
1

NATURAL PRODUCTS

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1.1	INTRODUCTION	12
1.2	HISTORY AND BACKGROUND OF THE USE OF NATURAL PRODUCTS AS THERAPEUTIC AGENTS	12
1.3	NATURAL PRODUCTS RESEARCH AND DEVELOPMENT—AN UPDATE	14
1.4	DISCOVERY OF NATURAL PRODUCTS	22
	Literature Sources	22
	Environmental Sources	23
1.5	ESSENTIAL PHARMACODYNAMICS	33
	Protein Targets of Drug Action	33
	General Principles of Drug Action	34
	Molecular Aspects of Drug Action—Receptors	37
	Molecular Aspects of Drug Action—Ion Channels	39
	Molecular Aspects of Drug Action—G-Protein-Coupled Systems	40
	Molecular Aspects of Drug Action—Receptors as Enzymes	40
	Molecular Aspects of Drug Action—Transcription Factors	41
	Molecular Aspects of Drug Action—Other Targets	41
	Drug Tolerance	42
1.6	SCREENING FOR NATURAL PRODUCT ACTIVITY	43
1.7	ISOLATION AND PURIFICATION OF NATURAL PRODUCTS	47
1.8	STRUCTURE IDENTIFICATION OF NATURAL PRODUCTS	51
1.9	SYNTHESIS OF NATURAL PRODUCTS	53
1.10	DEVELOPMENT OF NATURAL PRODUCTS	55
	Regulatory Guidelines and Nonclinical Development	55
	Learning from the Mistakes of the Past in the Development of Natural Products	58
1.11	FUTURE OF NATURAL PRODUCTS	60
	References	63

1.1 INTRODUCTION

By definition, the word *natural* is an adjective referring to something that is present in or produced by nature and not artificial or man-made. When the word *natural* is used in verbiage or written, many times it is assumed that the definition is something good or pure. However, many effective poisons are natural products [145]. The term *natural products* today is quite commonly understood to refer to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine [72]. That will not be the case in this chapter. The information presented here will be restricted to the discovery and development of modern drugs that have been isolated or derived from natural sources. While in some cases, such discovery and development may have been based on herbs, folklore, or traditional or alternative medicine, the research and discovery of, along with the development of, herbal remedies or dietary supplements typically present different challenges with different goals [93, 152]. So while the stories of herbs and drugs are very much intertwined, it needs to be fully appreciated that the use of herbs as natural product therapy is different than the use of herbs as a platform for drug discovery and further development.

1.2 HISTORY AND BACKGROUND OF THE USE OF NATURAL PRODUCTS AS THERAPEUTIC AGENTS

Natural products are generally either of prebiotic origin or originate from microbes, plants, or animal sources [115, 116]. As chemicals, natural products include such classes of compounds as terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acid bases, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and so forth. Natural products are not just accidents or products of convenience of nature. More than likely they are a natural expression of the increase in complexity of organisms [76]. Interest in natural sources to provide treatments for pain, palliatives, or curatives for a variety of maladies or recreational use reaches back to the earliest points of history.

Nature has provided many things for humankind over the years, including the tools for the first attempts at therapeutic intervention [115, 116]. Neanderthal remains have been found to contain the remnants of medicinal herbs [72]. The *Nei Ching* is one of the earliest health science anthologies ever produced and dates back to the thirtieth century BC [115, 116]. Some of the first records on the use of natural products in medicine were written in cuneiform in Mesopotamia on clay tablets and date to approximately 2600 BC [29, 30, 115, 116]. Indeed, many of these agents continue to exist in one form or another to this day as treatments for inflammation, influenza, coughing, and parasitic infestation. Chinese herb guides document the use of herbaceous plants as far back in time as 2000 BC [72]. In fact, *The Chinese Materia Medica* has been

repeatedly documented over centuries starting at about 1100 BC [29, 30]. Egyptians have been found to have documented uses of various herbs in 1500 BC [29, 30, 72]. The best known of these documents is the Ebers Papyrus, which documents nearly 1000 different substances and formulations, most of which are plant-based medicines [115, 116]. Asclepius (in 1500 BC) was a physician in ancient Greece who achieved fame in part because of his use of plants in medicine [72]. A collection of Ayurvedic hymns in India from 1000 BC and earlier describes the uses of over 1000 different herbs. This work served as the basis for *Tibetan Medicine* translated from Sanskrit during the eighth century [29, 30]. Theophrastus, a philosopher and natural scientist in approximately 300 BC, wrote a *History of Plants* in which he addressed the medicinal qualities of herbs and the ability to cultivate them. The Greek botanist Pedanius Dioscorides in approximately AD 100 produced a work entitled *De Materia Medica*, which today is still a very well-known European document on the use of herbs in medicine. Galen (AD 130–200), practiced and taught pharmacy and medicine in Rome and published over two dozen books on his areas of interest. Galen was well-known for his complex formulations containing numerous and multiple ingredients. Monks in monasteries in the Middle Ages (fifth to the twelfth centuries) copied manuscripts about herbs and their uses [29, 30, 72]. However, it should not go unrecognized that it was the Arabs who were responsible for maintaining the documentation of much of the Greek and Roman knowledge of herbs and natural products and expanding that information with their own knowledge of Chinese and Indian herbal medicine [29, 30]. The Persian philosopher and physician Avicenna produced a work entitled *Canon Medicinæ*, which is considered to be the definitive summarization of Greek and Roman medicine. Li Shih-Chen produced a Chinese drug encyclopedia during the Ming Dynasty entitled *Pen-ts'as kang mu* in AD 1596, which records 1898 herbal drugs and 8160 prescriptions [115, 116]. John Wesley, the founder of Methodism, had a profoundly negative view on the status of physicians within society and in 1747 wrote a book entitled *Primitive Physic*, which was a popular reference book of the time detailing numerous natural cures [72]. When the colonists originally came to America, they lacked trained physicians and so turned to the Native Americans for advice in healing practices. Such a lack of conventional medicine and physicians in early America spawned the production of various types of almanacs and other publications that contained various natural product-based recipes and assorted tidbits of medical information. Indeed, in an effort to curry favor with commoners, physicians themselves turned to the production of self-treatment guides for the general public. Various types of societies and botanical clubs held meetings and published different types of communiqués to educate the public with regard to the availability of natural products and how they could be helpful to an individual's health. Samuel Thompson's *Thompson's New Guide to Health* was one very popular publication. For a variety of different reasons, the interest in natural products continues to this very day [6, 8, 17, 39, 72, 81, 88, 90, 104]. The first commercial pure natural product introduced for therapeutic use is gen-

erally considered to be the narcotic morphine, marketed by Merck in 1826 [118]. The first semisynthetic pure drug based on a natural product, aspirin, was introduced by Bayer in 1899.

1.3 NATURAL PRODUCT RESEARCH AND DEVELOPMENT—AN UPDATE

The World Health Organization estimates that approximately 80 percent of the world's population relies primarily on traditional medicines as sources for their primary health care [44]. Over 100 chemical substances that are considered to be important drugs that are either currently in use or have been widely used in one or more countries in the world have been derived from a little under 100 different plants. Approximately 75 percent of these substances were discovered as a direct result of chemical studies focused on the isolation of active substances from plants used in traditional medicine [29, 30]. The number of medicinal herbs used in China in 1979 has been estimated to be numbered at 5267 [115, 116]. More current statistics based on prescription data from 1993 in the United States show that over 50 percent of the most prescribed drugs had a natural product either as the drug or as the starting point in the synthesis or design of the actual end chemical substance [118]. Thirty-nine percent of the 520 new drugs approved during the period 1983 through 1994 were either natural products or derivatives of natural products [65]. Indeed, if one looks at new drugs from an indication perspective over the same period of time, over 60 percent of antibacterials and antineoplastics were again either natural products themselves or based on structures of natural products. Of the 20 top-selling drugs on the market in the year 2000 that are not proteins, 7 of these were either derived from natural products or developed from leads generated from natural products. This select group of drugs generates over 20 billion U.S. dollars of revenue on an annual basis [60, 65].

Drug development over the years has relied only on a small number of molecular prototypes to produce new medicines [65]. Indeed, only approximately 250 discrete chemical structure prototypes have been used up to 1995, but most of these chemical platforms have been derived from natural sources.

While recombinant proteins and peptides are gaining market share, low-molecular-weight compounds still remain the predominant pharmacologic choice for therapeutic intervention [60]. Just a small sampling of the many available examples of the commercialization of modern drugs from natural products along with their year of introduction, indication, and company are: Orlistat, 1999, obesity, Roche; Miglitol, 1996, antidiabetic (Type II), Bayer; Topotecan, 1996, antineoplastic, SmithKline Beecham; Docetaxel, 1995, antineoplastic, Rhône-Poulenc Rorer; Tacrolimus, 1993, immunosuppressant, Fujisawa; Paclitaxel, 1993, antineoplastic, Bristol-Myers Squibb.

The overwhelming concern today in the pharmaceutical industry is to improve the ability to find new drugs and to accelerate the speed with which

new drugs are discovered and developed. This will only be successfully accomplished if the procedures for drug target elucidation and lead compound identification and optimization are themselves optimized. Analysis of the human genome will provide access to a myriad number of potential targets that will need to be evaluated [60, 65]. The process of high-throughput screening enables the testing of increased numbers of targets and samples to the extent that approximately 100,000 assay points per day are able to be generated. However, the ability to accelerate the identification of pertinent lead compounds will only be achieved with the implementation of new ideas to generate varieties of structurally diverse test samples [60, 65, 66]. Experience has persistently and repeatedly demonstrated that nature has evolved over thousands of years a diverse chemical library of compounds that are not accessible by commonly recognized and frequently used synthetic approaches. Natural products have revealed the ways to new therapeutic approaches, contributed to the understanding of numerous biochemical pathways and have established their worth as valuable tools in biological chemistry and molecular and cellular biology. Just a few examples of some natural products that are currently being evaluated as potential drugs are (natural product, source, target, indication, status): manoalide, marine sponge, phospholipase- A_2 Ca^{2+} -release, anti-inflammatory, clinical trials; dolastatin 10, sea hare, microtubules, antineoplastic, nonclinical; staurosporine, streptomyces, protein kinase C, antineoplastic, clinical trials; epothilone, myxobacterium, microtubules, antineoplastic, research; calanolide A, B, tree, DNA polymerase action on reverse transcriptase, acquired immunodeficiency syndrome (AIDS), clinical trials; huperzine A, moss, cholinesterase, alzheimer's disease, clinical trials [60].

The costs of drug discovery and drug development continue to increase at astronomical rates, yet despite these expenditures, there is a decrease in the number of new medicines introduced into the world market. Despite the successes that have been achieved over the years with natural products, the interest in natural products as a platform for drug discovery has waxed and waned in popularity with various pharmaceutical companies. Natural products today are most likely going to continue to exist and grow to become even more valuable as sources of new drug leads. This is because the degree of chemical diversity found in natural products is broader than that from any other source, and the degree of novelty of molecular structure found in natural products is greater than that determined from any other source [31, 65, 142].

Where are these opportunities? Well, research into the use of plant-derived natural products alone in just the field of medicine covers a broad spectrum of activities [35, 67, 166, 168, 169]. Examples of such biological activity profiles would include, but are not limited to, nootropics, psychoactive agents, dependence attenuators, anticonvulsants, sedatives, analgesics, anti-inflammatory agents, antipyretics, neurotransmission modulators, autonomic activity modulators, autacoid activity modulators, anticoagulants, hyoplipidemics, antihypertensive agents, cardioprotectants, positive ionotropes, antitussives,

antiasthmatics, pulmonary function enhancers, antiallergens, hypoglycemic agents, antifertility agents, fertility-enhancing agents, wound healing agents, dermal healing agents, bone healing agents, compounds useful in the prevention of urinary calculi as well as their dissolution, gastrointestinal motility modulators, gastric ulcer protectants, immunomodulators, hepato-protective agents, myelo-protective agents, pancreato-protective agents, oculo-protective agents, membrane stabilizers, hemato-protective agents, antioxidants, agents protective against oxidative stress, antineoplastics, antimicrobials, antifungal agents, antiprotozoal agents, antihelminthics, and nutraceuticals [35]. Many frontiers remain within the field of natural products that can provide opportunities to improve our quality of life.

Fungal disease has historically been a difficult clinical entity with which to effectively deal. Fungal diseases can include more than just a mycosis and can also include allergic reactions to fungal proteins and toxic reactions to fungal toxins. Mycoses as a group include diseases that are significantly more serious and life-threatening than nail infestations, athlete's foot, or "jock-itch." Indeed, increasing numbers of overtly healthy individuals are becoming victims of the complications of fungal infestation. The reasons for this are that increasing numbers of people are receiving immunomodulatory treatment for an organ transplant or some underlying chronic systemic pathology, antineoplastic chemotherapy for cancer, or have been the recipients of proper or improper use of powerful antibiotics. Additionally there are a number of individuals within society that are infected with the human immunodeficiency virus (HIV). The available drugs to treat mycoses have been limited [5]. Furthermore, in this armamentarium, there are problems with dose-limiting nephrotoxicity, the rapid development of resistance, drug-drug interactions of concern, and a fungistatic mechanism of action. Thus there is an urgent need for the development of more efficacious antifungal agents with fewer limitations and less side effects. Ideally such compounds should possess good distribution characteristics, a novel mechanism of action, and a broad-spectrum candidal antifungal activity. The discovery and isolation of an echinocandin-type lipopeptide (FR901379) and lipopeptidolactone (FR901469) from microbes has been a significant achievement. These compounds are water soluble and inhibit the synthesis of 1,3- β -glycan, a key component of the fungal cell wall. Furthermore, since the cell wall is a feature particular to fungi and is not present in eukaryotic cells, such inhibitors certainly have the potential to demonstrate selective toxicity against the fungi and not against the animal or human host. The ultimate modifications of the lipopeptide and lipopeptidolactone referenced above led to the discovery of micafungin (FK463), which is currently in phase III clinical trials. This work along with the relatively recent approval of caspofungin (Merck) as a therapeutic agent for the treatment of disseminated aspergillosis are significant achievements in that they demonstrate that a melding of the proper research to identify and develop appropriate targets with the chemical and biological diversity found in natural products can be very rewarding.

Much ado has been made over recent years about endocrine disruptors and their effects on humans [33]. It needs to be recognized that endocrine disruptors are not just synthetic chemicals but can also be natural products. The use of natural product endocrine disruptors may provide significant insight into our understanding of the mechanisms by which the evolution of the genome can protect transactivation of the sex hormone receptors and aid in the development of drugs, which can protect the embryo during its development from hormone disruptive effects.

Diabetes is a multisystemic affliction, having impact on nearly every body organ. As a disease, it kills more individuals on a per annum basis than AIDS and breast cancer combined [148]. The impact on the quality of life of an individual suffering with diabetes is profound. A number of natural products currently exist that demonstrate hypoglycemic activity. Indeed, depending upon the source that one might use, there are approximately 800 to 1200 plants that exhibit hypoglycemic activity. While research and development efforts in this particular area thus far are largely restricted to traditional medicine uses, future research may well identify a potent antidiabetic agent.

The incidences of neuropsychiatric disorders are steadily increasing as our population increases in size and age. Such disorders include, but are not limited to, seizure disorders, schizophrenia, dementia, mania, aggression, memory loss, psychoses, age-related cognitive decline, depression, anxiety states, mood disorders, substance abuse, and substance dependence. There is a large body of data available that suggests the use of many natural products as potential treatments for these conditions and other neuropsychiatric disorders [18, 91, 92]. Indeed, a number of plant extracts have been associated with the treatment of various categories of mental symptoms and various types of receptor selectivity [18]. A very controversial potential psychotherapeutic agent is *Ginkgo biloba* [52]. A lack of understanding of mechanism of action, misidentification of materials, contamination of materials, intrinsic toxicity, and absence of standardization all contribute to this controversy. Further fractionation, isolation, and characterization of active components of these and other plants will undoubtedly lead to the discovery of novel neuropsychiatric agents as well as the debunking of other alleged therapies.

There are numerous blood-based diseases that afflict humans. These would include, but are not limited to, anemia, blood group incompatibility, blood protein disorders, bone marrow diseases, hemoglobinopathies, hemorrhagic diatheses, leukemia, disorders of leukocyte dysfunction, platelet disorders, and erythrocyte aggregation disorders. A number of natural products have been reported in the literature to be of value in the treatment of Epstein-Barr virus infection, leukemia, thrombosis and coagulopathy, malaria, anemia, and bone marrow diseases [113]. Extracts from the fungus *Trichothecium roseum*, the sea cucumber *Cucumaria japonica*, the legume *Amorpha fruticosa*, the tree *Magnolia officinalis*, and others may be useful in the therapeutic management of Epstein-Barr virus infection. Extracts from the basidiomycetes *Mycena pura* and *Nidula candida* may be useful in the treatment of leukemia. Com-

pounds isolated from *Streptomyces platensis* may be useful in the treatment of thrombocytopenia. Compounds obtained from the marine sponge *Aplysina archeri* have been reported to inhibit the growth of the feline leukemia virus. Scalarane-type bishomo-sesterterpenes isolated from the marine sponge *Phyllospongia foliascens* have been reported to exhibit cytotoxic, antithrombotic, and vasodilation activities. It should be noted that a number of natural products are based on the coumarin nucleus and as such may exhibit antithrombotic and antiplatelet activities. A number of blood-sucking animals have small, low-molecular-weight proteins in their salivas that interfere with the clotting of blood and therefore might be of value as potential anticoagulants. *Streptomyces hygroscopicus ascomyceticus* manufactures a macrolide that has been reported to have immunosuppressant activity and may prove to be beneficial in preventing transplant rejection in humans. It is entirely possible that these compounds and others offer sufficient structural diversity, range of biological activities, and differing mechanisms of action that new, safer, and more efficacious drugs to treat blood-based disorders could well burgeon from this library.

A wide variety of natural products are claimed to possess immunosuppressant activity, but it is often difficult to dissect this activity away from associated cytotoxicity [101]. Since the first heart transplant in the late 1960s, medicine has progressed to the point where most organ transplants have become relatively routine procedures. The survival of individuals with transplants is owed in large part to the discovery of the fungal metabolite cyclosporine A in 1970 and its widespread use starting in 1978. Indeed, cyclosporine A has achieved such success that it is currently being evaluated for value in the treatment of Crohn's disease, systemic lupus erythematosus, and rheumatoid arthritis. Research efforts abound in the area of natural products and immunosuppression. A methyl analog of oligomycin F isolated from *Streptomyces ostreogriseus* has been reported to quite effectively suppress B-cell activation and T-cell activation in the presence of mitogens at concentrations comparable to that of cyclosporine A. Concanamycin F first isolated from *Streptomyces diastatochromogenes* in 1992 has been found to possess a wide array of biological activities including immunosuppressive and antiviral activities. The experimental immunosuppressant (+)-discodermolide isolated from the marine sponge *Discodermia dissoluta* exhibits relatively nonspecific immunosuppression, causing the cell cycle to arrest during G₂ and M phases. This compound's current primary interest is as a potential antineoplastic agent since it stabilizes microtubules and prevents depolymerization, effectively causing cell cyclic arrest during the metaphase to anaphase transition. This same mode of activity is shared with Taxol (Paclitaxel), the epothilones, eleutherobin, and the sarcodictyins. The didemnins, cyclic peptides, were first isolated from the marine tunicate *Trididemnum solidum* and exhibit immunosuppressive activity through a generalized cytotoxicity mediated by inhibition of progression through the G₁ phase of the cell cycle by an unknown mechanism. The trichopolyns I to V from the fungus *Trichoderma polysporum* are

lipopeptides that suppress the proliferation of lymphocytes in the murine allogeneic mixed lymphocyte response assay. Triptolide from the plant *Tripterygium winfordii* demonstrates immunosuppressant activity through the inhibition of IL-2 receptor expression and signal transduction. The novel heteroaromatic compound lymphostin, obtained from *Streptomyces* KY11783 has demonstrated immunosuppressant activity through its potent inhibition of the lymphocyte kinase p56^{lck}. Over the last decade, research activities on immunosuppressants of natural product origin have focused on the mechanisms of inhibition of T-cell activation and proliferation. This approach has been fruitful, leading to the generation of significant information about signaling pathways between T cells, greater detail about the roles of T cells in immune function, and the discovery of Tacrolimus (Prograf) from the soil fungus *Streptomyces tsukubaensis*. As immunological research progresses, increasingly more potential targets will be elucidated for immunomodulatory therapeutic intervention. Natural products will undoubtedly provide a sound platform for the delivery of natural-product-based therapeutic agent candidates.

Natural-products-based anticancer drug discovery continues to be an active area of research throughout the world [34, 102, 112, 147]. While cancer incidences and the frequencies of types of cancer may vary from country to country, the most common sites for the development of neoplasia are generally considered to be the breast, colon/rectum, prostate, cervix/uterus, esophagus/stomach, pancreas, liver, lung, urinary bladder, kidney, ovary, oral cavity, and blood (leukemia and non-Hodgkin lymphoma) [147]. Currently, the chemotherapeutic management of these tumors involves a variety of different plant-based chemicals that are either currently in use or in clinical trials and include such drug classes as the vinca alkaloids, lignans, taxanes, stilbenes, flavones, cephalotaxanes, camptothecins, and taxanes. Despite the wide range of organ structure, type, and function, great similarities exist between the organs with regard to the pathogenesis of cancer. As more and more details of the molecular biology of cancer are revealed, more targets will present themselves for possible therapeutic chemical intervention in the growth and development of neoplasms. A somewhat new approach is that of cancer chemoprevention, where chemoprevention is defined as the prevention, delay, or reversal of carcinogenesis [112]. A few of the more promising cancer chemopreventive agents are (compound, plant source, target): brusatol, *Brucea javanica*, differentiation; zapotin, *Casimiroa edulis*, differentiation and apoptosis; apigenin, *Mezoneuron cacullatum*, antimutagenesis; deguelin, *Mundelea sericea*, inhibitor of ornithine decarboxylase; brassinin, *Brassica* spp., inducer of quinone reductase; and resveratrol, *Cassia quinquangulata*, cyclooxygenase inhibitor. A final note with regard to this approach is that it is important to appreciate that the distinction between chemopreventive agent and chemotherapeutic agent can become quite blurred.

A recurrent theme in neoplasia is the alteration of cell cycle control. One therapeutic approach to the treatment of neoplasia is the development of a treatment that would return to normal the altered cell cycle [143]. Cyclin-

dependent kinases (CDKs) control the progression of a cell through its growth cycle. CDKs are regulated through a series of site-specific complex mechanisms, and the components of such mechanisms include activating cyclins and endogenous CDK inhibitors. Processes of such mechanisms involve regulatory phosphorylation. There are natural products such as butyrolactone and staurosporine that are currently known to be able to provide such activity. These compounds and others generated from their platform are adenosine 5'-triphosphate (ATP) site-directed inhibitors and directly antagonize the activity of CDKs. Further research should more fully elucidate the most efficacious endpoint of CDK inhibition and lead to the control of neoplastic growth and possibly even bring about cytostasis or apoptosis.

The introduction of active agents derived from natural sources into the anti-cancer weaponry has already significantly changed the futures of many individuals afflicted with cancer of many different types. Continued research into natural sources will continue to deliver newer and more promising chemicals and chemical classes of anticancer agents with novel mechanisms of action that will improve survival rates to even higher degrees.

Human immunodeficiency virus infection is a devastating, globally widespread disease that consumes significant health-care dollars in the due course of management of patients [79]. Most of the currently useful anti-HIV agents are nucleosides and are limited in use due to severe toxicity and emerging drug resistance. Natural products, with their broad chemical structural diversity, provide an excellent opportunity to deliver significant therapeutic advances in the treatment of HIV [167]. Many natural products with novel structures have been identified as having anti-HIV activities [79, 167]. Betulinic acid, a triterpenoid isolated from *Syzygium claviflorum*, has been found to contain anti-HIV activity in lymphocytes. The quassinoid glycoside isolated from *Allanhus altissima* has been found to inhibit HIV replication. Artemisinin, isolated from *Artemisia annua*, is a sesquiterpene lactone that is of special interest because of its novel structure, potent antimalarial activity, and activity against *Pneumocystis carinii*. A novel phorbol ester isolated from *Excoecaria agallocha* has been reported to be a potent inhibitor of HIV-1 reverse transcriptase. Indeed, most of the natural product chemicals that are attracting interest in this area of research are secondary metabolites such as terpenes, phenolics, peptides, alkaloids, and carbohydrates and are also inhibitors of HIV reverse transcriptase. Other target opportunities in the life cycle of the human immunodeficiency virus available for exploitation are: (1) attachment of virus to cell surface, (2) penetration and fusion of the virus with the cell membrane, (3) reverse transcription via reverse transcriptase, (4) integration into the host genome, (5) synthesis of viral proteins including zinc fingers, and (6) processing of viral polypeptide with HIV protease and assembly of viral proteins and DNA into a viral particle, maturation, and extrusion of the mature virus [167].

Infectious viral diseases remain a worldwide problem. Viruses have been resistant to therapy or treatment longer than most other forms of life because

their nature is to depend on the cells that they infect for their multiplication and survival [41]. Such a characteristic has made the development of effective antiviral chemotherapeutic agents difficult. Today there are few effective antivirals available for use. In order to confidently wage the war against viruses, research efforts are now turning to the molecular diversity available from natural products. For the period 1983 to 1994, seven out of 10 synthetic agents approved by the Food and Drug Administration (FDA) for use as antivirals were based on a natural product. These drugs are famciclovir, stavudine, zidovudine, zalcitabine, ganciclovir, sorivudine, and didanosine. The viral genome can be composed of either RNA or DNA and HIV, which was discussed earlier is an RNA containing virus. The general potential targets of antiviral chemotherapy are: (1) attachment of virus to host cell, (2) penetration of the host cell by the virus, (3) viral particle uncoating, release, and transport of viral nucleic acid and transport proteins, (4) nucleic acid polymerase release/activation, (5) translation of mRNA (messenger RNA) to polypeptides (early proteins), (6) transcription of mRNA, (7) replication of nucleic acids, (8) protein synthesis (late proteins), (9) viral polypeptide cleavage into polypeptides necessary for maturation, (10) assembly of viral capsids and precursors, (11) encapsidation of nucleic acid, (12) envelopment, and (13) release. Early antiviral research focused on compounds that inhibited viral DNA synthesis, purine, and pyrimidine nucleoside analogs. Today most current antiviral agents target RNA-based viruses and the inhibition of reverse transcriptase in order to block the transcription of the RNA genome to DNA. Such inhibition would prevent the synthesis of viral mRNA and proteins. Protease inhibitors affect the synthesis of late viral proteins and viral packaging activity. There are no currently available drugs that target early viral protein synthesis. Antiviral compound research has included alkaloids, carbohydrates, chromones, coumarins, flavonoids, lignans, phenolics, quinines, xanthenes, phenylpropanoids, tannins, terpenes, steroids, iridoids, thiopenes, polyacetylenes, lactones, butenolides, phospholipids, proteins, peptides, and lectins. While plants have been a common hunting ground, many other sources are now starting to be explored, especially the marine environment. The use of natural products in the field of antiviral research appears to be limited only by the imagination of the researcher.

This review has demonstrated that natural products are indeed viable sources and resources for drug discovery and development [3]. Indeed, without natural products, medicine would be lacking in therapeutic tools in several important clinical areas such as neurodegenerative disease, cardiovascular disease, solid tumors treatment, and immunoinflammatory disease [4, 64, 122]. Furthermore, the continual emergence of new natural product chemical structure skeletons, with interesting biological activities along with the potential for chemical modification and synthesis bode well for the utility of natural products. Finally, the uses of natural products need to be by no means restricted to pharmaceuticals but can also be expanded to agrochemicals. For example, the use of pyrethrins obtained from *Chrysanthemum* spp. as insecti-

cides has been very popular over the years and persists today. Research continues into the use of natural products as pesticides. While the success stories have not been as numerous or spectacular for herbicides as they have been for drugs and pesticides, there have been victories along the way and the future holds strong potential for this field also [40, 99].

1.4 DISCOVERY OF NATURAL PRODUCTS

Literature Sources

Natural products can come from anywhere. People most commonly think of plants first when talking about natural products, but trees and shrubs can also provide excellent sources of material that could provide the basis of a new therapeutic agent. Animals too, whether highly developed or poorly developed, whether they live on land, sea, or in the air can be excellent sources of natural products. Bacteria, smuts, rusts, yeasts, molds, fungi, and many other forms of what we consider to be primitive life can provide compounds or leads to compounds that can potentially be very useful therapeutic agents. Suffice it to say that natural products can come from any point or level on the phylogenetic tree. When searching for natural products, one should never feel that a form of life is too low, simple, or grotesque to provide a compound of interest. However, before one goes marching out into the woods, sailing out into the sea, climbing the highest mountains, or descending into the deepest caves, it is appropriate to perform a little bit of research, and hence a visit to the library becomes the first step in any search for a natural product. Remember that the use of a natural product as a therapeutic agent requires that one match some particular characteristic of the compound with a disease or condition. This matching process involves a two-tiered process. The first tier can be comprised of a thorough evaluation of the pathophysiologic condition of interest including any pertinent history, etiology, clinical manifestations, biochemistry, clinical chemistry, hematology, physiology, pathology, and therapeutics. With the therapeutic target in mind and a complete understanding of the pathophysiology of the condition, one can then begin a search for a natural product that has some particular characteristic that might suggest that it has utility as a potential therapeutic agent. Alternatively, one could take the approach of observing or finding a particular characteristic of a natural product and then searching for a useful disease or condition to treat with the material. The choice of which path to follow is a personal one, with either selection being equally useful. Admittedly, in the past and not infrequently the search for and investigation of a natural product arose out of serendipity.

There has been an explosion of information in the biomedical sciences over the last 25 years, and attempting to find information about natural products can be a challenging task [39]. Ideally, it is important to know the history, folk-

lore, origin of use, source, chemical structure, availability, method of preparation, pharmacology, toxicology, and therapeutics of any natural product. However, the reality is that many times even for compounds or preparations that have been used for centuries, there are significant gaps in this portfolio of desired information. Nonetheless, a trip to the library can be very useful. It is amazing what research projects individuals have become engaged in over the years. Excellent sources of information on natural products can be readily found on the Internet. Some of these sites available on the Internet are shown in Table 1.1. Information retrieval services or search engines are also quite useful tools. These, however, can be expensive and require the aid of someone skilled in their use. Some of the more common sources are shown in Table 1.2. A number of helpful books have been published on natural products over the years, but the preponderance of them dwells on the topic of plants. The more common of these references are shown in Table 1.3. Finally, periodicals probably represent the most current and timely sources of research and information on natural products. Some of the better and more prominent journals are shown in Table 1.4.

Environmental Sources

Myriad opportunities abound throughout nature that can provide natural products with significant therapeutic potential [20, 29, 30, 61, 77, 83]. These opportunities can present themselves from almost any niche of nature and most likely some that have not even yet been discovered.

TABLE 1.1 Internet Sites

American Botanical Council <i>http://www.herbalgram.org</i>	American Herbalists Guild <i>http://www.americanherbalistsguild.com/top.htm</i>
Complementary & Alternative Methods <i>http://www.cancer.org/eprise/main/docroot/eto/eto_5?sitearea=eto</i>	Dr. Duke's Phytochemical and Ethnobotanical Databases <i>http://www.ars-grin.gov/duke</i>
Herb Research Foundation <i>http://www.herbs.org</i>	Herbal Education Services <i>http://www.botanicalmedicine.org</i>
Herbal Medicine: Internet Resources: Alternative Medicine <i>http://www.pitt.edu/~cbw/herb.html</i>	HerbMed <i>http://www.herbmed.org/</i>
International Herb Association <i>http://www.iherb.org</i>	MEDLINEplus: Alternative Medicine <i>http://www.nlm.nih.gov/medlineplus/alternativemedicine.html</i>
National Center for Complementary and Alternative Medicine <i>http://nccam.nih.gov/</i>	World Health Organization Publications <i>http://www.who.int/dsa/cat98/trad8.htm</i>

Adapted from DerMarderosian and Beutler [39].

TABLE 1.2 Information Retrieval Services

BIOSIS <i>http://www.biosis.org</i>	Chemical Abstracts Service <i>http://www.cas.org/</i>
Current Contents and Science Citation Index <i>http://www.isinet.com/isi</i>	<i>Excerpta Botanica. Section A, Taxonomica et Chorologica</i> , International Association for Plant Taxonomy, G. Fischer, Stuttgart, New York.
The Herb Research Foundation <i>http://www.herbs.org/</i>	<i>IPA (International Pharmaceutical Abstracts)</i> , <i>http://info.cas.org/ONLINE/DBSS/ipass.html</i> . Database contains international coverage of pharmacy and health-related literature.
<i>Lynn Index</i> , Massachusetts College of Pharmacy	<i>Medicinal and Aromatic Plants Abstracts</i> , Publications and Information Directorate, Council of Scientific and Industrial Research (CSIR), New Delhi, India.
MEDLINE, MEDLARS <i>http://www.nlm.nih.gov/</i>	NAPRALERT (NAtural PRoducts ALERT) <i>http://www.aq.uiuc.edu/~ffh/napra.html</i>
NAPRONET <i>http://ccl.net/chemistry/resources/tips/list/NAPRONET/index.shtml</i>	Poisindex System <i>infor@mdx.com or http://www.micromedex.com/products/poisindex</i>
Toxicology Information Response Center (TIRC) <i>http://www.ornl.gov/TechResources/tirc/hmepg.html</i>	TOXLINE <i>http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE</i>

Adapted from DerMarderosian and Beutler [39].

Microbes The observation of the effects of microbial secondary metabolites on pathogenic fungi and bacteria spawned the antibiotic era [29, 30, 38]. Since its inception, humankind has grown to take for granted the wonders of antibiotics. Indeed, results of antibiotic use were so impressive that compounds of this general type were for the most part the only chemicals used against pathogenic microorganisms. Due to escalating research and development costs, the difficulties in identifying novel structures and the problems in finding new mechanisms of action, the golden era of antibiotics appeared to be meeting its own demise. Many individuals even professed that the use of antibiotics might even be passé, with modern medicine choosing more modern techniques for treatment. However, this same library of antibiotics had, over the same time, also been found to exhibit other biological properties that might be beneficial to humankind. Accordingly, research into the complete biological activity profiles of antibiotics began with the intent of identifying the utility of these compounds for various pharmacological or agrochemical applications. This shift in focus expanded the search for new natural products from microbes, where microbial metabolites might be used to treat diseases other than those caused

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TABLE 1.3 *Continued*

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TABLE 1.3 *Continued*

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M. Sweet. <i>Common Edible and Useful Plants of the East and Midwest</i> . Healdsburg, CA: Naturegraph Publishers, 1975.	M. Sweet. <i>Common Edible and Useful Plants of the West</i> . Healdsburg, CA: Naturegraph Publishers, 1976.
V. E. Tyler, L. R. Brady, and J. E. Robbers. <i>Pharmacognosy</i> , 9th ed. Philadelphia: Lea and Febiger, 1988.	World Health Organization. <i>WHO Monographs on Selected Medicinal Plants</i> , Vol. 1. Geneva: World Health Organization, 1999.
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Adapted from DerMarderosian and Beutler [39].

by bacteria and fungi. Microorganisms have proven to be an excellent source of novel natural products including polyketide and peptide antibiotics as well as classes of other biologically active compounds [123]. Today, microbial metabolites are used as antineoplastic agents (e.g., mitomycin), immunosuppressive agents (e.g., rapamycin), hypocholesterolemic agents (e.g., pravastatin), enzyme inhibitors (e.g., desferal), antimigraine agents (e.g., ergot alkaloids), herbicides (e.g., bialaphos), antiparasitic agents (e.g., salinomycin), bioinsecticides (e.g., tetranactin), and ruminant growth promoters (e.g., monensin) [38]. It is noteworthy that some of these compounds when originally discovered failed in their development for their original uses as either antibiotics or as agricultural fungicides. Bacteriocins are ribosomally produced antibiotic peptides and proteins that can be subdivided into different categories, lantibiotics, and microcins. Lantibiotics are produced by Gram-positive bacteria and microcins are produced by Gram-negative bacteria. Both lantibiotics and microcins possess an ability to form pores or punch holes in membranes of susceptible microorganisms. This property is of interest to the food industry, as bacteriocins are produced by *Lactococcus* spp., which are used in the preservation of various foodstuffs [123].

The cyanobacterium *Nostoc ellipsosporum* has been found to produce a novel protein (CV-N), which has generated interest because of its viricidal activity and apparent potential as an anti-HIV therapeutic agent. The antiviral activity of this chemical is reported to be mediated through specific interactions with the HIV envelope glycoproteins gp120 and possibly gp41.

TABLE 1.4 Periodicals

<i>American Journal of Natural Medicine</i> http://www.impakt.com	<i>Botanical Review</i> http://www.nybg.org/bsci/spub/botr/frntpg3b.html
<i>Bulletin on Narcotics</i> http://www.odccp.org/bulletin_on_narcotics.html	<i>Canadian Journal of Botany</i> http://www.nrc.ca/cgi-bin/cisti/journals/rp/rp2_desc_e?cjb
<i>Canadian Journal of Herbalism</i> http://www.herbalists.on.ca/journal/	<i>Economic Botany</i> http://www.econbot.org
<i>European Journal of Herbal Medicine</i> http://www.ejhm.co.uk/	<i>Herb Companion Press</i> http://www.interweave.com/
<i>Herb Quarterly</i> http://www.herbquarterly.com	<i>International Journal of Aromatherapy</i> http://www.harcourt-international.com/journals/ijar
<i>Journal of Ethnopharmacology</i> http://www.elsevier.com/locate/jethpharm	<i>Journal of Natural Products</i> http://pubs.acs.org/journals/jnprdf/index.html
<i>Medical Anthropology: Cross Cultural Studies in Health and Illness</i> http://www.sfu.ca/medanth	<i>Medical Herbalism: A Journal for the Clinical Practitioner</i> http://medherb.com/MHHOME.SHTML
<i>Natural Health</i> http://www.naturalhealth1.com	<i>Natural Product Letters</i> http://www.tandf.co.uk/journals/titles/10575634.html
<i>Natural Product Reports</i> http://www.rsc.org/is/journals/current/npr/nprpub.htm	<i>Pharmaceutical Biology</i> http://www.szp.swets.nl/szp/frameset.htm
<i>Phytochemistry: The International Journal of Plant Biochemistry and Molecular Biology, The Journal of the Phytochemical Society of Europe and the Phytochemical Society of North America</i> http://www.elsevier.nl/locate/inca/273	<i>Phytomedicine: International Journal of Phytotherapy and Phytopharmacology</i> http://www.urbanfischer.de/journals
<i>Phytotherapy Research</i> http://www3.interscience.wiley.com/cgi-bin/jtoc?ID=12567	<i>Plant Foods for Human Nutrition</i> http://www.wkap.nl/jrnltoe.htm/0921-9668/contents
<i>Planta Medica: Natural Products and Medicinal Plant Research</i> http://www.thieme.de/plantamedica/fr_inhalt.html	<i>Toxicon: Official Journal of The International Society on Toxicology</i> http://www.elsevier.com/locate/toxicon
<i>Veterinary and Human Toxicology, American Academy of Veterinary and Comparative Toxicology, Comparative Toxicology Laboratories</i>	<i>Z Naturforsch</i> http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

Adapted from DerMarderosian and Beutler [39].

Research has further revealed that CV-N is a new class of antiviral agent because of its unique interaction with envelope glycoproteins.

Biologically active proteins produced from fungi should not be ignored. *Trichoderma viride* produces a polypeptide, alamethicin, that has demonstrated ion-gating activity. Some fungal ribotoxins such as mitogillin have been found to act as specific ribonucleases. The edible mushroom *Rozites caperata* has been found to produce a compound, RC-183, which inhibits herpes simplex virus (HSV-1 and HSV-2) in a murine animal model.

Various compounds isolated from microbes and fungi have gained favor in their utility as tools to investigate such activities as nerve growth and mechanisms of microbial infiltration and pathogenesis. The fruiting body of *Pleurotus ostreatus* has been reported to produce a lectin that demonstrates potent antitumor activity in mice. Similarly, another lectin from *Volvariella volvacea* shows antiproliferative activity against various tumor cell lines via the mediation of a concentration-dependent stimulation of the expression of cyclin kinase inhibitors resulting in cell cycle arrest in the G₂/M phase. Many other fungal lectins have been isolated, but complete determinations of their biological activity profiles remain to be completed.

The ingenious application of molecular and cellular biology to detect receptor agonistic and antagonistic activities of compounds has tremendously aided drug research activity. In addition to the many opportunities that exist for microbial secondary metabolites with regard to medicine and agrochemicals, time has also demonstrated the increased need for the development of novel antibiotics because of the development of resistant strains of pathogens, the appearance of new diseases, and the inadequacy or toxicity of current drugs [37]. The diversity of microorganisms is of a staggering quantity, and only an extremely small proportion of bacteria and fungi have been examined for the production of potentially useful secondary metabolites.

Plants The higher plants produce a variety of different types of compounds, including biologically active proteins. Some of these types of compounds are even shared with other organisms, and they include such chemical families as lectins, defensins, cyclotides, and ribosome-inactivating proteins [123]. Ribosome-inactivating proteins are a group of proteins exhibiting a wide spectrum of biological activities, including a ribonucleolytic activity for which the group is named. These compounds can be obtained from *Panax ginseng* and other plants and have been reported to demonstrate antifungal and antiviral activities. Ribosome-inactivating proteins from *Phytolacca americana* have been reported to be active against HIV and from *Saponaria officinalis* to possess antineoplastic activity. Plant antimicrobial peptides comprise another large group of biologically active compounds. This group of compounds can be further subdivided into thionins, defensins, cyclotides, and lectins. Thionins are small proteins that selectively form disulfide bridges with other proteins or form ion channels in membranes. This ability to make membranes more permeable suggests the potential for antimicrobial activity. Defensins are

cysteine-rich peptides that also permeabilize membranes but appear to be very specific in their activity, targeting fungal cell membranes and not mammalian or bacterial cell membranes. Cyclotides are a protein family, whose mechanism of action has not yet been elucidated, but have demonstrated inhibitory activity against HIV-1. Finally, the lectins are proteins that have a noncatalytic domain that binds reversibly to specific carbohydrates. This activity encompasses potentially a wide spectrum of biological activities including antineoplastic activity, immunostimulation activity, immunosuppression activity, antimicrobial activity, and antimicrobial activity.

Higher plants have been over time an extremely popular source of natural products [23, 95, 123, 127, 130]. Since 1961 approximately nine different compounds derived from plants have been approved in the United States as antineoplastic agents [95]. These drugs include vinblastine, vincristine, vinorelbine, etoposide, teniposide, paclitaxel, docetaxel, topotecan, and irinotecan. The mechanisms of action of these compounds ranges from that of tubulin inhibition to the inhibition of the essential DNA enzymes topoisomerase I and topoisomerase II or both topoisomerases I and II.

Podophyllum peltatum reportedly has curative properties on the venereal wart, *Condyloma acuminatum*, along with other therapeutic benefits [130]. The active glycoside component of extracts from *P. peltatum* has been found to inhibit mitosis in vitro. Furthermore, derivatives of this compound have been found to be capable of arresting cells in either late S phase or early G₂ phase, without inhibiting microtubule assembly. The latex from the plant *Euphorbia lateriflora* has been used both as a purgative and a cure for ringworm [23]. The Pacific yew tree yielded the antineoplastic, paclitaxel [29, 30]. *Newboutonia vellutina*, a Euphorbiaceae, has been reported to have utility both as a parasiticide as well as a treatment for gastric distension [23]. Compounds isolated and identified from this source will undoubtedly continue to make strong contributions to modern therapeutics.

Insects Various polypeptides of interest have been isolated from the venoms of arachnids and arthropods that prey on insects [123]. Indeed a variety of reviews have been published on ion channel toxins from scorpions and specific venom proteins and neurotoxins from arthropod venoms and their effects on the cardiovascular system. The caterpillar *Lonomia achelous* has been reported to be the source of a biologically active protein that causes a coagulopathy that is mediated via specific interactions with Factor V in the coagulation cascade. While this discovery requires further work to evaluate its therapeutic potential, the discovery may open new opportunities in thrombosis research. Insect peptides have been the subject of research into the immune defense system of insects but have not yet been investigated for effects and potential benefit in humans. Compounds from this peptide group include such sources as the termite (*Pseudacanthotermes spiniger*), the mosquito (*Anopheles gambiae*), the moth (*Heliothis virescens*), and the beetle (*Oryctes rhinoc-*

eros). Insect-derived natural products offer another strong potential avenue for the development of future drugs.

Vertebrates Research into a variety of antimicrobial peptides, such as magainins, defensins, cathelicidins, and protegrins generated by vertebrates has over recent time become very popular. Cathelicidin-type peptides are a broad range of antimicrobial proteins that have been isolated from rabbits, mice, sheep, and humans. Cathelicidins are composed of two different domains, the cathelin and antimicrobial domains. The cathelin domain becomes bactericidal after cleavage from the antimicrobial domain. Purportedly, these materials bind to lipopolysaccharide and neutralize its activity. The pit viper, *Bothrops jaracaca*, produces a compound that spurred the synthesis of the angiotensin-converting enzyme (ACE) inhibitors captopril and enalapril [29, 30]. The skin of the poisonous frog, *Epipedobates tricolor*, produces epibatidine. This substance has ultimately led to the creation of a new class of analgesics. Other vertebrates produce compounds that have been the subjects of substantial research, which offer potential opportunities to identify useful compounds in the areas of cardiovascular function, immune function, and central nervous system function.

Marine Organisms The marine environment, arguably the original source of all life, is a rich source of bioactive compounds [14, 45–48, 84, 89, 96, 144, 157]. More than 70 percent of our planet's surface is covered by the oceans, and some experts feel that the potentially available biodiversity on the deep seafloor or coral reefs is greater than that existing in the rainforests [62].

Consider the fact that many marine organisms have soft bodies and lead a sedentary lifestyle, making a chemical system of defense almost essential for survival. Marine organisms have evolved the ability to synthesize such toxic compounds or extract or convert pertinent compounds from other marine microorganisms. Natural products from marine organisms are released into the water and therefore are rapidly diluted, accordingly they must be very potent materials to have the desired end effect. The richly available marine biodiversity that is available to us has to this point only been explored to an extremely limited extent. Furthermore, the primary chemical diversity available from marine organisms is most likely capable of delivering an even greater abundance of secondary metabolites for research use. For all of these reasons it is believed that the natural products that are available from the seas and oceans provide a tremendous opportunity for the discovery of novel therapeutic agents [62].

The first discovery of a marine-based biologically active compound of therapeutic interest was really quite by accident approximately 10 years after the end of the World War II [29, 30]. The C-nucleosides isolated from the Caribbean sponge *Cryptotheca crypta* were found to possess antiviral activity. This discovery eventually led to the development of cytosine arabinoside, a

useful antineoplastic agent. Biologically active marine proteins derived from the venom of marine snails of the *Conus* genus have attracted a significant level of research over the years [123]. These conotoxin peptides interact in a unique fashion with voltage-gated ion channels to induce a wide spectrum of pharmacological effects. Such effects include anesthesia, analgesia, and anti-convulsant activity. The conotoxin ziconotide is currently under review in the United States for use in the treatment of chronic, opiate-resistant pain. According to some estimates, there are most likely approximately 1000 different *Conus* snails. Each snail produces up to approximately 200 different venoms. The broad spectrum of biological activities manifested by each of these venom components multiplied by the number of snails and venom components available suggests significant opportunity for new drugs from the snail alone. The mussel *Mytilis edulis* has been reported to produce antibacterial peptides and cytotoxic lectins. Horseshoe crabs produce a variety of different antibacterial peptides and proteins. Indeed, *Limulus polyphemus* produces an interesting group of antimicrobial peptides referred to as polyphemusins, and a synthetic peptide based on the sequence of polyphemusin II has been reported to strongly inhibit the cytopathic effect of infection with HIV.

It has been reported that the tunicate *Styela clava* produces α -helical antimicrobial peptides called clavaniins that are homologous to the magainins produced by certain types of frogs. The marine worm *Cerebratulus lacteus* produces neurotoxic polypeptides, which have been found to contain the ability to make membranes more permeable. Sponges have been found to produce a wide variety of interesting secondary metabolites. For example, lectins isolated from *Chondrilla nucula* have achieved utility in the histochemical labeling of melanoma and breast and thyroid carcinomas. Another compound, the protein mapacalcine, produced by *Cliona vastifica*, has been reported to specifically block non-L-type calcium channels in murine duodenal myocytes, while at the same time not exhibiting any affect on T-type calcium flux or potassium or chloride currents. Various proteins from sponges have been reported to selectively kill human tumor cells. For example, a protein from the sponge *Tethya ingalli* lyses ovarian cancer cells; a glycoprotein from *Pachymatisma johnstonii* has been discovered to inhibit cell growth at the G₀/G₁ phase via a unique mechanism in a non-small-cell-bronchopulmonary carcinoma line. Interestingly, this same chemical has been found to have potential as an antiparasitic in the treatment of leishmaniasis, by demonstrating cytotoxic activity against the parasite in the promastigote and amastigote stages of the life cycle. The protein niphatevirin isolated from the sponge *Niphates erecta* has been discovered to inhibit HIV-induced cytopathic effects, cell-to-cell fusion, and syncytium formation via interacting directly with the CD4 cellular receptor. Finally, a compound isolated from the sponge *Microciona prolifera* has been reported to bind to gp120 and resultantly protect T-lymphoblastoid cells from infection with HIV.

The so-called mining of the sea for potential drugs did not start in earnest until the mid-1970s because of a technical inability to effectively gain access

to the biodiversity that exists within the seas and oceans. Despite this slow start, the potential for contribution of new drugs is staggering, a vision that undoubtedly will become reality over the next decade. Examples of such research activity and fulfillment of this future hope are (chemical, source, chemical class, chemical target, therapeutic Indication): AM336 (AMRAD), cone snail, peptide, ion channels, chronic pain; GTS21 (Taiho), nemertine worm, anabaseine-derivative, ion channels, Alzheimer's disease and schizophrenia; LAF389 (Novartis), sponge, amino acid derivative, methionine aminopeptidase inhibitor, cancer; OAS1000 (OsteoArthritis Sciences), soft coral, diterpene-pentoseglycoside, PLA₂ inhibitor, wound healing and inflammation; ILX651 (Ilex Oncology), sea slug, peptide, microtubule-interference, cancer; Cemadodin (Knoll), sea slug, peptide, microtubule interference, cancer; Yondelis, sea squirt, isoquinolone, DNA-interactive agent, cancer; Alipidin, sea squirt, cyclic depsipeptide, oxidative stress inducer, cancer; Kahalalide F, sea slug/alga, cyclic depsipeptide, lysosomotropic compound, cancer; KRN7000 (Kirin), sponge, α -galactosylceramide, immunostimulatory agent, cancer; squalamine lactate, shark, aminosteroid, calcium-binding protein antagonist, cancer; IPL512602 [Inflazyme/Aventis], sponge, steroid, unknown, inflammation [62].

1.5 ESSENTIAL PHARMACODYNAMICS

Modern science has provided a detailed understanding of the interaction of many therapeutic drugs with biological systems at a biochemical or molecular biological level. The proper use of such information can provide a wealth of tools for the discovery of new drug opportunities by providing a framework to permit comparison of the mechanisms of action, biological activities, therapeutic indices, and the therapeutic potentials of drugs along with the ability to forecast possible problems [7, 87, 121, 131, 134, 137, 159].

Protein Targets for Drug Action

The actions of drugs can be divided into those occurring at specific sites and those that are nonspecific. Nonspecific effects are typically mediated through a generalized effect in many organs, and the response observed depends upon the distribution of the drug. The response is usually associated with the organ or organs having the highest concentrations of the drug. Specific effects are produced by an interaction of the drug with a specific site or sites either on the cell membrane or inside of the cell. The protein targets for drug action on mammalian cells can be broadly divided into receptors, ion channels, enzymes, transcription factors, and other nonspecific sites of action. The term *target* can also be broadly defined to include such things as microorganisms and cancer cells, but this discussion will be limited to normal mammalian systems or cells. It should be noted that while there are other types of protein that are known

to function as drug targets, it must be appreciated that there exist many drugs whose sites of action have not yet been elucidated in detail. Furthermore, many drugs are known to bind to plasma proteins as well as to various cellular constituents, without producing any obvious physiological effect. Nevertheless the above generalization that most drugs act on one of the five types of protein targets listed above is a reasonable initial working classification system.

General Principles of Drug Action

There are a variety of different types of drug action. Accordingly, drugs can be classified into specific categories such as agonists, antagonists, partial agonists, inverse agonists, allosteric modulators, enzyme inhibitors or activators, and those having nonspecific action.

Agonists bind to a receptor or site of action and produce a conformational change in the receptor or that site, which mimics the action of the normal physiologic binding ligand. At low concentrations, the activity of the drug is additive with the natural ligand. Drugs can differ in both their affinity or strength of binding and the rate of association and dissociation from the receptor or binding site. The affinity or strength of binding of the drug to the receptor ultimately determines the concentration necessary to produce a response and therefore is directly related to the potency of the drug. For some compounds a maximal response may of necessity invoke the contribution of all available receptors, but for most drugs a maximal response is produced while some receptors remain unoccupied. The presence of spare receptors becomes an important point when considering changes in the numbers of available receptors resulting from adaptive responses occurring in response to either chronic exposure to a drug or the irreversible binding of an antagonist. The rate of binding or dissociation of a drug to a receptor or site of action is generally of little importance in determining the rate of onset or termination of a drug's elicited effect *in vivo* because such behavior depends mostly on the rate of delivery to and removal from the target organ, in other words, the rates of absorption and elimination of the drug from the body. The effect of changes in the numbers of receptors on the dose–response curve for an agonist depends on the potency of the agonist, receptor occupancy by the agonist, and the efficacy of the agonist.

Antagonists bind to a receptor but do not elicit the necessary conformational change required to produce the normal response–effect. These types of compounds will block access to the receptor or binding site by the normal physiologic ligand. It is important to keep in mind that antagonist-induced effects may only be observable when the normal agonist or ligand is present. The binding of most clinically useful antagonists is both reversible and competitive. Consequently, typically a receptor or site of action blockage can be overcome by increasing the concentration of the natural ligand or another agonist drug. Most antagonists shift the dose–response

curve to the right but do not alter the magnitude of the maximum possible response.

Partial agonists demonstrate both agonist and antagonist activities. For these types of drugs, the activity demonstrated by the drug is a function of the concentration of the natural ligand or agonist. Maximal binding of the partial agonist to a receptor will produce only a submaximal response. This is most likely the result of incomplete amplification of the receptor signal via G proteins. A partial agonist will demonstrate agonist activity at low concentrations of the natural ligand, but the dose-response will not attain maximal activity even when all of the receptors are occupied. Alternatively, at high concentrations of the natural ligand a partial agonist will behave as an antagonist because it will prevent the access of the natural ligand to the receptor, thereby preventing a maximal response.

Inverse agonists act in such a fashion on receptors as to produce a change opposite to that caused by an agonist. While not totally understood, the discovery of this phenomenon has given rise to the theory that receptors exist in equilibrium between active and inactive forms in the absence of an agonist ligand. The presence of an agonist will increase the proportion of active receptors, but the presence of an inverse agonist will shift the balance toward the more inactive receptors, thereby reducing the level of basal activity. Antagonists, when bound to a receptor will block the activity not only of agonists but also of inverse agonists. The mechanism of action of inverse agonists is not well characterized but may involve the destabilization of receptor-G protein coupling. Inverse agonists may preferentially bind to the inactivated form of the receptor, shifting the equilibrium away from the active form. An additional complication to this whole concept is the fact that compounds that are normal antagonists for some tissue receptors act as inverse agonists for the same receptor in a different tissue. The concept of inverse antagonism and its use in the therapeutic utility of drugs remains to be fully understood and better characterized.

Allosteric modulators do not act directly on a ligand/receptor site but may bind elsewhere on the receptor to enhance or decrease the binding of the natural physiologic ligand to the receptor. Some drugs have a target site of action that is an enzyme, and these compounds can effect their action on either the catalytic site or elsewhere on the molecule at an allosteric site. Finally some compounds merely elicit a broad and generalized effect that causes the desired therapeutic outcome. Examples of such activity might be that of osmotic diuretics and their ability to induce diuresis through the general action of osmosis or general anesthetics, which modulate neuronal cell membrane fluidity.

Drug action also involves the demonstration of a number of different important properties. These are specificity, selectivity, potency, and efficacy. Specificity refers to the fact that many drugs act only at one type of receptor (e.g., cholinergic versus noradrenergic). Drugs that are not specific in their action for one type of receptor display a wide variety of not necessarily desirable side effects.

The site of action of a drug may in some cases involve one or more members of a family or group of receptors. In such a situation, where the drug may act preferentially with one member of the group, it is said to be selective in its action. Alternatively, the drug may show a similar affinity for more than one member of a group or family of receptors and is therefore referred to as being nonselective. Drugs may demonstrate a predilection for a particular receptor type or subtype and may bind to a different receptor type or subtype to different degrees. In these situations, it is possible to determine the individual dose–response relationships for each receptor type or subtype. The selectivity of a drug is a measure of the degree of separation of the individual dose–response curves for different receptor types or subtypes. Ultimately, the expression of selectivity is dependent upon the dose and the concentration of the drug at the different receptors. High concentrations of an agonist drug will typically generate a maximal occupancy of all of the involved and available receptor types or subtypes with little to no discrimination or selectivity. High concentrations of an agonist drug can even create a blockade of the receptor. One should always be careful to include the appropriate qualifications when referring to selectivity and nonselectivity because populations of receptors can be related or unrelated to each other. For example, one can speak of the selectivity of a drug for α -adrenoceptors as compared to β -adrenoceptors or β_1 -adrenoceptors as compared to β_2 -adrenoceptors.

The *in vitro* potency of a drug is determined by the strength of its binding to a receptor. This is also referred to as the affinity of the drug for the receptor. The more potent a drug is, the lower will be the concentration required to affect its binding to a receptor and to provide a response for an agonist or to block a response for the case of an antagonist. Potencies of different drugs are generally compared or contrasted using ratios of the different doses required to either elicit or block an equivalent response. Dose–response curves are typically S-shaped. If the mechanism of action is identical or very similar for the drugs being compared, the linear or midportions between the lower and upper plateaus of the dose–response curve are commonly found to be parallel. To make comparisons between different points on distinct dose–response curves more obvious and facile, a linearizing transform can be utilized for each sigmoid curve. A popular technique for linearizing data is the logit plot. Potencies are generally not determined by comparing responses at identical or similar doses. This is because with the selection of a single administered dose, inherent differences between individual drugs will cause too large of a difference in drug responses. Rather, relative potencies are determined by an evaluation of the ratio of the doses for different drugs that produce equivalent responses on the respective drug dose–response curves. For example, the doses that cause 50 percent inhibition of the target activity on various dose–response curves could be compared for different drugs. Drugs that demonstrate *in vitro* the highest affinities for a receptor are generally the most potent. However, the *in vivo* dose–response relationship will be a function of the delivery of the drug to the site of action. Such delivery is related to the absorption, distribu-

tion, and elimination (pharmacokinetics) of a drug. Therefore, it is important to keep in mind that for a series of related drugs in vivo behavior may not always reflect in vitro receptor binding properties.

The efficacy of a drug is its ability to produce the maximum possible response. For example, drugs can be classified as full agonists or partial agonists. Full agonists produce an increase in response with increase in concentration up to that point at which the maximum possible response is elicited. A partial agonist will produce an increase in response with an increase in concentration, but it cannot produce a maximal possible response.

Molecular Aspects of Drug Action—Receptors

The activities of most cellular processes are highly controlled in order for the cell to exist under optimum homeostatic conditions not only when at rest but also in response to the myriad of physiological and metabolic demands that are placed upon it. These reflex actions require specific responses appropriate for a given cell type to signals elicited as a result of a change in physiological state. These signals most commonly are specific chemicals that are released into the general circulation or are locally released but then are recognized by a targeted or specific cell. Ultimately the condition of homeostasis is based upon (a) the generation of a chemical signal, (b) the recognition of a chemical signal, and (c) the generation of an appropriate response or cellular change(s).

A chemical signal eventually binds to a specific type of macromolecule of a cell. This binding in and of itself then triggers a cellular response. The chemical signal is referred to as the ligand. The cellular macromolecule is referred to as the receptor. Receptors can be located in different places. They may be located in the cell membrane in order to accept extracellular ligands that cannot cross or only cross with difficulty the cell membrane. Alternatively, they can be located in the cytoplasm, where they will react with lipid-soluble ligands that can pass through the cell membrane. The binding produces a receptor–ligand complex that will then generate an appropriate cellular response. There are three general types of responses: direct, indirect, and second messenger. A direct response might be the inhibition of a specific process. An indirect response might involve the interaction of the receptor–ligand complex with another moiety (e.g., macromolecule) to continue or complete the process. A second-messenger response would involve the production of an additional chemical signal (second messenger), which would ultimately control a cellular process.

There are different structural and functional classifications of receptors, but generally speaking there are just a few functional families whose members share both common mechanisms of action and similarities in molecular structure. There are at least four main types of receptors: types 1 through 4. The classification of receptors is based on molecular structure and the nature of the receptor–effector linkage. Type 1 receptors are typically located in a mem-

brane and are directly coupled to an ion channel. Type 2 receptors are located in a membrane and are coupled by a G protein to an enzyme or channel. Type 3 receptors are located in membranes and are directly coupled to an enzyme. Finally, type 4 receptors are located in the nucleus or cytosol and are coupled via DNA to gene transcription.

Although there are some exceptions, ligands typically bind to receptors in a reversible fashion. Accordingly, the intensity and duration of the response generated from the binding of the ligand to the receptor is a function of the lifetime of the ligand–receptor complex. The interaction(s) between a receptor and a ligand most commonly do not involve the formation of permanent covalent bonds, but rather do involve the formation of weaker and reversible forces, such as ionic bonding, hydrogen bonding, van der Waals forces, and hydrophobic interactions.

Within a physiologic entity, there are myriad possible extracellular and intracellular chemical signals that are produced that can affect multiple different processes. Subsequently, a very important property of a receptor is its specificity or the extent to which a receptor can recognize, discriminate, and respond to only one signal. Some receptors demonstrate a very high degree of specificity and will bind only a single endogenous ligand, while other receptors are less specific and will bind a number of different endogenous ligands. This ability of receptors to recognize, discriminate, and bind to a given ligand depends on the degree and type of interaction between the receptor molecule and specific chemical structural characteristics of the ligand. Chemical structural differences between ligands may be very subtle in nature. However, receptor specificity occurs because the generation of a fully functional receptor–ligand complex requires the formation of reversible binding interaction between various different molecular sites on the ligand and on the receptor in a special three-dimensional spatial relationship. Receptors themselves are proteins folded into a three-dimensional configuration so that the specific arrangement of reversible interaction sites on the receptor itself is consolidated into a very small volume, referred to as the receptor binding site.

As receptors and ligands each have three-dimensional configurations, the ligand must be presented to the receptor in a very specific three-dimensional configuration to bind and produce a functional receptor–ligand complex. Many drugs exist in different stereoisomeric forms, and each of these various stereoisomers may well exhibit different receptor binding behavior and resulting response. Different isomers can be equally active or some active, some partially active and some even toxic. Furthermore, it is important to realize that if a particular drug exists in equal concentrations of two different isomeric forms, only one of which is active and the other of which is inactive, then only 50 percent of the drug mixture is therapeutic. This has led over the years to the recognition of the value of the separation and purification of individual isomers of drugs for development as potential therapeutic agents.

Different types of receptors recognize and bind to different ligands. Furthermore, there may be different subtypes of a given receptor, each of which

recognizes or binds to the same specific ligand but generates different intracellular responses. Various receptor subtypes are often found in different tissues, organs, or distribution patterns in an organ and hence can produce different end effects. The existence of receptor subtypes creates the opportunity for specific drugs to produce extremely selective actions with fewer unwanted side effects.

At any given time, the number of receptors in a cell is not static but rather is a dynamic. There is a high turnover of receptors as they are continuously removed and replaced. Drug treatment itself can either increase the number of receptors, a phenomenon that is called upregulation, or decrease the number of receptors, a phenomenon referred to as downregulation. This potential change in numbers of receptors can be an important aspect of drug treatment and management of a clinical case to achieve a desired therapeutic response.

Molecular Aspects of Drug Action—Ion Channels

Receptors for several neurotransmitters exist as agonist-regulated, ion-selective channels in the plasma membrane of a cell and are referred to as ligand-gated ion channels. These receptors send their signals by altering a cell's membrane potential or its ionic composition. This group of receptors includes nicotinic cholinergic receptors, γ -aminobutyric acid receptors (subtype A), glycine receptors, aspartate receptors, glutamate receptors, nicotinic cholinergic receptors, and 5-hydroxytryptamine (subtype 3) receptors. These receptors are all multiple subunit proteins. Each protein subunit spans the cell membrane, and the subunits are arranged symmetrically in such a fashion as to form a channel. This channel opens and closes upon proper stimulation as a result of specifically induced molecular structural changes.

While some ion channels are linked to a receptor and open only when the receptor is occupied by an agonist or ligand, there are other types of ion channels, which themselves serve as targets for drug action. This type of interaction can be indirect, involving other intermediates, but the interaction can also be direct with the behavior of the channel being modulated by the binding of a drug directly to a part or parts of the channel protein. The simplest type of ion channel blocking involves a physical chemical barricade of the channel opening by the drug itself. More complex types of direct ion channel blocking involve drug-channel protein interactions. Normally, an ion channel opens in response to membrane depolarization. In these situations, a drug, by binding with ion channel protein alters or modulates the probability of the channel opening. The degree of inhibition or facilitation of the opening of the ion channel is a function of the chemical structure of the drug itself. In this latter case, a drug actually affects the gating of the channel, while in the former case the drug actually blocks penetration of the channel. The modulation of ion channels by drugs is a very important mechanism through which pharmacologic effects are mediated at the cellular level.

Molecular Aspects of Drug Action—G-Protein-Coupled Systems

There is a large family of receptors that utilize heterotrimeric guanosine 5'-triphosphate (GTP)-binding regulatory proteins. These regulatory proteins, known as G proteins, behave as transducers to send signals to their effector proteins. Ligands for G-protein-coupled receptors include eicosanoids, a variety of lipid signaling molecules, various peptides, different proteins, and a host of biogenic amines. Effectors for G-protein-regulated receptors include adenylyl cyclase, phospholipase C_β , Ca^{2+} currents, rho GTP exchange catalysts, inward-rectifying K^+ currents, and phosphatidylinositol-3-kinase. G-protein receptors occur widely in nature and are widely used drug targets.

G-protein-coupled receptors span the cell membrane and exist as a bundle of seven α helices. A cleft exists in the three-dimensional configuration of these seven α helices, which binds agonists on the extracellular face of the receptor. Alternatively, agonists can also bind to a globular ligand-binding domain located on the extracellular face of the G-protein-coupled receptor. The G proteins themselves bind to the cytoplasmic face of the receptor. These receptors respond to the binding of an agonist by promoting the binding of GTP to the G protein. The binding of the GTP to the G protein activates the G protein, which in turn activates the effector protein. The G protein remains in an activated state until it hydrolyzes the bound GTP to guanosine 5'-diphosphate (GDP). G proteins are composed of an α -GTP-binding subunit, which is specifically recognized by the receptor and an associated dimer of β and γ subunits. G proteins are defined by α -subunit composition, such as α_s , α_i , α_o , α_q , and α_{13} . The activation of the G_α subunit by GTP permits the regulation of an effector protein and promotes the release of $G_{\beta\gamma}$ subunits, which in turn regulate their own group of effectors.

There are many different G-protein-coupled receptor types in a cell. Each type is specific for one of many different G proteins. Each G_α subunit can in turn regulate one or more effectors. Therefore, receptors that bind to multiple different ligands can focus their signals through a single G protein. Alternatively, a G-protein-coupled receptor is also capable of sending multiple signals through activation of more than one G protein species. It should be appreciated that receptor G-protein effector systems are intricately versatile and complex networks.

Molecular Aspects of Drug Action—Receptors as Enzymes

Receptors with inherent enzymatic activity are most commonly cell surface protein kinases. These receptors demonstrate their regulatory activity by phosphorylating various effector proteins at the inner face of the cell membrane. The biochemical reaction of phosphorylation changes the molecular structure, biological properties, and hence the biological activities of an effector or its interactions with other protein molecules. Catalytic activities include tyrosine kinase, tyrosine phosphatase, guanyl cyclase, serine kinase, and threonine

kinase. However, tyrosine residues are the most common substrate. Basic receptor protein kinases are composed of an agonistic-binding domain on the extracellular membrane surface, a single element that spans the cell membrane and a protein kinase domain on the cytoplasmic face of the cell membrane. Other variations exist of this basic model and include oligomerization and/or the elaboration of other regulatory or protein binding domains to the cytoplasmic portion of the receptor. Some protein kinase receptors lack the attached intracellular enzymic domain, and in response to agonist binding, attract, attach, or link to and activate a distinct soluble and mobile protein kinase on the cytoplasmic side of the cell membrane.

There can be further variation on the above structural theme for receptor enzymes. For example, tyrosine phosphatases possess an extracellular enzymatic domain. In other receptor enzymes, the intracellular domain is not a protein kinase, but rather a guanyl cyclase, which synthesizes a second messenger, adenosine-3',5'-cyclic monophosphate (cAMP). Other variations of this receptor type may also be possible.

Before leaving this category of receptor, it is appropriate to mention second messengers. A variety of signals within a cell are sent via second-messenger pathways. While there are few cytoplasmic second messengers, their release and presence can affect many different activities. Recognized second messengers include cAMP, nitric oxide, diacylglycerol, Ca^{2+} , inositol phosphates, and guanosine-3,5'-cyclic monophosphate (cGMP). Second messengers can exert control on each other either directly or indirectly. Directly, they can alter the metabolism of other second messengers. Indirectly, they can share intracellular targets. Such a complex pattern of regulatory activity permits a cell to respond to the presence of an agonist with an integrated and concerted expression of second messengers and responses. Cyclic AMP is the best known of the second messengers and is synthesized by adenylyl cyclase under the control of different G-protein-coupled receptors.

Molecular Aspects of Drug Action—Transcription Factors

There are receptors for steroid hormones, thyroid hormones, retinoids, vitamin D, and other molecules that are soluble DNA binding proteins that regulate the transcription of specific genes. These receptors are part of a larger family of transcription factors that are regulated by phosphorylation, by association with other proteins, binding to metabolites, or binding to regulatory ligands. These receptors exist as homo- and heterodimers, and their actions and activity can also be regulated by higher order oligomerization with other regulatory molecules.

Molecular Aspects of Drug Action—Other Targets

Enzymes can also serve as targets for drugs. Typically, the drug is a substrate that is structurally related to the normal substrate and acts as a competitive

inhibitor of the enzyme. Another type of enzyme–drug interaction occurs when a drug is a false substrate. In this type of situation, the drug undergoes the enzyme-mediated chemical transformation to form an atypical product, which then undermines the normal metabolic pathway. This altered function of the metabolic pathway occurs as a result of the creation of a substrate that is not usable for the next step in the metabolic process or ultimately generates a partially functional or nonfunctional end product. It should be mentioned that drugs can also be metabolized by enzymes in such a fashion as to either convert the drug into a toxic reactive intermediate causing significant cellular damage or convert the drug from a totally inert moiety (prodrug) into an active compound with therapeutic benefit.

In addition to the sites and mechanisms of action discussed above, drugs may also bind to and either activate or inhibit other specific sites. These could include specific types of cells, cellular organelles, transport proteins, specific ion pump, and the like. A discussion of all of these additional targets is beyond the scope of this chapter.

Drug Tolerance

An observed decrease in drug response with repeated doses is commonly referred to as the development of tolerance. Tolerance may occur as a result of a decrease in the concentration of the drug at the receptor site or through a decrease in response of the receptor to the same concentration of the drug. Some drugs can stimulate their own metabolism, are therefore eliminated more rapidly with repeated dosage, and accordingly less drug is available to elicit a response. However, the most clinically important and relevant examples of tolerance result from changes in numbers of receptors and subsequent quantitative changes in concentration–response relationships.

Desensitization is a term that is used to describe changes in the dose–response relationship, irrespective of time, arising from a decrease in response of the receptor. Desensitization can result from decreased G-protein coupling, decreased receptor binding affinity, downstream modulation of the initial signal, or the downregulation (decreased numbers) of receptors. This latter process is slow and takes hours to days to become effective. However, extracellular receptors coupled to G proteins can show rapid desensitization within minutes during continued activation that can occur through two different mechanisms. The first of these mechanisms is homologous desensitization, in which enzymes are activated as a result of ligand binding to a receptor–G protein complex. These enzymes include G-protein-coupled receptor kinases (GRKs), which interact with the $\beta\gamma$ subunit of the G protein and inactivate the occupied receptor protein by phosphorylation. The second of these mechanisms is heterologous desensitization, in which the receptor, whether occupied or not, is inactivated through phosphorylation by a cAMP-dependent kinase, which can be switched on by a variety of signals that increase cAMP. Both α_1 -adrenoceptors and muscarinic receptors, which are

linked to phospholipase C, may also undergo desensitization via receptor phosphorylation, which uncouples the G protein from the receptor. Regardless of the mechanism, ultimately the phosphorylated receptor protein is eventually internalized and subjected to intracellular dephosphorylation before reentering the cytoplasmic membrane. Finally, the downstream modulation of a signal may occur through either a feedback mechanism or through simple depletion of an essential cofactor.

1.6 SCREENING FOR NATURAL PRODUCT ACTIVITY

What is a screen? A screen is an assay or biological assay that provides a tool that can be used to test for or establish the presence and level of a target activity in a specific sample. Bioassays in a screening program should be rapid, simple to conduct, relevant, capable of being automated, cost effective, and of the potential to deliver high throughput [15, 16, 69]. Appropriate technology should be used to permit low limits of detection. This last point is important because the concentration of desirable compounds is unknown in each sample and so it behooves one to strive for the lowest possible limit of detection. Screens should also be specific for the molecular or cellular therapeutic target of choice. Appropriate additional discriminatory tests outside of the focus of the chosen target activity, such as cytotoxicity measurement for cell-based assays, or isotype specificity tests for molecular assays, are valuable in that they provide additional information relative to the overall value of a potential hit. Furthermore, data generated from all screens in which samples are tested should be compared so that selective hits can be identified at an early rather than a late stage. Such a combination of specific screens, data comparisons, and discriminatory assays makes possible the earliest selection of the best hits for continued work and success. The screens must work in the presence of the compounds to be tested and accordingly must be compatible with a given molecule's physico-chemical characteristics. Accordingly, natural product screens need to be operational in the presence of solvents and buffered against extremes of pH and ionic strength and should not be affected by the presence of color. Screens should always incorporate the proper use of controls, both positive and negative. Screens should be bidirectional with regard to their output and have a defined and easily interpretable endpoint. Screens need to be capable of delivering quantifiable data. Screens can be designed in such a fashion as to monitor only a single biological activity. However, there is value to the approach of coupling biological screens to evaluate multiple general biological activities in addition to the target activity [38]. This is because a compound that does not provide a target hit may well generate value for itself by demonstrating some other unrelated and unexpected activity. Alternatively, compounds that do provide hits may well expand their value by demonstrating other unexpected types of biological activity. Additional considerations of the construction of screens, screening programs, and screen design are beyond

the scope of this chapter, and the reader is instead referred to an excellent reference on the subject by Gad [54]. What will be addressed here are some of the common features of screens and screening specific to research on the development of natural products as drugs.

Before one can begin to screen, it is important to know for what one wants to screen. Drug discovery begins with basic ideas, ideas relevant to therapeutic targets and sources of compounds [40, 66, 164]. Therapeutic targets can arise from genomics, the molecular cloning of receptors and signaling molecules, a detailed understanding of physiology, biochemistry, and pathology, research into folklore or ethnomedicine, and knowledge of the traditional uses of natural products. Sources of compounds can present from existing chemical libraries, historical compound collections, natural product libraries, combinatorial libraries, rational chemical synthesis, general or targeted literature searches based upon existing knowledge or leads, and antisense oligonucleotides. The simultaneous consideration of all or at least several of these areas is essential to the original design of a screening program.

A totally random approach in the selection of a source can be coupled with mass screening, but such a path is typically not successful. But to be fair, the random approach is more likely to generate compound novelty [41]. Generally, approaches utilizing literature searching, existing chemical libraries and folklore, ethnomedicine, or traditional medicine are the most popular because of their cost effectiveness. It is worth noting that programs and selections based on or incorporating ethnomedicine or folklore are five times more effective in the ultimate generation of leads. The most effective approach is generally considered to be a mixing of as many components of the therapeutic arm with the compound source arm as opposed to the selection of any single aspect.

It is worth noting at this point that a new approach is being used in the field of natural products that leads to the generation of “unnatural natural product compounds” [40]. This approach is termed *biochemical combinatorial chemistry*. In this technique, appropriate secondary metabolic enzymes are isolated from a crude natural product mixture. These enzymes are then used to generate unnatural metabolites, which are isolated and then subjected to a bioassay procedure that couples a bioassay to an analytical procedure permitting structure elucidation.

What does one put into a screen? It is very tempting to consider the purification of natural products into their individual components before embarking on screening activities. However, this is a very economically challenging and financially unrealistic approach [65]. The classical approach is to design a screen or screening program that will permit the use of the assay or assays to provide guidance to successive steps in purification. An advantage of this is that the effectiveness and efficiency of purification can also be simultaneously evaluated at the same time as biological activity is being enhanced. Ultimately, after sufficient purification, a chemical structure can be determined for the active moiety. However, Grabley and Sattler [60] are of the opinion that the

use of cost-effective physico-chemical and chemical screening procedures will facilitate biological screening because of an ability to provide purer extracts if not pure compounds for biological screening.

Some objectives should be kept in mind when preparing natural product extracts for their ultimate introduction into the screening process [70]. First, every attempt should be made to stop ongoing biological processes. Second, steps should be taken to provide chemical stability of the compounds in the extract. Third, efforts need to be made to minimize losses of material. Fourth, sample preparation costs need to be minimized. When trudging through the iterations required to purify mixtures, one should be sure to: (1) focus on the activity of interest, (2) focus on the compound(s) of interest, (3) eliminate nuisance materials such as cell parts, biopolymers, and other compounds not of interest, and (4) be sure to enrich the composition of compounds of interest.

What types of screens, assays, or bioassays should be used? Assays for activity can be performed at a variety of levels ranging from the molecular level to the whole organism. While it is true that the high-throughput screening of synthetic compounds generated by combinatorial methods may be best achieved at the molecular level, Duke et al. [40] are of the opinion that natural product screening should be performed at the highest level possible since more effort per compound has been invested in the discovery of each compound.

Historically, natural products have been subjected to what is termed bioassay-directed isolation. In this approach, a crude natural product mixture is subjected to fractionation and the individual fractions then bioassayed for specific biological activity. This process continues repetitively with comparison of individual fraction assay data to a bioassay database. When the data is shown to match a previously known profile, the process is terminated and the sample is discarded. If the data is shown to provide a new profile, the structure of the compound is determined. However, this approach can lead to the rediscovery of previously known compounds after significant effort has been expended.

Low-molecular-weight natural products from a variety of sources represent unique structural diversity. In order to more adequately and efficiently access this diversity, various new strategies improving targeting and direction have been developed [60]. Modern separation/chemical characterization approaches can eliminate much of this problem by identification of the compounds before they are subjected to bioassay. Indeed the coupling of such techniques to biological screens can improve the quality of the assay result and shorten research and development time frames. These new tandem approaches are termed fractionation-driven bioassays. While biological screening directly correlates to a predefined biological effect, physico-chemical and chemical screens do not. In this latter case, lead selection is based on either physico-chemical properties or chemical reactivities. In both cases, the first step is the chromatographic separation of compounds from the complex source mixture. In the second step, the physico-chemical properties or chemical reactivities of the separated compounds are analyzed. Both of these chemical-based strategies have proven to be of value as auxiliary or

supplemental methods to biological approaches. Data generated from physico-chemical and/or chemical screening is very helpful in the de-replication or early identification and exclusion of known or otherwise unsuitable compounds that occur during high-throughput biological screening programs. Furthermore, the use of physico-chemical and chemical screening will aid in the establishment and building of natural-product-based compound libraries, which could then be used more broadly in testing programs.

There are two general types of new tandem assays. The first of these is referred to as the fractionation-driven bioassay. In this method, a crude natural product mixture is subjected to fractionation and the individual fractions then subjected to nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry/mass spectrometry (MS/MS). The structures of the compounds in the individual fractions are identified; and, if they are known, their biological activity profiles are evaluated from an existing database. If the structures are unknown, then the compounds are subjected to bioassay. In an alternative approach, termed the isolate and assay approach, a crude natural product mixture is subjected to automated fractionation and purification. The individual fractions are then subjected to bioassay. Desirable biological activity serves as the trigger to subject the sample to NMR or MS/MS and ascertain the structure(s). If the material is a novel compound, the structure can be optimized. If the material is a previously known compound, it may well be discarded, depending on its biological activity or toxicological profile.

A broad range of screening technologies are currently available for use in screening for natural-product-based drugs [69]. For molecular targets, such procedures would include generalized solution-phase assays, immobilized substrate assays, scintillation proximity assays, and time-resolved fluorescence assays. For cell based targets, cell signaling, cell communication, cell receptor, and reported gene assays are available. Isolated subcellular systems are also available [156]. Bioassays can, if desired, incorporate lower level organisms, isolated vertebrate organs, or whole animals. In short, systems are limited only by the creativity and design of the screener.

Examples of screening programs can be readily found in the literature. Just to reference a few, Quinn [128, 129] has reported on his efforts on prospecting the biodiversity available in Queensland; Mehta and Pezzuto [112] have published on their program to identify cancer preventive agents from plants; El Sayed [41] has reported on his screening program for antiviral agents; Barrett [5] has written on his program to find novel antifungal agents; Yang and co-workers [167] has reported on his search for anti-HIV compounds from natural sources; and Bindseil and co-workers [9] have published on their experiences on screening with pure compounds.

The emergence of high-throughput screening (HTS) has permitted the rapid screening of extremely large collections of structurally diverse synthetic compounds against a variety of novel and diverse disease targets [19, 128, 129, 150]. However, despite initial hopes that HTS was the final solution for drug discovery, for reasons that will not be discussed here, HTS has not achieved

that distinction. Nevertheless, HTS is still a powerful tool. HTS strategies focus on the ability to screen large libraries of compounds. However, the limiting factor in HTS is the ability to access large numbers of chemically diverse substances. Natural products are undoubtedly the greatest source of structural diversity. Accordingly, HTS of the unparalleled diversity that exists in natural product extracts offers the highest probability for discovery of novel lead compounds and should therefore be viewed as being complementary to compounds generated from combinatorial chemistry alone. The synergistic melding of HTS and natural products has started, and, as it progresses along its development path, exciting new breakthroughs will undoubtedly be presented.

As a compound generates interest through a variety of screening assays and progresses down the drug discovery path, certain questions need to be asked [5]. Is the chemical structure of the compound novel? Is the mechanism of action of the moiety novel or of utility? Is the biological activity of the compound useful? Is the potency of the compound reasonable? Is a proof of concept available? Is chemical modification of the structure possible? Is solubility a problem? Can the material be synthesized on a large scale? The ability to ask and answer these questions effectively early on in the process will be highly predictive of the ultimate success of a particular line of research. While there are no “correct” answers to these questions, as answers will be different depending on the therapeutic indication and other available therapeutic alternatives, they still need to be addressed to provide proper program focus.

Before ending this section, it is important to emphasize the necessity and importance of keeping detailed records as one initiates the screening process [70]. Maintenance of a secure physical inventory with a controlled environment and adequate records is another important detail. The use of bar code identifiers is very desirable for samples and relevant computer programs are readily available. It should be considered to be essential to establish a complete database, which should include the source, source location, isolation details, any relevant taxonomic information (kingdom, genus, and species), and any other relevant information (third-party suppliers of reagents, potential pathogenicity or toxicity, relevance to any international biodiversity treaties), preservation methods, age, position or location within a freezer, and the like. Other considerations in the design and management of a successful screening program could be limitation of access, storage of reference or backup samples, storage of a backup copy of inventory and database, establishment of tracking procedures, writing of standard operating practices (SOP) for sample handling, and temperature alarms.

1.7 ISOLATION AND PURIFICATION OF NATURAL PRODUCTS

Why do scientists working with natural products isolate and purify them? For one of two reasons: (1) to ascertain what the natural product is and (2) to carry

out sufficient experimental work necessary to biologically characterize or profile the compound. It can be quite a sobering experience to look at a flask full of dark-colored, inhomogeneous sludge and liquid and realize that one is going to attempt to isolate just one particular type of molecule from all of the other materials that are present. To put this in perspective, typically the material sought after represents only about 0.0001 percent of the total biomass in the flask [15, 16]. Then, just to make things even more challenging, the desired molecules can also be bound with other materials and molecules present in the mixture, making the desired compound(s) even harder to purify. It is important to keep in mind that the isolation of natural products differs from that of the more prevalent biological macromolecules. This is because natural products are typically secondary metabolites and as such are smaller in size, chemically more diverse in structure, and present in smaller concentrations than the more homogeneous proteins, carbohydrates, lipids, nucleic acids, and the like.

This section will not present a condensed work on separation science and procedures as that is best left to any of the myriad analytical chemistry textbooks. What will be attempted here is to provide sufficient guidance on the isolation and purification of natural products so that proper focus can be assured in the design and implementation of a successful isolation and purification program.

Before initiating an isolation and purification, there are a number of basic questions that need to be asked and answered. First, what are you trying to isolate and purify? There are a number of different possible targets: (1) an unknown compound associated with a particular biological activity, (2) a previously known compound present in a specific organism, (3) a group of compounds within an organism that are all structurally related to each other, (4) all of the metabolites produced by one natural product source that are not produced by another closely related source, or (5) all of the molecules of a particular organism.

Second, why are you trying to isolate this material? While the asking of such a question might appear to be superficially inane, it is important to know why you want something so that you know how much of it you might need. Possible reasons for carrying out an extraction might be: (1) the generation and supply of larger amounts of an already known compound so that more extensive biological testing such as pharmacology and toxicology can be performed on the material, (2) the purification of a small amount of material for initial biological and chemical characterization to be performed, or (3) to purify sufficient material in order to conduct complete structural studies and further biological activity characterization.

Third, what type of purity is desired? If a natural product compound is to be used for biological testing, it is important to know not only the degree of purity of the material but also the nature of the impurities. It needs to be appreciated that the impurities themselves can contribute significantly to any biological activity observed in the screening program. If the material is to be

used in more refined pharmacological or pharmacokinetic testing, then the material should generally be at least 99 percent pure. If, on the other hand, the material is to be used only for chemical characterization, the acceptable level of purity can range from 95 to 99 percent. Such a range of purity will generally be sufficient for the determination and assignment of a complex chemical structure via such techniques as NMR spectroscopy, infrared (IR) spectroscopy, and MS/MS spectrometry. It should be noted that if the compound under consideration is present in a high concentration in the starting material and a standard for that compound already exists, then structural confirmation can be achieved with less pure material and the associated purification scheme will be composed of fewer steps. Depending upon one's goals, varying degrees of purification may be acceptable. X-ray crystallographic studies will demand material of 99.9 percent purity, while detection of the presence or absence of a specific structural feature via analysis of the ultraviolet spectrum may tolerate purities down to a level of 50 percent. An important concept of purification is that the relationship between purity of compound achieved during natural product extraction and the amount of effort expended to achieve such a level of purity is almost exponential in nature. When starting with a crude, complex mixture, it is very easy to eliminate large components of unwanted material. However, as the purity begins to escalate, it can become infinitely more challenging to improve purity levels. For example, the effort required to go from 50 percent purity to 90 percent purity can pale in comparison to the effort required to go from 99.5 to 99.9 percent purity. In concordance with this, it is fair to state that the relationship between purity level and yield are also exponentially related. In a purification scheme no step delivers the desired material in 100 percent yield. Each extraction step results in the loss of material, and when working to attain very high levels of purity, losses can be extreme. While it may be necessary to take only very "centralized" cuts in a purification step, keep in mind that the "tail" cuts can themselves be later subjected to reprocessing.

Fourth, what type of fractionation should be used in the isolation and purification scheme? All separation processes involve the division of a mixture into a number of discrete fractions. This process is called fractionation. Such fractions can be physically separate such as the two phases of a liquid-liquid extraction or they may not be physically separate such as the continuous eluate from a chromatography column. The eluate from a chromatography column can then be artificially divided into fractions via the use of a fraction collector. The method of fractionation depends on the sample and the goals of the separation. Fractions are typically equal in size and can be large or small in volume. The collection of a large number of small fractions improves the probability that each fraction might contain pure compound. However, such an approach requires significant work in the analysis of each fraction. Additionally, this approach may spread the desired compound over so many fractions that if the target molecule(s) was present originally only in low levels, it may prove undetectable in any one of the fractions. Alternatively, if the separation

process is cruder, employing the collection of only a few large volume fractions, a more rapid and facile tracking of the desired compound and its activity is possible.

Fifth, what is the nature of the compound? The answer to this question depends on how much is already known about the compound. General features that are useful at this stage of the project are acid/base properties (pK_a , pK_b), molecular charge, stability, and solubility (hydrophilicity/lipophilicity). If the target molecule is an unknown moiety, it is very likely that little of this information is known and all of it will have to be determined along the way. If one is isolating a known compound, much of this information will already be available. Finally, if the goal is to isolate a number of secondary metabolites rather than a single molecule, then the value of this step is less important, but an appreciation of the relative values of these parameters can still be useful with regard to understanding the characteristics of the mixture.

Sixth, where is the desired activity localized? Each potential source of a natural product source—whether it is plant, tree, moss, bacteria, vertebrate, invertebrate, insect, terrestrial, or marine based—has components or parts in which the desired activity or compound is present in greater concentration as opposed to other parts in which the compound is present in lesser amounts. To obviate any problems associated with dilution of the compound and its activity, the initial biomass should be selected on the basis of its content of the target biological activity.

Only with the thoughtful provision of answers to the above questions can one have a clear idea of what one is attempting to achieve and how to successfully secure the project goals. It should be obvious that there is no correct or incorrect protocol or standard operating procedure for the isolation and purification of natural products. Indeed, the final method or scheme itself is most likely to vary with the answers to the above questions as well as the natural product source and the specifics of the assays and biological assays that are to be used in the screening program. However, a consult of the literature is essential during the design and construction of an isolation and purification program for any natural product compound [149, 165]. While it is possible that extensive data may have already been published on the compound one is trying to isolate or compounds related to it, it is also entirely possible that nothing is known. Regardless, proper use of the natural products literature can facilitate the effort invested into the design and implementation of a specific isolation and purification program.

A variety of different techniques can be used for the isolation and purification of natural product compounds [15, 16, 82]. These techniques include, but are not limited to, solid-phase extraction [15, 16], high-performance liquid chromatography (HPLC) [15, 16], gradient high-performance liquid chromatography [15, 16], bioautography [165]; thin-layer chromatography (TLC) [15, 16], countercurrent chromatography [2, 49, 50], droplet countercurrent chromatography [74], vacuum column chromatography [165], desalting [149], liquid-liquid chromatography [75], paper chromatography [15, 16], ion

exchange chromatography [15, 16], size exclusion chromatography [73], affinity chromatography [15, 16], acid–base switching technology [42], centrifugal partition chromatography [74], liquid–solid chromatography [15, 16], microwave-assisted extraction [80], pressurized solvent extraction [80], large-scale solvent extraction [42], and supercritical fluid extraction [94, 126]. While the theories along with the relative advantages and disadvantages behind each one of these procedures have not been discussed here, the listing will serve as a catalog of potential techniques available to the researcher. Specific details on any of these procedures can be obtained from any number of books on separation.

A debate still exists as to the timing of isolation and purification in the drug discovery process [65]. It is always tempting to isolate and purify before screening, but understandably this can present a challenge. Classical approaches have used a successful marriage between purification steps and bioassay activity assessment to isolate, identify, and fully characterize natural product compounds. It should be appreciated that with the advances in chromatographic and analytical techniques that have taken place over the last 15 years, the time required to proceed from an initial hit to an identified active compound should take no longer than for the resynthesis and purification of a potential active compound from a combinatorial library. Accordingly, the timing of isolation and purification in the natural product drug research and development timeline should not persist as such a point of contention as previously [9].

1.8 STRUCTURE IDENTIFICATION OF NATURAL PRODUCTS

The chemical structures of natural product compounds are tremendously diverse and can be very elegant in their nature [98, 115, 116, 170]. Such diversity can present a challenge to the analytical or medicinal chemist attempting to unravel the mystery of the chemical structure of an unknown material presented to him or her. However, modern technology has made structure identification simpler and faster. Today, scientists take for granted such techniques as MS, MS/MS, IR, Fourier transform infrared spectroscopy (FTIR), NMR, Fourier transform nuclear magnetic resonance spectroscopy (FTNMR), and others. It is beyond the scope of this chapter to discuss the theory and relative merits of each of these techniques, and the reader is urged to consult appropriate textbooks on analytical chemistry. However, it is worth mentioning that some particularly exciting developments in structure determination pertinent to the area of natural products have come from the field of computer-assisted structure elucidation (CASE). Several computer programs have become available for scientists to use and a number of publications reporting on the utility of this technological advancement have been forthcoming [154]. As has been previously described, the elucidation of the structure of a natural product begins with the collection of a crude material. This material is then

subjected to a series of separation steps, usually involving chromatography, delivering in the end pure compound(s). Finally, a set of spectroscopic and spectrometric experiments are performed on the pure compound to delineate the structural characteristics. Such analysis may even reveal the two-dimensional or three-dimensional structure of the isolated chemical.

Time is money in the drug development business and in order to accelerate activities, the following steps of the structural elucidation process should be considered to be targets amenable to automation: (1) the choice of the smallest group of procedures that is most likely to reveal the unknown structure [NMR, FTNMR, MS, MS/MS, IR, FTIR, ultraviolet (UV), etc.], (2) the acquisition of data from the selected procedures, (3) the analysis of data from the selected procedures, and (4) the use of a computer program to construct the structure from collected spectroscopic and spectrometric data. Historically, structure identification has occurred after purification was complete. However, not infrequently in these cases, structural identification revealed that all of the previous laborious steps of purification had produced a compound that was already known or of an undesirable type. Now, the coupling of liquid chromatography (LC) or high-pressure liquid chromatography (HPLC) with such technologies as MS, MS/MS, and NMR has permitted the construction of devices that allow the injection of a crude sample, separation of the sample using automatically determined optimized conditions, and on the fly spectrometry or spectroscopy followed by CASE for each set of acquired spectra. The identification of unwanted compounds or de-replication should occur as early as possible in the natural product isolation and purification process to avoid the loss of time and funds. The development of coupled techniques has permitted the achievement of that goal. Indeed, coupled techniques such as LC/NMR/MS have now evolved and have potent application in the pharmaceutical field. As the sensitivity of instrumentation continues to improve, the value of these coupled techniques will increase even more.

A number of powerful aids for NMR spectroscopic interpretation have emerged and are now readily available from instrument manufacturers and include programs such as Auralia and AMIX [117]. However, any comprehensive CASE program will be based on a quality structure generator. To this point, only a few high-quality, pure structure generators have been developed. While over the years there have been a variety of programs that have been developed, they have been limited to those researched and developed by small, private groups. Now there are some highly capable programs that are commercially available, such as Assemble (Upstream Solutions GmbH, Zurich, Switzerland) and MOLGEN (<http://www.molgen.de>). A well-known deterministic CASE system that is often cited in the literature is CISOC-SES, which is now commercially available under the acronym NMR-SAMS (Spectrum Research, Madison, WI, USA). Another deterministic CASE program, COCON has been relatively recently introduced, and several examples demonstrating its value in structural elucidation have been reported [97, 154].

Deterministic algorithms have a limit to the size of the molecule with which they can work. To overcome this, a stochastic structure generator has been published for the computer-assisted structure elucidation of organic molecules [153]. The name of the program is SENECA. This program is written in Java and therefore is platform independent and allows for a simple plug-in mechanism for new spectroscopic data types. Theoretically, many different kinds of properties can be plugged into this system, as long as the property can be reliably calculated from a generated molecule.

Classical CASE systems can at least attempt to provide the two-dimensional structure of an unknown molecule. Now, with the greater exposure of, availability of, capabilities of, and demand for CASE programs, efforts are being made toward incorporation of the ability to confidently determine the three-dimensional structure of unknown molecules [68].

Advancements in chromatography, spectrometry, and spectroscopy together with breakthroughs in the coupling of these technologies are important steps in the production of a fully automated and integrated natural products structure determination instrument, which will provide significant advantage to the early, rapid, and facile identification of new natural-product-based drug opportunities.

1.9 SYNTHESIS OF NATURAL PRODUCTS

Once a natural product compound has been screened for biological activity, isolated, purified, its structure identified, and the pharmacological profile refined, the journey is not over. The molecule may turn out to be too complex in nature and too expensive to be synthesized. Indeed, when compared to a purely man-made synthetic alternative, many times the natural product compound is quickly eliminated from further consideration because of considerations of time and potential costs of synthetic production.

Any given natural product compound may possess unacceptable physicochemical, pharmacodynamic, pharmacokinetic, or bioavailability properties or demonstrate excessive toxicity and will therefore require optimization of its chemical structure. Optimization involves a dissection of the lead molecule and the synthetic addition, removal, replacement, or modification of substituent groups so as to enhance the utility and efficacy of the molecule. The synthesis of a complicated molecule is a very difficult task since every group and atom must be placed in a proper position and with the correct stereochemistry.

Such chemical structure modification or synthesis has been performed over decades by what might be termed more classical means. Indeed, the complete synthesis of natural products has been an area of interest for a long time, and the efforts to produce man-made natural products has provided significant challenge and learning opportunity over the years [21, 43, 53, 103, 115, 116, 125, 133, 151]. Because of the widespread chemical diversity that can be found

in natural products, an ever-expanding collection of fascinating natural product compounds will continue to be presented to chemists for synthesis [162]. If one compares the chemical diversity of man-made synthetic products with the chemical diversity of natural product compounds, it quickly becomes apparent that there are significant qualitative differences between synthetics and natural products [114]. Natural product compounds contain more alcoholic and ether groups, while pure synthetic compounds possess more aromatics, amines, and amides. If one looks at group combinations, there are higher percentages of alcohol/ether, alcohol/ester, arene/alcohol, arene, alcohol, or ether functionalities in natural product compounds when compared to the synthetic compounds. However, pure man-made synthetic compounds are found to have combinations such as arene/amine, amine/amide, or amine/arene/amide in higher frequencies than natural product type of compounds. Finally, natural product compounds are found to more commonly possess bridgehead atoms and contain a greater number of rotatable bonds per molecule, chiral centers per molecule, and rings per molecule than pure man-made synthetic compounds. The importance of these differences is that they reveal and emphasize the complementarity of natural product compounds as a group with man-made synthetic compounds. Despite all of the knowledge and achievements that have been gained and the advances that have been made, classical synthetic organic chemistry will not alone unlock and open the potential of natural products to the pharmaceutical marketplace. Instead, the future lies in the synergistic union of classical organic chemistry with microbiology, biochemistry, combinatorial chemistry, and other fields to provide new synthetic strategies to generate natural-product-based drugs.

The history and specific techniques of combinatorial chemistry are beyond the scope of this chapter, and the reader is referred to appropriate textbooks for discussions on that topic. While it should be recognized that combinatorial chemistry is a perfect match for high-throughput screening because of its ability to produce large numbers of compounds in a short period of time. The promise of combinatorial chemistry to deliver more drug candidates within a shorter period of time has remained unfulfilled. What has been lacking in combinatorial chemistry is the skeletal structural novelty that natural products can provide [1, 12, 13, 59, 63, 111, 119, 120, 155, 158, 161, 163].

Over time, organic synthetic chemists have become interested in enzymes and their potential role in natural product synthesis [85, 109, 136]. Enzymes have great power as catalysts for regiospecific and stereocontrolled synthesis. These biological catalysts are very capable at room temperature of converting inexpensive substrates into value-added products at a significantly high throughput. However, barriers remain to the more expanded use of enzymes in organic synthetic chemistry. The most important of such obstacles includes the inability of enzymes to work with unnatural substrates.

Combinatorial biosynthesis utilizes enzymes from various natural product source biosynthetic pathways to create novel chemical structures [10, 27, 135].

The engineering of polyketide synthases has thus far been the central point of this activity and led to the production of several erythromycin analogs. The end result of such research activity will be the development of more rational and faster methods of production of new compounds for the development of therapeutic agents from natural products.

The research on and screening of natural products today is focusing on many different therapeutic indications. Fermentation broths and plant extracts have done well in delivering leads and genomics and molecular biology have done well in delivering targets. Regardless of the type of compounds involved, improved efficiencies in the design and synthesis of natural-product-based agonists and antagonists will be key to a realization of the full potential of natural products as drugs [1, 139, 140].

1.10 DEVELOPMENT OF NATURAL PRODUCTS

Regulatory Guidelines and Nonclinical Development

The classical model of drug development is composed of three phases: discovery, development, and marketing [108]. Discovery is the first of these phases and is composed of two essential components, drug discovery and drug design. Development is the second of these phases and is composed of two large components, preclinical studies and clinical studies [30, 56–58, 95, 108, 146]. The development phase, although lengthy, expensive, and time consuming is meant in its design and conduct to cull out undesirable compounds and ensure the safety and efficacy of compounds that successfully run the developmental gauntlet and decrease the hazard and risk of exposure for humans. We have touched on the drug discovery and design in earlier sections of this chapter. In the course of these phases, therapeutic targets have been selected and screens were designed to identify drug candidates. These same candidates were then isolated, purified, identified, and then structurally optimized in consideration of the molecule's biological activity profile. This process of optimization attempts to minimize toxicity and maximize therapeutic value, efficacy, and pharmacokinetic characteristics. Finally, research was performed to elucidate a mechanism of action for the potential drug and animal models identified to establish efficacy of the material. Pharmacology studies, a component of the discovery phase, confirm the efficacy of a potential drug. The availability of analytical methods is essential to establish the exposure of the test system to the drug candidate and the presence of the drug in various tissues and fluids. The absorption, distribution, metabolism, and excretion and elimination of the compound also need to be determined. Human *in vitro* P-450 studies are necessary to be performed to fully understand the hepatic metabolic pathways. These studies may also herald the potential for serious drug interactions that may have to be addressed during the development cycle.

Safety pharmacology studies are part of the development phase and the preclinical development program but can be performed either as part of the pharmacologic profiling of a drug candidate or as a prelude to the toxicology studies. These types of studies are a preliminary hazard assessment of the test article in key organ systems such as the central nervous system, cardiovascular system, gastrointestinal system, renal system, and pulmonary system and essential in the planning of human clinical trials. For an excellent and detailed discussion of safety pharmacology, the reader is referred to Gad [54].

Preclinical studies begin some of the more rigorous testing that a potential candidate must successfully endure and survive. It needs to be recognized that a decision to proceed with preclinical studies represents a major commitment of time, resources, effort, and money. The major objectives of any preclinical development program should be: (1) development of a Good Manufacturing Practices (GMP) synthetic method to produce the test article, (2) synthesis of a supply of test article that is adequate to permit the performance of all preclinical work and the initiation of clinical studies, (3) the creation of a usable and tolerable preclinical formulation(s) and efficacious clinical formulation(s), (4) complete pharmacologic and pharmacokinetic profiling of the test article, (5) performance of proper toxicology studies to support an Investigational New Drug (IND) application, and (6) construction of a complete and detailed informational platform to permit the recommendation of an initial human dose in phase I studies.

Nonclinical or preclinical studies are the same thing and are composed of studies on drug processes, pharmacology, and toxicology. Drug processes involve the scaling up of synthetic procedures or the bulk preparation of the drug [32, 146]. This means the development of a synthetic method that permits the synthesis of compound at a reasonable cost, in a reasonable amount of time in such a fashion that regulatory and GMP guidelines are satisfied. A major obstacle in the performance of preclinical studies is often the ability to merely “have drug.” Drug supply is often overlooked in the drug development program and can cause costly delays. Related to this is the existence of some sort of formulation for the drug candidate that will permit effective dosing in preclinical studies. The lack of proper planning on a preclinical formulation (not the final clinical formulation) is oft overlooked, and this oversight can cause significant problems of delay or confounding toxicity. Also during the preclinical phase of the drug development paradigm, various analytical techniques must be developed and validated in a variety of species and biological milieu, analytical standards defined and made, compound stability profiles generated, and research into the proper human formulation initiated and conducted. Sometimes even radiolabeled drugs will be required to be made for some studies, a difficult, costly, and time-consuming project in and of itself.

Toxicology studies are performed to assess a drug candidate’s potential safety. These studies are so critical that there are established procedures called Good Laboratory Practices (GLP), which specify how studies are to be done.

Toxicology studies have a profound quality assurance component, which facilitate inspection and permit validation of the data by a regulatory agency or reviewer. There are numerous different types of toxicology studies and the details of them are beyond the scope of this discussion, but the most comprehensive, detailed, and descriptive source is by Gad [56]. These studies typically include mutagenicity, genotoxicity, and cytotoxicity studies; acute studies; subchronic studies; chronic studies; reproductive and developmental toxicity studies; carcinogenicity studies; immunotoxicity assessments; various worker safety studies; and certain specialized toxicity studies, the conduct of which is determined by compound-specific issues or dose route. Toxicology studies should in their core design determine the target organ(s) of any potential toxicity, characterize the shape of the dose–response curve, ascertain the reversibility of any potential lesion, and facilitate the selection of a human dose in phase I clinical trials. An excellent example of preclinical and clinical development programs for a natural-product-based drug has been reported by Rowinsky et al. on the antineoplastic agent Taxol [141].

When a drug candidate completes the preclinical studies, an IND is filed and clinical trials are initiated. Clinical trials are composed of phases I to IV. Phase I studies represent the first exposure of a drug to humans. These studies examine the effects of single and multiple increasing doses in small numbers of normal and/or patient volunteers. Tolerance to the compound is also evaluated. Basic pharmacokinetic studies are performed in conjunction with the studies to aid in the determination of later dosage regimens and to fully characterize the routes of metabolism, excretion, and elimination and assess the presence and amounts of active and inactive or toxic metabolites. Typically only a small number of volunteers are involved. Phase II studies represent the first time that a drug candidate is tested in humans for efficacy. However, safety and tolerance are still monitored in these studies concurrently. In phase II studies, different dose–range finding studies are performed to optimize the dose of the drug, to maximize its efficacy, and to minimize any compound-associated intolerance. These studies typically involve up to several hundred patients and generally provide the first indications of the potential benefit of the drug as compared to its risk of exposure. The last clinical studies that need to be performed before submitting a complete information package for regulatory approval are phase III studies [New Drug Application (NDA) for filing in the United States]. In these studies, the drug candidate is utilized at the optimum dose, in the target patient population, and in exactly in the same fashion that the drug will be used if eventually approved and marketed. These studies will verify efficacy and safety and detect adverse reactions and contraindications. These are very large studies composed of hundreds to thousands of volunteer patients, depending upon the therapeutic indication. Phase IV studies are postmarketing surveillance studies, which are conducted after a drug has been approved for sale. These studies typically involve adverse reaction reporting, surveys and general sampling, and testing evaluations.

The design of a proper and detailed preclinical program is a challenging exercise, and the successful navigation of the program with its on-time completion should not be taken for granted. Effective project management with a timely resolution to all obstacles that will present themselves along the way can save significant amounts of money. Accordingly, consideration should always be given to the retention of a professional and suitably experienced preclinical project manager. The expenses incurred with the design and implementation of a preclinical development program is more than offset by the avoidance of cost and/or time overruns.

As stated earlier in this chapter, natural products can be interpreted by different people to mean different things. For purposes of this discussion, the term *natural products* refers to drugs of natural product origin and not to botanicals, herbals, or traditional medicine products. The development of botanicals, herbals, dietary supplements, and over-the-counter products is not relevant to this chapter and is covered elsewhere [152]. There are no regulations or guidelines that are specific to the development of therapeutic agents developed from natural products. Indeed, the development of a natural-product-based therapeutic candidate is more than adequately covered in existing governmental guidelines. These guidelines are available from a variety of governmental sources [www.fda.gov (U.S. FDA), www.eudra.org (European Union), www.mhw.go.jp/english (Japan), www.ifpma.org (ICH)] or references on preclinical development [51, 55–58, 105, 106]. The most comprehensive and detailed of these references is by Gad [56]. However, it should be noted that according to a draft document entitled “Botanical Drug Products Docket Number: OOD-1932” issued by the Office of Nutritional Products, Labeling and Dietary Supplements (HFS-800) in 2003, if a natural product has been sold in a crude or semipurified form as an over-the-counter drug and a sponsor wishes to file an IND on a moiety purified from such a preparation, then reduced documentation may be acceptable to the FDA.

Learning from the Mistakes of the Past in the Development of Natural Products

The very first sample of bark from the Pacific yew (*Taxus brevifolia*) was originally collected in 1962 by the U.S. Department of Agriculture as part of a plant screening program established by the Cancer Chemotherapy National Service Center of the National Cancer Institute (NCI) [32]. It was not until 1964 that a positive response was found for the extract in the KB cytotoxicity assay. Taxol was then identified as the active component of the mixture in 1969. Not unlike many other compounds being tested at that time, paclitaxel originally demonstrated moderate in vivo activity against P388 and L1210 murine leukemia models and was not considered to be a promising candidate for further development. However, strong activity demonstrated by Taxol against the B16 melanoma line introduced by the NCI in 1975 caused a reevaluation

of the material. Indeed as a direct result of this additional research, Taxol was recommended as a candidate for preclinical development in 1977. Further work demonstrated strong activity against human tumor xenograft systems and stimulated hope of efficacious performance in the clinic. Taxol's mechanism of action was elucidated in 1979, formulation work completed, and toxicology studies started in 1980 [32, 100]. With the completion of nonclinical studies, approval was given for entry into phase I clinical trials in 1983 [28, 141]. The early clinical trials raised serious issues of toxicity. Indeed, further development of Taxol was almost discontinued. The problems, however, were determined to be related to the poor solubility of Taxol in aqueous systems and the necessity for a high dose, when compared to other antineoplastic agents of the time. The development of a suitable formulation required the use of Cremophor EL, a polyethoxylated castor oil derivative, which created a whole new set of challenges to be addressed [36]. Eventually, a safe clinical regimen was established reducing the incidence of allergic reactions, and Taxol proceeded into phase II clinical trials. However, at this point in development, faith and interest in the success of Taxol began to wane for a variety of reasons, and the priority of Taxol's development was again lowered. Indeed, the support for Taxol decreased to such a level that the production of bulk quantities of Taxol needed to proceed through clinical trials was severely cut back. This position forced the creation of a critical problem when Taxol demonstrated significant antitumor activity in phase II clinical trials in patients afflicted with ovarian cancer. The preliminary reports from clinical trials indicated an approximate 30 percent response rate, with some patients actually achieving remission [110]. Additional clinical trials supported the initial clinical response rate, and accordingly there was a strong demand for more Taxol. Concurrently, it was also discovered that Taxol demonstrated very favorable responses in patients afflicted with metastatic breast cancer [71]. Add to this the findings that favorable clinical responses to the administration of Taxol were also being found in patients afflicted with lung cancer, malignant melanoma, lymphomas, and cancers of the head and neck, and it is easy to appreciate the magnitude of the gap created between the supply of Taxol and the demand for Taxol [32]. Unfortunately, all of this favorable clinical performance was met with not just a poor supply of Taxol but an extremely disappointing inability to produce Taxol on a large scale. The crisis in the supply of paclitaxel was eventually resolved as a result of collaborative efforts between many groups [28, 32]. Such a crash collaborative effort, while admirable, came about as a result of poor planning.

Today, Taxol is viewed as a very important antineoplastic agent on the front line of cancer chemotherapy. Yet, despite its success, it must be sadly admitted that on several occasions during paclitaxel's development, Taxol almost did not make it to market. In an attempt to learn from the mistakes of the past, what lessons can be learned from the Taxol story? These are addressed in the next section when we talk of the future of natural products in drug discovery and development.

1.11 FUTURE OF NATURAL PRODUCTS

Current libraries providing fodder for the search for new therapeutic agents include, for the most part, but are not limited to, synthetic chemical libraries of purified natural products from a variety of different sources [27]. When combinatorial chemistry made its entry onto the drug discovery stage, many people considered this technique to be the ultimate solution for the discovery of potential new therapeutic agents. Accordingly, the perceived value of natural products in the drug discovery process began to pale. While combinatorial chemistry has demonstrated that it is certainly of value in the process of lead optimization, nature itself presents the most diverse and complete source of leads. A relatively recent approach that unites the strengths of combinatorial chemistry and natural product identification is a process referred to as combinatorial biosynthesis [10, 27, 135]. In this technique, the pathway leading to the production of a natural product is identified and the genetic basis of it elucidated. Genetic modifications of the organism are then made causing the production of different biologically active products. These products can then be evaluated for target therapeutic potency in various screens. While this approach has been utilized by some biotechnology companies, widespread acceptance in the pharmaceutical industry has been lacking to this point [27]. However, as acceptance develops, so will the identification of many new potential drug candidates.

To fully capitalize on the extensive biodiversity available to us in natural products, high-throughput screening processes need to be improved upon so that they can provide a higher degree of quantifiable rather than quantal (active versus not active) results, permitting a more adequate description of whether or not any given compound may be considered to be potentially active.

It is also important to realize that one should not rely on just one either *in vitro* or *in vivo* screening assay in a discovery program. Obviously, there has to be an economic component to screening programs with some degree of cost containment, but a realistic balance can be achieved, which provides a realistic cost-to-benefit ratio. The discovery and development of paclitaxel is a good example of this concern [32].

The early conduct of studies leading to the elucidation of the mechanism of action of a compound is important. The delineation of a novel mechanism of action can provide considerable impetus to the continued development of a chemical moiety. Irrespective of novelty, the establishment and correlation of a chemical structure with a mechanism of action can permit the categorization of a compound with other chemicals, potential therapeutic agents, and drugs and more rapidly facilitate a compound's potential for therapeutic use.

The efforts to identify potential leads for the treatment of a constellation of maladies and diseases are increasing at a hectic pace and the growth of numbers of actual compound candidates is markedly outpacing the availabil-

ity of screening procedures. In order to accommodate the requisite testing associated with the generation of chemical leads, advances will need to be made in the methods of and approaches to the synthesis of natural products. Critical demands will be placed upon synthetic and medicinal chemists to produce pure chiral products in significant amounts. These products will undoubtedly be the result of complex synthetic pathways, which will incorporate catalysts and biocatalysts, soluble and immobilized elements, and biologically generated as well as man-made moieties. These multistep processes will need to be functional on both a low bench scale as well as a commercial scale. The development of paclitaxel suffered a severe developmental program setback because of an inability to produce adequate supplies at an appropriate time. In association with the development of effective synthetic pathways leading to the production of natural products, attention must also be paid to the required formulation of such products permitting human use, as was well demonstrated with Taxol.

Genetic research will continue to elucidate an ever-increasing number of potential pathophysiologic targets, which can be mated with hopeful novel therapeutic agents. This coupled with the fact that little of nature's natural products archives have really been adequately researched portends great opportunity. How big is this opportunity? Admittedly, there are no sound estimates for the actual total numbers of species of plants, shrubs, trees, insects, fungi, smuts, rusts, marine organisms, and the like that exist on land and in the waters in this world [24]. But, with regard to microorganisms, less than 0.1 percent of the total microorganisms present in the soil to date have been evaluated, simply because they cannot be cultured [27, 138]. Only about 70,000 of 2 to 5 million different fungi are considered to have been identified [27]. Similarly, only about 800,000 of approximately 20 million different insects have been identified.

Structure-activity studies on leads generated from natural products sources combined with computerized graphic model building will become increasingly more prevalent. Such activity in turn will result in the discovery of molecules with optimal activity, improved bioavailability, fewer side effects, and very desirable therapeutic indices [156].

However, abuse of our natural resources will certainly limit our ability to learn from nature [27, 78, 132]. Lack of proper conservation, the expanding pall of pollution, and the wanton destruction of forests, especially the rain forests, will, for example, diminish the opportunity to gain valuable knowledge from a staggering amount of indigenous biodiversity and obviate the possibility to generate a valuable therapeutic agent. Indeed, it is estimated that approximately one-eighth of all plant species are currently near the point of extinction [78]. Alternatively, the highly focused and single-minded research into natural products can in and of itself lead to the depletion of sources of natural products and even the eventual extinction of species [22].

In the hunt for natural products another obstacle that will have to be more commonly addressed is the issue of intellectual property rights [3, 11, 27, 32,

107, 160]. As various biotechnology and pharmaceutical companies attempt to expand their libraries with compounds generated from various biodiversity sources, many countries are beginning to impose significant demands on the use of such raw materials and are limiting access without the expression of adequate consideration to the "landowners" [124]. Of course, ultimately there is the potential for significant economic reward for involved countries, but the various forms of life, which historically have been considered to be free for the taking, are no longer free. The potential conflict between host countries and prospecting organizations will undoubtedly be resolved with the establishment of contingent compensation plans making use of rights and royalties coupled with the attainment of specific milestones. The building of the requisite trust to establish meaningful, lasting, and functional business relationships will require time, patience, education, and respect for the different cultures involved [140]. History will color all negotiations and ethnic and cultural differences will serve to make the process painfully slow.

The search for and the development of natural products will result in the creation of a variety of different types of alliances between industry, government, individuals, universities, and hopefully even foster a spirit of international cooperation [25, 26, 32, 86]. Over the last few years, those involved in the pharmaceutical industry have already seen the increasing numbers of partnerships, alliances, agreements, and other types of relationships forged between large pharmaceutical companies and smaller biotechnology companies, all for the purpose of seizing opportunities of new technology. The collaborations that currently exist in the field of chemotherapy can set an example for other therapeutic areas as well as drug treatment in general. Another pertinent example has been the formation of the International Cooperative Biodiversity Group (ICBG) Program [32]. The ICBG is a collection of academic, industrial, U.S. governmental organizations, and developing countries. This program is also jointly sponsored by the National Science Foundation (NSF), parts of the National Institutes of Health (NIH), the National Cancer Institute (NCI), the Fogarty International Center, the National Institute of Allergy and Infectious Diseases (NIAID), the National Heart, Lung, and Blood Institute (NHLBI), and the National Institute of Mental Health (NIMH). The purpose of this program is to facilitate research and drug discovery from natural sources. This program is of extreme value and promotes the identification, establishment of an inventory, conservation of natural resources, and economic development of financially challenged countries.

Thought should be given to the more expanded establishment of repository programs. The NCI has established a Natural Products Repository (NPR), which is an extremely valuable resource in the search for and discovery of new drugs. The opportunities are there, they just need to be found.

In conclusion, drug discovery can be significantly improved through the use of the knowledge to be gained from research into natural products. However, despite the powerful resource that natural products present to us, the knowledge that is locked therein can only be fully realized with proper management

of the resources, the parallel development of ancillary technologies, and the fostering of open and shared communication.

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