate, a normal traditional Chinese medicine, has been conducted to investigate the anti-metastatic effects of artesunate on cervical cancer. The results demonstrated that artesunate inhibits cancer cell migration and invasion in vitro through suppressing HOTAIR and COX-2-mediated angiogenesis [70]. Likewise, similar inhibitory effects have also been observed in various extracts from Chinese medicines. The aqueous extract of Coptidis Rhizoma, a traditional Chinese medicinal herb with a long history, was observed to inhibit hepatocellular carcinoma cell migration both in vitro and in vivo through suppressing Rho/ROCK signaling pathway and inhibiting VEGF secretion [71, 72]. Gardeniae Fructus, a fruit obtained from *Gardenia jasminoides* Ellis, has been applied as traditional medicine and possesses various health benefits against multiple diseases. A recent study has shown that the ethanol extract of baked Gardeniae Fructus has an inhibitory effect on the angiogenic

and metastatic ability of melanoma cells both in vitro and in vivo via inhibiting the release of pro-angiogenic factors [73]. Lophatheri Herba, a dried leaf obtained from Lophatherum gracile Brongn, possesses inhibitory effects on the metastasis and angiogenesis of malignant cancer cells at noncytotoxic doses. It has been shown that ethanol extract of Lophatheri Herba (ELH) can inhibit the cancer cell metastasis both in vitro and in vivo through suppressing tumor-induced angiogenesis via decreasing the pro-angiogenic factors [74]. As an ancient Chinese medicine formula, Gegen Qinlian decoction was reported to suppress the neoangiogenesis in xenografted renal carcinoma cell tumor through inhibiting the enzyme activity of MMP-2 [75].

5. Anoikis regulation of circulating tumor cells

Anoikis, known as detachment-induced apoptosis, is a process of programmed cell death [76]. It can block metastasis by eliminating circulating cancer cells. However, in highly metastatic cancer cells, anoikis can be overcome and cancer cells can survive in a circulating condition until reaching a proper secondary site [77]. Anoikis is controlled by Bcl-2 family proteins. The pro-apoptotic proteins, such as Bax and the anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, interact during anoikis [78]. In addition, anti-apoptotic protein myeloid leukemia cell sequence-1 (MCL-1) and caveolin-1 (CAV-1) have also been demonstrated to suppress anoikis [79]. Anoikis has become potential therapeutic target, and discovering new natural products obtained from Chinese medicines targeting anoikis is of great interest [80].

The anti-metastatic study of arctigenin on colorectal cancer has been recently conducted. Arctigenin can induce anoikis via MAPKs signaling, inhibit EMT through increasing E-cadherin and decreasing N-cadherin, vimentin, β-catenin, and Snail, and downregulate MMP-2/9, so that inhibition on the tumor cell migration and invasion both in vitro and in vivo was achieved [81]. Imperatorin, an active furanocoumarin component obtained from the root of *Angelica dahurica*, has been demonstrated to sensitize anoikis by downregulating Mcl-1 protein and upregulating Bax in lung cancer [82]. As a major dietary flavonoid, quercetin was reported to induce apoptosis through the MAPKs pathway, regulate EMT markers including E-, N-cadherin, β-catenin, and snail and modulate MMPs and TIMPs in colorectal cancer [83]. Curcumin, a compound derived from the rhizome of turmeric, was reported to inhibit the migratory and invasive ability of lung cancer cells through sensitizing anoikis, which was associated with downregulation of Bcl-2 [84]. Such anoikis regulation was also observed in lung cancer cells challenged other single compounds obtained from Chinese medicine, such as artonin E [85], ecteinascidin 770 [86], renieramycin M [87], Oroxylin A [88], and so on. In addition, regulation on anoikis was also observed in tumor cells treated with various extracts from Chinese medicines. Annona muricata Linn from Annonaceae family has long been applied to treat different diseases. Recently, its leaf aqueous extract (B1 AMCE) has been reported to exhibit anti-metastatic property in breast cancer [89]. B1 AMCE can significantly suppress the metastasis of 4T1 breast cancer cells in vitro and in vivo via inducing their apoptosis. The aqueous extract of *Andrographis paniculata* was demonstrated to inhibit anoikis resistance in esophageal cancer [89]. The bibenzyl compounds from *Dendrobium pulchellum*

[90] and the mixture of flavonoids extracted from Korean *Citrus aurantium* have been shown to induce apoptosis and inhibit metastasis of lung cancer cells [91].

6. Regulation of miRNA-mediated gene expression

As negative regulators of gene expression, microRNAs (miRNAs) have been shown to modulate multiple biological functions, such as immune response, metabolism, and metastasis [92]. miRNAs mediate the expression of target protein through degrading its mRNA or inhibiting the translation of mRNA via binding to mRNA three prime untranslated region (3′UTR). There is a dual action of miRNAs in cancers, either functioning as cancer promoters or inhibitors. Nearly, all human tumors have the characteristic of miRNAs dysregulation [93]. Since miRNAs play an important role in the metastatic behaviors of cancer cells, developing natural products obtained from Chinese medicines targeting miRNAs may be a promising strategy to treat metastatic cancers.

Recently, the anti-metastatic property of arsenic trioxide (ATO) in chondrosarcoma has been elucidated. It was reported that ATO attenuate the metastasis of chondrosarcoma cells through inhibit miR-125b/Stat3 axis [94]. As a common antioxidant obtained from cruciferous plants, sulforaphane has been reported to inhibit the migratory and invasive ability of lung cancer both in vitro and in vivo. This action is mediated by miR-616-5p [95]. In addition, ginsenoside Rd (Rd), one of the chemical compounds in Panax Notoginseng Saponins, has been investigated for its anti-metastatic property recently. The results showed that Rd treatment inhibited the migratory and invasive ability of breast cancer both in vitro and in vivo via suppressing miR-18a-mediated Smad2 expression [96].

7. Conclusion and future challenges

Accumulating evidence has demonstrated that Chinese medicine is an excellent source for the development of novel therapies for metastatic cancer. As mentioned above, the molecular mechanisms underlying the effects of potential anti-invasive and metastatic Chinese medicines include suppression of EMT (e.g., epithelial and mesenchymal markers), suppression of proteases expression (e.g., MMPs, uPA and Rho GTPases), suppression of cancer-induced angiogenesis (e.g., pro-angiogenic and anti-angiogenic factors), anoikis regulation of circulating tumor cells (e.g., pro-apoptotic and anti-apoptotic proteins), and regulation of miRNA-mediated gene expression (e.g., miR-125b, miR-616-5p and miR-18a). The chapter summarized the potential anti-invasive and metastatic drug candidates, which provided scientific evidence for clinically used Chinese medicines in the field of cancer therapy. Understanding of the underlying molecular mechanisms may in turn lead to discovery and development of novel anticancer drugs. Although these findings show the anti-metastatic potential of Chinese medicines, studies to evaluating the marked efficacies and determining the appropriate therapeutic doses of anti-metastatic Chinese medicines in animal models and clinical trials are still

badly necessary in the future. In addition, the modern techniques such as nanoparticles which may improve the anti-cancer properties via better cellular uptake, enhanced bioavailability, and localization to targeted sites should also be studied in the future.

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The evidence of cancer in humans, animals and plant species suggests that it is as old as multicellular life on Earth. Why is it so difficult to understand and fight? Because cancer begins from the organism's own mutated single cell focused on its own survival. It would be naive to expect that cancer could be ever entirely eliminated, but there is still hope for finding effective treatments. The book is to give a view of selected aspects of cancer like its spread in nature, novel anticancer drugs based on Chinese herbs or birch bark, novel promising targets of annexins and kinases and progress in immunotherapy. It is our hope that you will find in this book interesting, inspiring and stimulating information concerning cancer research.

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