Natural products as drug source biology essay

Science, Biology

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Natural products as drug source

1. 1 Introduction

The use of natural products, especially plants, for healing is as ancient and universal as medicine itself. The therapeutic use of plants certainly goes back to the Sumerian civilization, and 400 years before the Common Era, it has been recorded that Hippocrates used approximately 400 different plant species for medicinal purposes. Natural products played a prominent role in ancient traditional medicine systems, such as Chinese, Ayurveda, and Egyptian, which are still in common use today. According to the World Health Organization (WHO), 75% of people still rely on plant-based traditional medicines for primary health care globally. A brief summary of the history of natural product medicine is presented in Table 1 (Gray, 2006). About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from Digitalis spp., quinine and quinidine from Cinchona spp., vincristrine and vinblastine from Catharanthus roseus, atropine from Atropa belladonna and morphine and codeine from Papaver somniferum. Papaverine, useful as a smooth muscle relaxant, provided the basic structure for verapamil, a drug used to treat hypertension. Galegine was isolated as an active anti-hyperglycemic agent from the plant Galega officinalis L. This plant was used ethnomedically for the treatment of diabetes. Galegine provided the template for the synthesis of metformin and opened up interest in the syn-thesis of other biguanidine-type antidiabetic. It is estimated that

60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin. It is estimated that, in 1997, the world market for over-the-counter phytomedicinal products was US$ 10 billion, with an annual growth of 6. 5%. China and India have a well-established herbal medicines industry and Latin American countries have been investing in research programs in medicinal plants and the standardisation and regulation of phytomedicinal products, following the example of European countries, such as France and Germany. In Germany, 50% of phytomedicinal products are sold on medical prescription, the cost being refunded by health insurance. In North America, where phytomedicinal products are sold as " health foods", consumers and professionals have struggled to change this by gathering information about the efficacy and safety of these products, and new guidelines for their registration are now part of FDA policy. In 1997, the North American market for products of plant origin reached US$ 2 billion. The NCI (National Cancer Institute, USA) has tested more than 50, 000 plant samples for anti-HIV activity and 33, 000 samples for anti-tumour activity. In 1993, the International Program of Co-operation for Biodiversity (IPCB) was launched in order to promote natural products in Latin America and Africa, linking universities, industries and governments in a multidisciplinary programme for the sustained development and preservation of the environment. Large pharmaceutical companies, such as Merck, CIBA, Glaxo, Boehringer and Syntex, now have specific departments dedicated to the study of new drugs from natural sources (Rates, 2001).

Table 1

Period Type DescriptionBefore Ayurveda (knowledge of life) Introduced medicinal properties of3000 BC Chinese traditional medicine plants and other natural products1550 BC Ebers Papyrus Presented a large number of crude drugfrom natural sources(e. g., castor seeds and gum arabic)460–377 BC Hippocrates, Described several plants and animals‘‘ The Father of Medicine’’ that could be sources of medicine370–287 BC Theophrastus Described several plants and animalsthat could be sources of medicine23–79 AD Pliny the Elder Described several plants and animalsthat could be sources of medicine60–80 AD Dioscorides Wrote De Materia Medica, which describedmore than 600 medicinal plants131–200 AD Galen Practiced botanical medicines (Galenicals)and made them popular in the West15th century Kräuterbuch Presented information and pictures(herbals) of medicinal plants

1. 2 Historic role of natural products in drug discovery

Natural product preparations have historically been the major source of pharmaceutical agents. Analysis of FDA new-drug approvals from 1981 to 2010 reveals that natural products continued to play a pivotal role during that time, even if the industry had turned to other discovery strategies. Indeed, more than 90% of current therapeutic classes derive from a natural product prototype and interestingly, even today, roughly two-thirds to three quarters of the world’s population relies upon medicinal plants for its primary pharmaceutical care. Those ‘‘ medicinal plants’’ are either preparations of or natural product substances from plants that have potential utility as pharmaceutical agents (McChesney, Venkataraman, & Henri, 2007).

1. 3 The decline in interest in natural products as pharmaceuticals

In spite of the obvious successes of the natural products approach to drug discovery, in recent years it has lost some favor, particularly within the pharmaceutical industry. The reasons for this are complex, but can be summarized as being due to a combination of factors, including the incompatibility of crude extracts with the high throughput assays used in the pharmaceutical industry, the cost of sample collection, problems with the lack of reproducibility and the presence of artifacts in some extracts, the difficulty in isolating active compounds, the long resupply times for active extracts, problems with large scale supply if a drug should emerge, the difficulty of complying with the Rio Treaty on Biodiversity, and last but not least, the diversion of resources to combinatorial chemical approaches to drug discovery. However, there is evidence that some people now realize that the move to discontinue natural products research in favor of combinatorial chemistry may have been a mistake. It has now been concluded with these trenchant observations: " The early years of combinatorial chemistry suffered from an excess of hype, and a major victim was natural-product screening. Many organizations went through an irreversible shift in policy, and prematurely discontinued their efforts in this area. We are now seeing the backlash from this knee-jerk reaction. The early combinatorial strategies were flawed and unproven, and have yet to deliver any blockbuster drugs. Meanwhile, we have lost the uniqueness of screening natural-product space as a complement to synthetic compounds. If past indicators are any guide, there are undoubtedly many more unique and

potent biologically active natural products waiting to be discovered" (Kingston, 2005)

1. 4 The challenges for traditional medicine

Historically, the study of traditional medicines has been, and remains, a very neglected aspect of global health care. As a result, a vast array of challenges face all those who venture into this financial backwater of global health, and these have been discussed elsewhere. 7 Some of the challenges are mentioned below, and a few of those based in science have been touched on in this article. Many of these same challenges also apply to the appropriate development of dietary supplements:• Nations typically have no policies or regulations relating to all of the aspects of traditional medicine as an integral part of their overall health care system. This results in a minimal commitment to research and development funding.• The basic information on health care needs, on the economic issues relative to investment and development, and on the cost-effectiveness of health care outcomes is not available for various traditional medicine practices. There is little respect from most Western medicine physicians for traditional medicine in its various forms in the health care system.• The breadth and depth of the issues related to the quality control of traditional medicine products and practices may not be known to regulators, producers, and scientists.• Global attention (fiscal and human resources) is insufficient to enhance the basic, applied, and clinical sciences behind traditional medicine. This results in major deficiencies in the scientific evidence regarding the quality, safety, effectiveness, and/or health benefits of traditional medicine. Costs of traditional medicines may increase as investment is made to enhance

product validity.• Formal training programs and associated standards for learning and licensing of practitioners may not be available. Regulations regarding practitioner training are quite different between nations.• Standards for traditional medicine products and practices, including terminology and philosophical approaches, are highly varied. This limits communication and efforts to harmonize systems between nations.• Patients may be unaware that the plant-based products they are buying are not regulated for quality, safety, and effectiveness. There may be limited awareness of the results of traditional medicine research with respect to safety and effectiveness.• Mechanisms may not be in place to report and act on issues related to adverse drug events involving traditional and allopathic medicines within and between nations.• Conservation of medicinal plants, as a component for assuring long-term access to health care resources, may not be a government priority.• Intellectual property issues regarding access to indigenous knowledge and to natural resources for research may be complex and highly bureaucratic within a country and are typically different between countries.• The literature and knowledge regarding traditional medicine are highly scattered, or are in library collections and databases that are not easily accessible.• Scientific and clinical research on traditional medicines does not always fit the Western model for medical research, which may make publication of results difficult. Health insurance coverage is very difficult to justify if traditional medicine products and practices are not evidence based. Addressing these challenges in a strategic manner is a critical aspect for the development of traditional medicines as an integral

component of validated health care practices and products for the benefit of a global population (Cordell & Colvard, 2012).

1. 5 Future scenario of herbal drugs

Inspection of the data shows the continued important role for natural products in spite of the current greatly reduced level of natural products-based drug discovery programs in major pharmaceutical houses. Inspection of the rate of NCE approvals demonstrates that, even in 2010, the natural products field is still producing or is involved in ca. 50% of all small molecules in the years 2000−2010 (Newman & Cragg, 2012). 23 new drugs derived from natural sources have been launched on the market during 2000 – 2005, even though many pharmaceutical companies have discontinued their programs of drug discovery from natural sources. These new drugs have been approved for the treatment of cancer, neurological diseases, infectious diseases, cardiovascular and metabolic diseases, immunological, inflammatory and related diseases, and genetic disorders, which encompass many of the common human diseases. Besides new drugs launched on the market from 2000 to the present, there are a variety of new chemical entities from natural sources undergoing clinical trials. Further research on these compounds at industrial, governmental, and academic institutions is seen as vital for the enhancement of human health (Chin, Balunas, Chai, & Kinghorn, 2006). In some middle-income countries, governments, academic institutions, and corporations are working together, with little external support, to examine local resources and indigenous knowledge in order to discover new drugs for both global and local diseases and to develop processes for the validation of the safety and efficacy of traditional

medicines. In the People’s Republic of China, very significant government investments in the diverse aspects of examining quality, safety, and efficacy are a central component of national health care policy. Major efforts are under way to enhance research and industrial production facilities and to close those not meeting GMP standards. Dedicated ultraperformance liquid chromatography systems examine each batch of each traditional medicine product in one major company visited (GAC) in 2010. This company is also responsible for the first phase III clinical trial to be approved by the United States Food and Drug Administration based on a standardized traditional medicine. The product consists of the root of Salvia miltiorrhiza Bunge (Dan-shen) and includes Panax notoginseng (Burkill), Feng (Sanchi) and borneol. Chinese government and corporate expectations are that tight quality control will ensure regulatory acceptance and enhance the future global marketing of evidence-based traditional Chinese medicine products (Cordell & Colvard, 2012)

. Natural product isolation

2. 1 Introduction

Products of natural origins can be called ‘‘ natural products.’’ Natural products include: (1) an entire organism (e. g., a plant, an animal, or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation (e. g., drying), (2) part of an organism (e. g., leaves or flowers of a plant, an isolated animal organ), (3) an extract of an organism or part of an organism, and exudates, and (4) pure compounds (e. g., alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, sugars, terpenoids, etc.) isolated from plants, animals, or

microorganisms. However, in most cases the term natural products refers to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism. Concepts of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, shunt metabolism produced during idiophase, defense mechanism regulator molecules, etc. Natural products can be from any terrestrial or marine source: plants (e. g., paclitaxel [Taxol®] from Taxus brevifolia), animals (e. g., vitamins A and D from cod liver oil), or microorganisms (e. g., doxorubicin from Streptomyces peucetius). Strategies for research in the area of natural products have evolved quite significantly over the last few decades. These can be broadly divided into two categories: 1. Older strategies: a. Focus on chemistry of compounds from natural sources, but not on activity. b. Straightforward isolation and identification of compounds from natural sources followed by biological activity testing (mainly in vivo). c. Chemotaxonomic investigation. d. Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses. 2. Modern strategies: a. Bioassay-guided (mainly in vitro) isolation and identification of active ‘‘ lead’’ compounds from natural sources. b. Production of natural products libraries. c. Production of active compounds in cell or tissue culture, genetic manipulation, natural combinatorial chemistry, and so on. d. More focused on bioactivity. e. Introduction of the concepts of dereplication, chemical fingerprinting, and metabolomics. f. Selection of organisms based on ethnopharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.

Extraction

The choice of extraction procedure depends on the nature of the source material and the compounds to be isolated. Prior to choosing a method, it is necessary to establish the target of the extraction. There can be a number of targets; some of these are mentioned here. 1. An unknown bioactive compound. 2. A known compound present in an organism. 3. A group of compounds within an organism that are structurally related. 4. All secondary metabolites produced by one natural source that are not produced by a different ‘‘ control’’ source, e. g., two species of the same genus or the same species grown under different conditions. 5. Identification of all secondary metabolites present in an organism for chemical fingerprinting or metabolomics study. It is also necessary to seek answers to the questions related to the expected outcome of the extraction. These include: 1. Is this extraction for purifying a sufficient amount of a compound to characterize it partially or fully? What is the required level of purity? 2. Is this to provide enough material for confirmation or denial of a proposed structure of a previously isolated compound? 3. Is this to produce as much material as possible so that it can be used for further studies, e. g., clinical trial? The typical extraction process, especially for plant materials, incorporates the following steps: 1. Drying and grinding of plant material or homogenizing fresh plant parts (leaves, flowers, etc.) or maceration of total plant parts with a solvent. 2. Choice of solventsa. Polar extraction: water, ethanol, methanol (MeOH), and so on. b. Medium polarity extraction: ethyl acetate (EtOAc), dichloromethane (DCM), and so on. c. Nonpolar: n-hexane, pet-ether, chloroform (CHCl3), and so on. 3. Choice of extraction method includes

maceration, boiling, soxhlet, supercritical fluid extraction, sublimation, steam distillation and fractionation. A crude natural product extract is literally a cocktail of compounds. It is difficult to apply a single separation technique to isolate individual compounds from this crude mixture. Hence, the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes. These fractions may be obvious, physically discrete divisions, such as the two phases of a liquid–liquid extraction or they may be the contiguous eluate from a chromatography column, e. g., vacuum liquid chromatography (VLC), column chromatography (CC), size-exclusion chromatography (SEC), solid-phase extraction (SPE), etc. For initial fractionation of any crude extract, it is advisable not to generate too many fractions, because it may spread the target compound over so many fractions that those containing this compound in low concentrations might evade detection. It is more sensible to collect only a few large, relatively crude ones and quickly home in on those containing the target compound. For finer fractionation, often guided by an on-line detection technique, e. g., ultraviolet (UV), modern preparative, or semipreparative high-performance liquid chromatography (HPLC) can be used.

2. 3 Isolation

The most important factor that has to be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. The general features of the molecule that are helpful to ascertain the isolation process include solubility (hydrophobicity or hydrophilicity), acid–base properties, charge, stability, and molecular size. If

isolating a known compound from the same or a new source, it is easy to obtain literature information on the chromatographic behavior of the target compound, and one can choose the most appropriate method for isolation without any major difficulty. However, it is more difficult to design an isolation protocol for a crude extract where the types of compounds present are totally unknown. In this situation, it is advisable to carry out qualitative tests for the presence of various types of compounds, e. g., phenolics, steroids, alkaloids, flavonoids, etc., as well as analytical thin-layer chromatography (TLC), or HPLC profiling. The nature of the extract can also be helpful for choosing the right isolation protocol. For example, a MeOH extract or fractions from this extract containing polar compounds are better dealt with using reversed-phase HPLC (RP-HPLC). Various physical properties of the extracts can also be determined with a small portion of the crude extract in a series of small batch-wise experiments. Some of these experiments are summarized below. 1. Hydrophobicity or hydrophilicity: An indication of the polarity of the extract as well as the compounds present in the extract can be determined by drying an aliquot of the mixture and trying to redissolve it in various solvents covering the range of polarities, e. g., water, MeOH, acetonitrile (ACN), EtOAc, DCM, $CHCl_3$, petroleum ether, n-hexane, etc. The same information can be obtained by carrying out a range of solvent partitioning, usually between water and EtOAc, $CHCl_3$, DCM, or n-hexane, followed by an assay to determine the distribution of compounds in solvent fractions. 2. Acid–base properties: Carrying out partitioning in aqueous solvents at a range of pH values, typically 3, 7, and 10, can help determine the acid–base property of the compounds in an extract. It is

necessary to adjust the aqueous solution or suspension with a drop or two of mineral acid or alkali (a buffer can also be used), followed by the addition of organic solvent and solvent extraction. Organic and aqueous phases are assessed, preferably by TLC, for the presence of compounds. This experiment can also provide information on the stability of compounds at various pH values. 3. Charge: Information on the charge properties of the compound can be obtained by testing under batch conditions, the effect of adding various ion exchangers to the mixture. This information is particularly useful for designing any isolation protocol involving ion exchange chromatography. 4. Heat stability: A typical heat stability test involves incubation of the sample at ~90oC for 10 min in a water bath followed by an assay for unaffected compounds. It is particularly important for bioassay-guided isolation, where breakdown of active compounds often leads to the loss or reduction of biological activity. If the initial extraction of natural products is carried out at a high temperature, the test for heat stability becomes irrelevant. 5. Size: Dialysis tubing can be used to test whether there are any macromolecules, e. g., proteins, present in the extract. Macromolecules are retained within the tubing, allowing small (<2000 amu) secondary metabolites to pass through it. The necessity of the use of any SEC in the isolation protocol can be ascertained in this way. The chromatographic techniques used in the isolation of various types of natural products can be broadly classified into two categories: classical or older, and modern. Classical or older chromatographic techniques include: 1. Thin-layer chromatography (TLC). 2. Preparative thin-layer chromatography (PTLC). 3. Open-column chromatography (CC). 4. Flash chromatography (FC). Modern

chromatographic techniques are: 1. High-performance thin-layer chromatography (HPTLC). 2. Multiflash chromatography (e. g., Biotage®). 3. Vacuum liquid chromatography (VLC). 4. Chromatotron. 5. Solid-phase extraction (e. g., Sep-Pak®). 6. Droplet countercurrent chromatography (DCCC). 7. High-performance liquid chromatography (HPLC). 8. Hyphenated techniques (e. g., HPLC-PDA, LC-MS, LC-NMR, LC-MS-NMR).

2. 4 Quantification

The yield of compounds at the end of the isolation and purification process is important in natural product research. An estimate of the recovery at the isolation stage can be obtained using various routine analytical techniques that may involve the use of a standard. In bioassay-guided isolation, the compound is monitored by bioassay at each stage, and a quantitative assessment of bioactivity of the compound is usually carried out by serial dilution method. Quantitative bioactivity assessment provides a clear idea about the recovery of the active compound(s) and also indicates whether the activity results from a single or multiple components. During the isolation process, if the activity is lost or reduced to a significant level, the possible reasons could be as follows: 1. The active compound has been retained in the column. 2. The active compound is unstable in the conditions used in the isolation process. 3. The extract solution may not have been prepared in a solvent that is compatible with the mobile phase, so that a large proportion of the active components precipitated out when loading on to the column. 4. Most of the active component(s) spread across a wide range of fractions, causing undetectable amounts of component(s) present in the fractions. 5. The activity of the extract is probably because of the presence of synergy

among a number of compounds, which, when separated, are not active individually.

2. 5 ‘‘ Poor-Yield’’ Problem

Poor yield or poor recovery is one of the major problems in natural product isolation. For example, only 30 g of vincristine was obtained from 15 t of dried leaves of V. rosea (or C. roseus). Similarly, to obtain 1900 g of Taxol®, the felling of 6000 extremely slow-growing trees, Taxus brevifolia, was necessary to produce 27, 300 kg of the bark. To tackle this poor-yield problem, especially in the case of Taxol®, a meeting was organized by the National Cancer Institute in Washington, D. C., in June 1990, where four suggestions were made: 1. Finding a better source for the supply of Taxol®, such as a different species or a cultivar of Taxus, or a different plant part or cultivation conditions. 2. Semisynthesis of Taxol® from a more abundant precursor. 3. Total synthesis of Taxol®. 4. Tissue culture production of Taxol® or a close relative. Out of these four ways, the most successful one was semisynthesis. While three successful total syntheses of Taxol® have been achieved, they have not been proven to be economically better than the semisynthetic approach.

2. 6 Structure Elucidation

In most cases of extraction and isolation of natural products, the end point is the identification of the compound or the conclusive structure elucidation of the isolated compound. However, structure elucidation of compounds isolated from plants, fungi, bacteria, or other organisms is generally time consuming, and sometimes can be the ‘‘ bottleneck’’ in natural product

research. There are many useful spectroscopic methods ofgetting information about chemical structures, but the interpretation of these spectra normally requires specialists with detailed spectroscopic knowledge and wide experience in natural product chemistry. With the remarkable advances made in the area of artificial intelligence and computing, there are a number of excellent automated structure elucidation programs available that could be extremely useful. If the target compound is known, it is often easy to compare preliminary spectroscopic data with literature data or to make direct comparison with the standard sample. However, if the target compound is an unknown and complex natural product, a comprehensive and systematic approach involving a variety of physical, chemical, and spectroscopic techniques is required. Information on the chemistry of the genus or the family of plant or microbe under investigation could sometimes provide additional hints regarding the possible chemical class of the unknown compound. The following spectroscopic techniques are generally used for the structure determination of natural products: 1. Ultraviolet-visible spectroscopy (UV-vis): Provides information on chromophores present in the molecule. Some natural products, e. g., flavonoids, isoquinoline alkaloids, and coumarins, to name a few, can be primarily characterized (chemical class) from characteristic absorption peaks. 2. Infrared spectroscopy (IR): Determines different functional groups, e. g.,-C= O, -OH, -NH2, aromaticity, and so on, present in a molecule. 3. Mass spectrometry (MS): Gives information about the molecular mass, molecular formula, and fragmentation pattern. Most commonly used techniques are: electron impact mass spectrometry (EIMS), chemical ionization mass spectrometry (CIMS),

electrospray ionization mass spectrometry (ESIMS), and fast atom bombardment mass spectrometry (FABMS). 4. NMR: Reveals information on the number and types of protons and carbons (and other elements like nitrogen, fluorine, etc.) present in the molecule, and the relationships among these atoms. The NMR experiments used today can be classified into two major categories: a. One-dimensional techniques: 1HNMR, 13CNMR, 13CDEPT, 13CPENDANT, 13C J mod., nOe-diff., and so on. b. Two-dimensional techniques: 1H-1H COSY, 1H-1H DQF-COSY, 1H-1H COSY-lr, 1H-1H NOESY, 1H-1H ROESY, 1H-1H TOCSY (or HOHAHA), 1H-13C HMBC, 1H-13C HMQC, 1H-13C HSQC, HSQCTOCSY, and the like. In addition to the above-mentioned spectroscopic techniques, X-ray crystallographic techniques provide information on the crystal structure of the molecule, and polarimetry offers information on the optical activity of chiral compounds.

2. 7 Assays

Chemical, biological, or physical assays are necessary to pinpoint the target compound(s) from a complex natural product extract. At present, natural product research is more focused on isolating target compounds (assay-guided isolation) rather than trying to isolate all compounds present in any extract. The target compounds may be of certain chemical classes, have certain physical properties, or possess certain biological activities. Therefore, appropriate assays should be incorporated in the extraction and isolation protocol. The following basic points should be borne in mind when carrying out assays of natural products: 1. Samples dissolved or suspended in a solvent different from the original extraction solvent must be filtered or centrifuged to get rid of any insoluble matter. 2. Acidified or basified samples

should be readjusted to their original pH to prevent them from interfering with the assay. 3. Positive and negative controls should be incorporated in any assay. 4. Ideally, the assay should be at least semiquantitative, and/or samples should be assayed in a series of dilutions to determine where the majority of the target compounds resides. 5. The assay must be sensitive enough to detect active components in low concentration. Physical assays may involve the comparison of various chromatographic and spectroscopic behaviors, e. g., HPLC, TLC, LC-MS, CE-MS LC-NMR, and so on, of the target compound with a known standard. Chemical assays involve various chemical tests for identifying the chemical nature of the compounds, e. g., FeCl3 can be used to detect phenolics, Dragendorff's reagent for alkaloids, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) for antioxidant compounds, and so on. Bioassays can be defined as the use of a biological system to detect properties (e. g., antibacterial, antifungal, anticancer, anti-HIV, antidiabetic, etc.) of a crude extract, chromatographic fraction, mixture, or a pure compound. Bioassays could involve the use of in vivo systems (clinical trials, whole animal experiments), ex vivo systems (isolated tissues and organs), or in vitro systems (e. g., cultured cells). In vivo studies are more relevant to clinical conditions and can also provide toxicity data at the same time. Disadvantages of these studies are costs, need for large amount of test compounds/fractions, complex design, patient requirement, and difficulty in mode of action determination. In vitro bioassays are faster (ideal for HTS), and small amounts of test compounds are needed, but might not be relevant to clinical conditions. The trend has now moved from in vivo to in vitro. Bioassays available today are robust, specific, and more sensitive to even as

low as picogram amounts of test compounds. Most of them can be carried out in full or semiautomation (e. g., using 96- or 384-well plates). There are a number of biological assays available to assess various activities, e. g., Drosophila melanogaster BII cell line assay for the assessment of compounds with ecdysteroid agonist or antagonist activity, antibacterial serial dilution assay using resazurin as indicator of cell growth, etc. Most of the modern bioassays are microplate based and require a small amount of extract, fraction, or compound for the assessment of activity. While it is not the intention of this chapter to discuss at great length various assays presently available, a summary of two typical assays used in natural product screening, the DPPH assay and antibacterial serial dilution assay using resazurin as indicator of cell growth, is presented here as an example (Gray, 2006).

Hyperlipidemia

3. 1 Introduction

Today in most of the developed and developing countries, hyperlipidemia and thereby atherosclerosis is the leading cause of cardiac illness and deaths. 1, 2 In 1984 it was demonstrated for the first time that there exists a link between serum cholesterol levels and risk to coronary heart disease (CHD). 3 Worldwide, it causes deaths almost twice as many as those caused by cancer and 10 times as many as those caused by accidents. Despite significant medical advances, heart attacks due to coronary artery disease (due to atherosclerosis, that affects the arteries supplying blood to the heart) and stroke (due to atherosclerosis that affects the arteries supplying blood to the brain) are responsible for more deaths than all other causes combined. A

1% drop in serum cholesterol reduces the risk for CHD by 2%. In addition to this, different cholesterol lowering drugs or non-pharmacological treatments can significantly reduce morbidity from CHD, thus providing a causal role for cholesterol in coronary events.

Hyperlipidemia and atherosclerosis

Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions such as coronary heart disease, ischemic cerebrovascular disease, and peripheral vascular disease. A causal relationship between the elevated plasma lipids and the development of atherosclerotic plaques has been well established. Hyperlipidemia is an elevation of lipids in the bloodstream and these lipids include fats, fatty acids, cholesterol, cholesterol esters, phospholipids, and triglycerides. CHD is caused by the narrowing of the artery that supplies nutrients and oxygen to the heart. The main reason for this narrowing is atherosclerosis. The word atherosclerosis is derived from the greek words, athero (meaning gruel or paste) and sclerosis (hardness). Atherosclerosis may be defined as degenerative changes in the intima of medium and large arteries. The degeneration includes accumulation of lipids, complex carbohydrates, blood and blood products, and cellular waste products, and is accompanied by the formation of fibrous tissues and calcium deposition in the intima of blood vessels. These deposits or plaques progressively decrease the lumen of the artery, reduce its elasticity, and may create foci for the thrombi and subsequent occlusion of blood vessels.

3. 3 Pathophysiology of hyperlipidemia

An understanding of the biology of the lipoproteins and the pathophysiology of hyperlipidemic states is essential to the rational choice of treatment regimen.

3. 3. 1 Exogenous pathway: route of uptake of dietary lipids

Chylomicrons (CM) are complexes of triglycerides (TG), cholesteryl esters (CE), and apoproteins. After the removal of triglycerides they become chylomicron remnants (Fig. 4). Chylomicrons are degraded by lipoprotein lipase on endothelial cells of adipose tissue and muscle. After removal of TG for storage, the CM remnants are transported to the liver. This results in dietary TG stored in adipose tissue and muscles.

3. 3. 2 Endogenous pathway: route for distribution of cholesteryl esters (CE) from liver to target cells

VLDL is secreted by the liver into plasma and transported to adipose tissue and muscles, where lipoprotein lipase extracts most triglycerides. The remnant IDL is either taken up by the liver or circulated until the remaining triglycerides are removed forming LDL particles, rich in cholesterol. LDL is cleared from plasma through LDL receptor-mediated endocytosis. This results in transfer of TG from liver to target cells via VLDL, as well as, transfer of CE from liver to target cells via LDL (Fig. 4).

3. 3. 3 Route for cholesterol recovery

Reverse cholesterol transport is a pathway where cholesterol is transported from atherosclerotic plaques or other lipids back to liver to be excreted into the faecus via bile. As cell dies and the cell membranes turnover, free

cholesterol is released into the plasma. It is immediately absorbed into HDL particles, esterified with a long chain fatty acid by lecithin cholesterol acyl transferase (LCAT), and transferred to VLDL or IDL by a cholesteryl ester transfer protein in plasma. Eventually, it is taken up by the liver as IDL or LDL, thus resulting in the recovery of cholesterol from cell membranes and reincorporation into LDL pool or return to liver.

3. 3. 4. De novo cholesterol biosynthesis

Liver synthesizes 2/3rd of the total cholesterol made in the body. The rate limiting enzyme is 3-hydroxy-3-methylglutaryl(HMG)-CoA reductase and provides feedback regulation by controlling the cholesterol concentrations in cells.

3. 3. 5 Cholesterol excretion by enterohepatic circulation

Bile salts are synthesized from cholesterol in the liver, released into the intestine, and recycled. A small amount of bile acid is excreted. This results in conversion of liver cholesterol to bile salts for excretion.

Types of hyperlipoproteinemias (Fredrickson/WHO classification)

The classification of hyperlipoproteinemias is presented in Table 3. Type I: Severe elevation of chylomicrons and TG due to congenital deficiency of lipoprotein lipase or apo C-II. Deposition of fats in skin represents clinical manifestations of the disorder. Type IIA: Elevation of LDL cholesterol. Genetic conditions responsible are familial hypercholesterolemias, polygenic hypercholesterolemias, familial combined hyperlipidemia, and familial defective apolipoprotein B 100. These individuals are at high risk of

developing premature coronary artery disease. Type IIB: Characterized by the elevation of both LDL cholesterol and triglyceride levels. Familial combined hyperlipidemia is the most common genetic cause of this disorder where both VLDL and LDL levels are elevated. Type III: Develops due to a defect in VLDL remnant clearance. Also known as familial dysbetalipoproteinemia. These individuals have difficulty in removing triglyceride rich VLDL remnant particles and this consequently leads to elevation of cholesterol and triglycerides. Type IV: Characterized by hypertriglyceridemia (250–500 mg/dl). Causes are multiple as well as genetic. Diseases contributing to this are diabetes, nephrosis along with administered medications. Type V: Elevated levels of chylomicrons and VLDL. Defective lipolysis and an overproduction of VLDL are responsible for this. Causes can be genetic or can occur secondary to diabetes mellitus, obesity or alcohol consumption.

Treatment of hyperlipidemia

3. 5. 1 Hydroxymethyl glutarate coenzyme A (HMG-CoA) reductase inhibitors (statins)

The statins (Lovastatin, Simvastatin, Pravastatin, Fluvastatin, Atorvastatin, Rosuvastatin, Pitavastatin) competitively inhibit HMG-coenzyme A reductase, which is involved in the rate limiting step of cholesterol biosynthesis in the liver. In addition, statins increase levels of HDL which has cardiovascular protective effects. Furthermore, statins reduce the susceptibility of lipoproteins to oxidation, both in vitro and ex vivo. Oxidative modification of LDL appears to play a key role in mediating the uptake of lipoprotein cholesterol by macrophages.

3. 5. 2 Bile acid sequestrants

Bile acid sequestrants (anion-exchange resins) are highly positively charged resins and bind to the negatively charged bile acids. Because of their large size, these resins are not absorbed and thus the bound bile acids are excreted in the stool. Consequently the liver's pool of bile acids depletes, leading to increased conversion of cholesterol to bile acid in hepatocytes. Decline in hepatic cholesterol content stimulates the production of LDL receptors and also increases synthesis of cholesterol in the liver. The increase in hepatic LDL receptors increases LDL clearance and lowers LDL-C levels. However this effect is partially offset by the enhanced cholesterol synthesis caused by the upregulation of HMG-CoA reductase.

3. 5. 3 Fibrates or lipoprotein lipase stimulants (fibric acid derivatives)

The fibrates (Clofibrate, Gemfibrozil, Fenofibrate, Ciprofibrate, and Benzafibrate) affect lipid metabolism as agonists of the enzyme peroxisome proliferator activated receptor alpha (PPARa). This results in the peripheral lipolysis of triglyceride rich lipoproteins through the stimulation of lipoprotein lipase, a reduction in apoprotein C-III, and an increase in apoprotein A-I production. Fibrate mediated increase in HDL-C is due to PPARa stimulation of apo A-I and apo A-II expression, which increases HDL levels. Though, the primary effect of fibrates is marked reduction in triglyceride levels, additionally moderate reduction in LDL cholesterol may also be seen. Four key mechanisms responsible for the effects of fibrates are; increased lipolysis, increased hepatic fatty acid uptake, reduced hepatic triglyceride production, and increased LDL uptake by LDL receptors. Stimulation of

reverse cholesterol transport results in increased HDL. Clofibrate (ethylester of p-chlorophenoxyisobutyrate) is the prototype of fibric acid derivatives.

3. 5. 4 Niacin

Niacin is a water-soluble vitamin of type B, used to treat dyslipidemia. Niacin inhibits the lipolysis of triglycerides by hormone-sensitive lipase, which reduces transport of free fatty acids to liver and decreases hepatic triglyceride synthesis. In liver niacin reduces triglyceride synthesis by inhibiting both the synthesis and esterification of fatty acids that increases apo B degradation. 35 Reduction of triglyceride synthesis reduces hepatic VLDL production, which accounts for the reduced LDL levels. Niacin also enhances lipoprotein lipase (LPL) activity, which promotes the clearance of chylomicrons and VLDL triglycerides. Niacin raises HDL-C levels by decreasing the fractional clearance of apo A-1 in HDL, rather than by enhancing HDL synthesis (Jain, Kathiravan, Somani, & Shishoo, 2007).