CHAPTER

2

The Drug Discovery Process: From Ancient Times to the Present Day

Throughout the course of history, there has been a near constant need for therapeutic intervention for the treatment of disease. Efforts to provide for this need can be traced to prehistoric times as evidenced in cave drawings from 7000 to 5000 BC that are suggestive of the use of hallucinogenic mushrooms. The concept that curing diseases or alleviating symptoms could be accomplished by eating, drinking, or applying substances to the body is ancient, but the methods used to discover therapeutic agents has changed dramatically over the course of human history. In its earliest form, from ancient times until the mid-nineteenth century, the identification of new drugs was primarily the result of serendipity, as the foundational science required for the systematic study of potential new therapeutic entities had not yet been established. Modern methods of drug discovery have evolved over the last two centuries, however, as a result of advances in basic science (e.g., chemistry, biology, pharmacology) and applied science (e.g., transgenic animal models, molecular modeling, robotics) leading to a process that is far less dependent upon the serendipitous identification of therapeutic agents. A third factor, governmental and regulatory oversight, which focuses primarily on ensuring the safety and efficacy of new medications, has also had a major impact on modern drug discovery over the last century. This chapter will review the evolution of the drug discovery process from ancient times to the modern age, focusing on key scientific advances and the regulatory environment that changed the way in which new drugs are identified.

Basic Principles of Drug Discovery and Development
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THE AGE OF BOTANICALS: PREINDUSTRIAL DRUG DISCOVERY

The search for effective methods and medications designed to improve the quality and length of life predates the age of modern discovery by several thousand years. While it is unclear exactly when humanity began to understand that ingestion of specific materials (i.e., drugs) could influence physiology, disease-related or otherwise, there is evidence suggesting that these concepts were beginning to evolve as early as prehistoric times. Plant remains from between 7000 and 5500 BC found in the Spirit Caves of north-western Thailand included seeds of the betel nut, a mildly psychoactive agent, indirectly suggesting its use in the Neolithic period.[1] Human consumption, perhaps for the alteration of perception, is also suggested by the presence of skeletal remains from 2680 BC in the presence of lime-containing betel nut shells found in the Duyong cave of the Philippines. Although not conclusive evidence of consumption, the presence of the lime and betel nuts is consistent with practices designed to aid in the absorption of the active ingredient (arecoline) while chewing that are still in practice in modern India.[2] The prehistoric use of hallucinogenic mushrooms is also implicated by Saharan cave drawings (c.7000–5000 BC),[3] suggesting that humanity became aware of the potency of these plants long before recorded history.

Direct evidence of the early identification of the most frequently consumed drug in history, alcohol, is far easier to obtain. Although it is unclear how the fermentation of alcoholic beverages was discovered, there is ample evidence indicating that its discovery occurred early in human history. Strong evidence exists indicating that alcoholic beverages were developed as early as the Neolithic period, and that its use was common across the ancient world.[4] Given that the effects of alcohol consumption occurs rapidly upon ingestion, it is not surprising that various alcoholic beverages were among the first drugs to be widely consumed for either recreational or medicinal purposes.

When humanity began to recognize the medicinal properties of various plants and chemicals is also an open question. It is clear, however, that the pursuit of treatments for diseases and symptom relief is not a phenomenon of the modern world. The Mesopotamians documented their medical methods and prescriptions on stone tablets. One of the oldest and largest collections from this civilization consists of a series of 40 tablets from around 1700 BC that are collectively known as "Treatise of Medical Diagnosis and Prognoses." Included among the writings are some of the earliest recorded uses of drugs for medicinal purposes (Figure 2.1(a)).[5] In a similar fashion, the Ebers Papyrus was written by the ancient Egyptians around 1550 BC and contains several hundred "prescriptions" for the treatment of disease or symptomatic

(a) (b) (c)

FIGURE 2.1 (a) Cuneiform clay tablets unearthed from the library of King Ashurbanipal at Nineveh describe Mesopotamian medical practices. It is estimated that they originated between 1900 and 1700 BC. *(Image from The Schoyen Collection, MS2670,* http://www.schoyencollection.com/smallercollect_files/ms2670.jpg*.)*. (b) A section of the Ebers Papyrus, a compilation of Egyptian medical knowledge composed around 1550 BC containing over 800 prescriptions for various conditions *(Image from the NIH U.S. National Library of Medicine Archives,* http://www.nlm.nih.gov/archive/20120918/hmd/breath/breath_exhibit/MindBodySpirit/IIBa18.html*.)*. (c) A page from the Pen-tsao Kang-mu, a compilation of traditional Chinese medicines written by Li Shih-chen. The completed text contains over 1800 Chinese medicines and 11,000 prescriptions *(Image from the U.S. National Library of Medicine, History of Medicine Division* http://www.nlm.nih.gov/exhibition/chinesemedicine/images/017c.jpg*.)*.

relief (Figure 2.1(b)).[6] The origin of traditional Chinese medicine is largely unknown, but it is estimated that the practices and methods are at least 2000 years old. The herbalist and acupuncturist Li Shih-chen completed the first draft of Pen-tsao Kang-mu, which is widely considered the most comprehensive text on traditional Chinese medicine, in 1587. The text describes hundreds of distinct herbs and thousands of combinations useful for treating disease and alleviating symptoms (Figure 2.1(c)).[7]

There are some commons threads that run through all preindustrial drug discovery efforts, irrespective of their country or region of origin. First, they depended almost exclusively on plants, plant-derived mixtures, or plant extracts, as the ability of preindustrial society to isolate or prepare pure chemicals with medicinal value was limited. Second, medications developed in the preindustrial ages were identified using empirical observation of the presence or absence of symptoms in patients, rather than an understanding of the disease or condition afflicting the patient. Third, and perhaps most importantly, all of the efforts to develop new medication in the preindustrial age of drug discovery

did so in the absence of the vast majority of the fundamental knowledge required to understand even the basic principles of disease progression. This almost certainly led to the use of any number of concoctions with little true medicinal value and some that were actually detrimental to the patients' well-being.

Despite these facts, there are a number of medications that were identified prior to the advent of modern drug discovery that still play an important role in modern medicine. The treatment of malaria caused by *Plasmodium falciparum*, for example, was revolutionized by the discovery of quinine, an alkaloid found in cinchona bark (Figure 2.2). Agostino Salumbrino

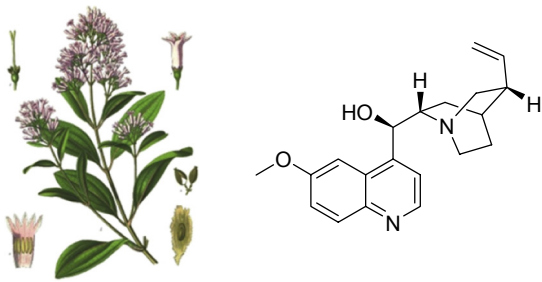

FIGURE 2.2 The cinchona tree (left) and quinine (right) were important players in the treatment of malaria infection for over 300 years. *Source:* http://upload.wikimedia.org/wikipedia/commons/e/e8/Koeh-179-cropped.jpg.

(1561–1642), a Jesuit living in Lima, Peru, observed the Quechua people chewing the bark from the cinchona tree in an effort to relieve shivering and fevers. Although Salumbrino certainly had no knowledge of the causative malaria parasite, he did recognize that the symptoms of the febrile phase of malaria might be positively impacted by the cinchona bark and arranged for a sample to be shipped to Rome for evaluation as a treatment for malaria. The cinchona bark, also known as Jesuit's bark or Peruvian bark, and the Quechua people thus became the source of the first successful antimalarial agent, a drug that was a first line treatment for malaria infection until 2006.[8]

In a similar fashion, cardiac glycosides were identified as an important treatment for congestive heart failure via the foxglove plant, which contains high levels of several cardiac glycosides in the leaves (Figure 2.3). The use of the foxglove plant as part of an herbal remedy for dropsy, swelling, and fatigue, all of which are symptoms of congestive heart failure, can be traced to medieval Europe. Although the structure and mechanism of action were clearly not known at the time, William Withering deduced that the foxglove plant was the source of the active ingredient of the herbal remedy in 1785 and unknowingly provided a primary treatment for congestive heart failure, digoxin that is still routinely used in modern medicine.[9]

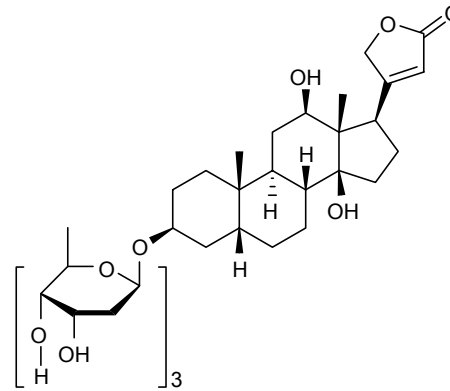

FIGURE 2.3 The common foxglove plant (*Digitalis purpurea*, left) contains cardiac glycosides such as Digoxin (right), which are known to increase cardiac contractility via inhibition of myocardial sodium/potassium ATPase. *Source: Kurt Stüber* http://en.wikipedia.org/wiki/Foxglove#mediaviewer/File:Digitalis_purpurea2.jpg.

PAUL EHRLICH: THE FATHER OF MODERN DRUG DISCOVERY[10]

There are many additional examples of useful drugs that were discovered in the preindustrial era, such as morphine,[11] cocaine,[12] and aspirin,[13] but it was Paul Ehrlich's (Figure 2.4) efforts that are most often cited as the

FIGURE 2.4 Paul Ehrlich (1854–1915), the founder of modern drug discovery was a physician and scientist noted for his discoveries in the fields of hematology, immunology, and chemotherapy. In 1908, he received the Nobel Prize in Physiology or Medicine in recognition of his work. *Source: NIH U.S. National Library of Medicine* http://ihm.nlm.gov/luna/servlet/view/search?q=B07744.

starting point for modern drug discovery methods. Ehrlich's early observations of differential affinities of biological tissues for various dyes, such as Trypan red, Trypan blue and methylene blue (Figure 2.5), lead him to

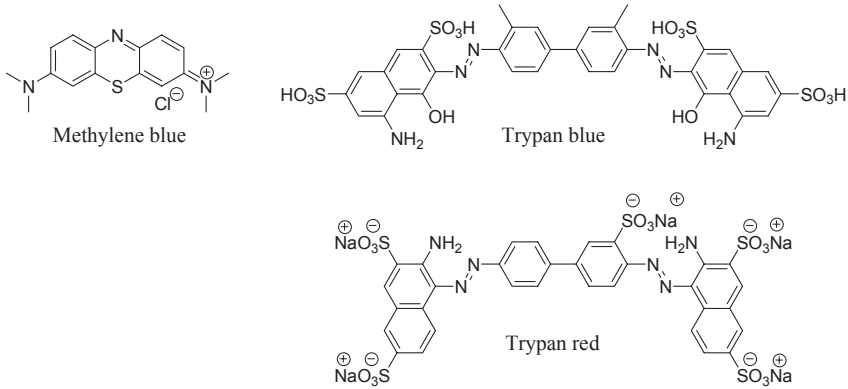

FIGURE 2.5 Methylene blue is used in Wright's stain, Jenner's stain, and northern blotting experiments. Trypan blue and Trypan red are commonly employed as stains that distinguish between viable and non-viable cells.

postulate the existence of "chemoreceptors" that influence the interaction of cells with the chemicals around them to produced a biological effect. He further theorized that "chemoreceptors" of infectious organisms or cancer cells would be different from those of the host and that the differences could be exploited to produce a therapeutic benefit (the "magic bullet" theory). These concepts, along with his hypothesis that the chemical composition of drugs controlled their mode of action in an organism, formed the basis of modern chemotherapy. His initial successful treatment of two malaria patients using methylene blue lead him to conclude that this dye possessed a clear affinity for the malaria parasite over the host, and that compounds previously used only as dyes might have therapeutic value. In an effort to capitalize on these theories, Ehrlich and his colleague began a systematic evaluation of hundreds of commercial synthetic dyes in mice infected with *Trypanosoma equinum*, also known as sleeping sickness. In 1904, these first attempts to develop structure–activity relationships (see Chapter 5 for a full discussion of this concept), led to the identification of Trypan red as an agent capable of killing this infections in mice. Unfortunately, resistant strains of the organism developed and eventually killed the mice, as well as rats and dogs that were also studied, marking setbacks in the research efforts, but also prompting Ehrlich to hypothesize the development of resistant organisms. More importantly, however, these efforts marked the first concerted effort to discern the relationship between chemical structure and biological activity in an effort to develop new therapeutic agents in conjunction with a chemical manufacturing company, also known as a pharmaceutical product pipeline.

Full validation of Ehrlich's methods came with the identification of Salvarsan, the first successful synthetic chemotherapeutic drug, and the first truly effective treatment of syphilis (Figure 2.6). Prior to the identification

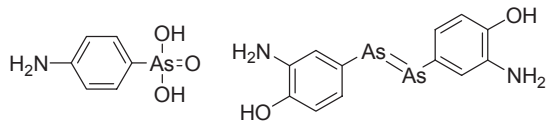

FIGURE 2.6 The discovery of Salvarsan from Atoxyl is one of the earliest examples of an effective drug discovery program. Salvarsan was the drug of choice for the treatment of syphilis until the mid-1940s, when it was replaced by penicillin.

of the causative agent of syphilis, *Treponema pallidum*, Ehrlich and his colleagues had prepared and tested a number of phenyl arsenide analogs in an attempt to improve upon the drug Atoxyl, a treatment for African sleeping sickness (African trypanosomiasis) with a high risk of blindness. Erich Hoffman (1868–1959), a contemporary scientist of Ehrlich's, noted the similarities between the causative agents of the two diseases, and at Hoffman's urging, Ehrlich reexamined the phenyl arsenide analogs in a rabbit model of syphilis developed by Sahachiro Hata (1873–1938). These efforts lead to the identification of arsphenamine in 1909 as a lead compound for the treatment of syphilis. Clinical results demonstrating its efficacy in patients were presented at the 1910 Congress for Internal Medicine, and Hoechst marketed the drug as Salvarsan. Thus, the age of modern drug discovery was born.

MILESTONES IN DRUG DISCOVERY

Ehrlich's research and methods provided much of the foundation of what eventually became modern drug discovery, but his efforts did not provide many of the important tools that are now commonplace. When Paul Ehrlich unknowingly launched the age of modern drug discovery, the ability to prepare, analyze, and screen compounds for biological activity was in its infancy. Over the course of the next 100 years, critical tools required to efficiently identify biologically active compounds and understand how they function, both in a whole organism and in isolated systems, were developed. The fields of animal modeling, X-ray crystallography, molecular modeling, high throughput screening, and high throughput chemistry, as well as biotechnology tools such as recombinant DNA and transfection technology grew as knowledge in basic sciences such as biology and chemistry expanded. In many cases, the development of new technology in one field led to advances in a related field. The advent of transfection technology, for example, provided the tools necessary to generate transgenic and knockout animal models. Advances in

X-ray crystallography led to advances in molecular modeling and computational chemistry, and the combination of increased computer capacity, automation science, and *in vitro* screening techniques led to the introduction of high throughput screening. There can be no question that a wide range of scientific disciplines influenced the development of modern drug discovery science. It is well beyond the scope of this text to provide a complete history of the various important fields that influenced the evolution of the drug discovery process. The history and growth of synthetic organic chemistry or *in vitro* biology, for example, would require many texts unto themselves. There are, however, some scientific advances that had foundational impact on drug discovery. An understanding of their history provides insight into how the process developed to its current status, and perhaps some guidance as to where the field may be going in the future.

Milestones in Animal Models: Breeding a Better Model

The Wistar Rat

While modern drug discovery research is performed using a wide assortment of standardized animals from any number of different species, this was not the case at the beginning of the twentieth century. Up until 1906, there were no standardized animal models available and the common house mouse, *Mus musculus*, was used for laboratory research. This changed in 1906, however, with the introduction of the Wistar rat (Figure 2.7),[14] a strain

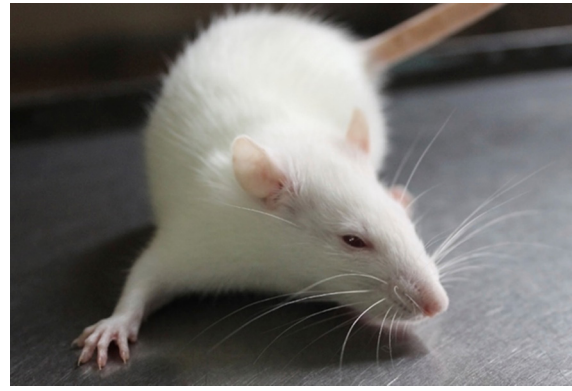

FIGURE 2.7 The Wistar rat is a product of research lead by Milton Greenman and Henry Donaldson at the Wistar Institute, the first independent biomedical research facility in the United States, which was founded in 1892. © istock.com/VseBogd

of albino rats belonging to the species *Rattus norvegicus*, which marked the first effort to develop a "pure strain" animal as a model organism for medical research. It is estimated that over 50% of all laboratory rat strains are descendants of the original colony established at Wistar Institute and it remains one of the most commonly employed rat strains in modern medical

research. While a full listing of rat models developed using the Wistar rat is well beyond the scope of this text, there is little doubt as to the importance of this watershed animal model. Wistar rats models include spontaneously diabetic rats,[15] spontaneous tumor formers (the Rochester strain),[16] high anxiety behavior rats, low anxiety behavior rats,[17] the Lobund-Wistar rat model of prostate cancer,[18] Wistar Kyoto rats (an important model of attention deficit disorder),[19] myelin deficient rats,[20] and the spontaneously hypertensive rat (SHR),[21] the most widely studied model of hypertension.

Immunocompromised Mice

The Nude Mouse[22]

The availability of the Wistar rat and the concept of using standardized animal strains led other research teams to examine their animal colonies more closely in an attempt to identify useful subpopulations. Thousands of useful animal models across a range of different species have been identified in the intervening time period, but few have had the impact of the nude mouse and the severe combined immune deficient mouse. Prior to the development of these two animal models, the ability to study human tumor progression in animals was limited by T-lymphocyte-mediated rejection of implanted human tumors. The nude mouse (Figure 2.8) was

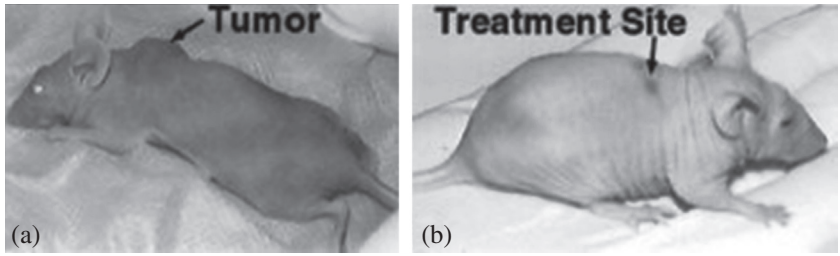

FIGURE 2.8 The nude mouse: Disruption of the FOXN1 gene in mice produces a strain of mice with a severely inhibited immune system due to the absence of the thymus, a major source of T-cells. The nickname "nude mouse" is based on the most obvious physical feature resulting from the disruption of the FOXN1 gene, a distinct lack of body hair. Nude mouse bearing subcutaneous tumor before (a) and after (b) high intensity focused ultrasound treatment. *Source: Reprinted from Vaezy, S.; Fujimoto, V. Y.; Walker, C.; Martin, R. W.; Chi, E. Y.; Crum, L. A. Treatment of uterine fibroid tumors in a nude mouse model using high-intensity focused ultrasound. Am. J. Obstet. Gynecol., 183 (1), 6–11, copyright 2000 with permission from Elsevier.*

originally identified in the Virus Laboratory, Ruchill Hospital, Glasgow in 1962,[23] and it was subsequently demonstrated that they were congenitally athymic.[24] In the absence of the thymus, nude mice are unable to generate mature T-lymphocytes, which severely limits their ability to mount an immune response. In the absence of a pathogen, the nude mice have a similar life span to their normal counterparts, but they are unable to reject

transplanted tissues such as human tumors. Both primary and metastatic tumors of human origin can be grown and studied in the nude mouse. As such, they were rapidly accepted as a major model for the study of cancer progression and therapeutic intervention. The nude mouse also facilitated the study of infectious disease, as it became possible to study pathogen progression and potential therapies in the absence of a full immune response.

The SCID Mouse

The development of immune compromised models was further advanced in 1983 with the introduction of the severe combined immune deficient (SCID) mouse.[25] An autosomal recessive mutation in mice was identified at the Fox Chase Cancer Center that, when homozygous, leads to animals that are severely deficient in B- and T-lymphocytes. This leaves them highly susceptible to infectious disease, irrespective of the nature of the pathogen, and, similar to nude mice, unable to reject transplanted tissues. The introduction of the SCID mouse model, and the variations that were developed as a result of its identification, provided an additional platform for the study of cancer and infectious disease that was previously unavailable to the research community.

Transgenic Animal Models

Up until 1974, the ability to develop new animal models was limited to selective breeding and depended on the natural occurrence of mutations, such as the nude mouse, to provide improved models for research. Direct manipulation of an animal's genetic codes was not possible. This changed, however, with the introduction of transgenic science. The initial breakthrough in this area was provided by Rudolf Jaenisch, who successfully inserted simian virus 40 DNA sequences into mice.[26] Although the genes were not passed onto offspring, these efforts marked the first successful transfer of foreign DNA into an animal suitable for drug discovery research. Subsequent efforts by Frank Ruddle (Yale),[27] Frank Constantini (Oxford), and Elizabeth Lacy (Oxford)[28] demonstrated that the addition of foreign DNA to single cell mouse embryos provided incorporation of the foreign DNA, and the new genes were passed on to subsequent generations (Figure 2.9). These efforts marked the beginning of a new era in both animal modeling and drug discovery. It was now possible to insert disease-related genes into animals that did not normally demonstrate the pathology in question. Mouse models of Alzheimer's disease, for example, were created by inserting DNA that induced the production of Aβ42 plaques, a hallmark of this disease, providing a new platform for the study of this important malady.[29] Similarly, models of human obesity have been generated in mice through the transgenic methods, providing significant insight into the mechanism of obesity.[30] The pathogenesis of viral infections such as HIV, hepatitis (B and C), polio, and measles

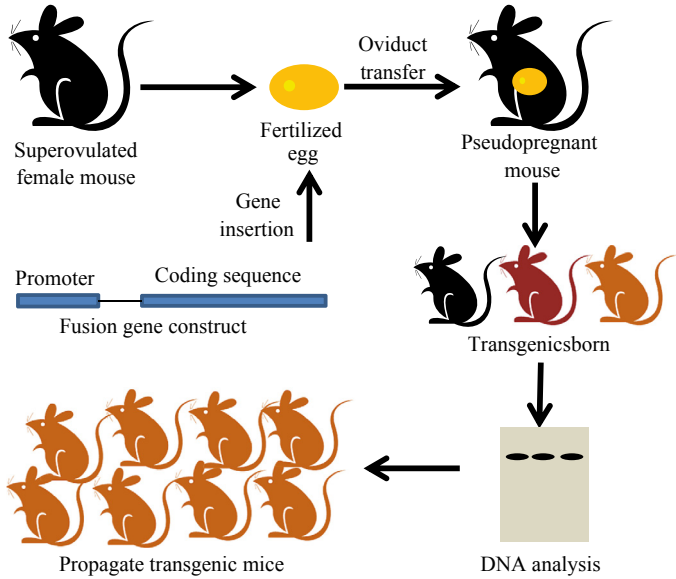

FIGURE 2.9 Transgenic animal models are developed through a combination of selective breeding and genetic manipulation. A gene construct suitable for insertion into an organism's DNA is prepared and then inserted into a fertilized egg via microinjection. The altered embryos are then implanted into a suitably pseudo-pregnant female and carried to term. After birth, genetic profiling is employed to identify offspring that are carriers of the transgene. Identification of transgene positive progeny is then followed by selective breeding to further the germ line.

have all been studied through the development of transgenic models through expression of either the human receptor for the virus or viral proteins important for pathogenesis.[31] Production of therapeutically relevant biomolecules has also been accomplished through the generation of transgenic animals.[32] Human antithrombin,[33] fibrinogen,[34] and monoclonal antibodies[35] have all been produced via transgenic science. It is well beyond the scope of this text to describe the wide array of transgenic animal models that has been developed since these initial experiments, but the impact of transgenic animals has been significant (Figure 2.10).

Knockout Animal Models

The advent of transgenic technology in animal models opened the door to knockout animal models. By the late 1980s, it had been well established that new animal models could be developed through the insertion and expression of foreign DNA in animal models. The next logical step, the suppression of normal gene function, was addressed by Capecchi, Evans, and Smithies in 1989 when they introduced the first knockout mouse.[36]

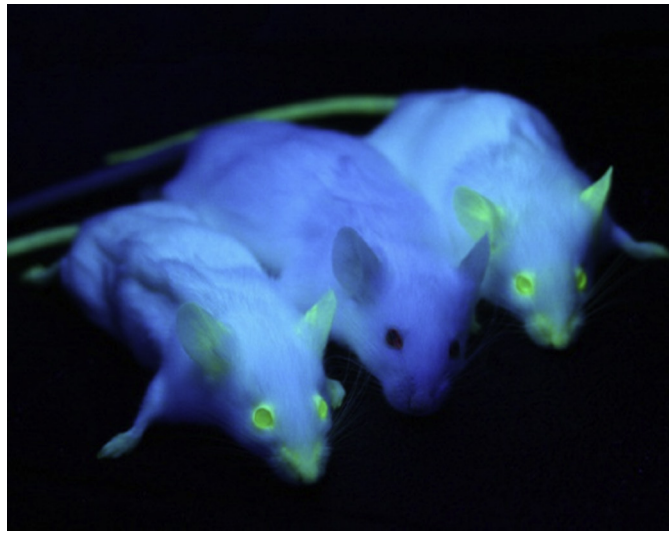

FIGURE 2.10 Transgenic insertion of the gene responsible for the production of green fluorescent protein (GFP) results in mice that fluoresce when exposed to ultraviolet light. The GFP gene has been successfully expressed in bacteria, fungi, plants, insects, and mammalian cells. Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien were awarded the Nobel Prize in Chemistry in 2008 in acknowledgement of their work on GFP technology. *Source: Moen, I.; Jevne, C.; Wang, J.; Kalland, K. H.; Chekenya, M; Akslen, L. A.; Sleire, L.; Enger, P.; Reed, R. K.; Yan, A. M.; Stuh, L. E. B. Gene expression in tumor cells and stroma in dsRed 4T1 tumors in eGFP-expressing mice with and without enhanced oxygenation. BMC Cancer 2012, 12:21.* http://doi:10.1186/1471-2407-12-21.

In their seminal experiments, they were able to eliminate functional hypoxanthine-guanine phosphoribosyl transferase genes (*hprt*) in mouse embryonic stems cells using either a sequence replacement targeting vector or a sequence insertion targeting vector (Figure 2.11). In both cases, the insertion of foreign DNA into the otherwise functional DNA segment led to the suppression of the *hprt* gene in viable embryonic stem cells, which were then implanted into the uterus of a healthy mouse and progressed to birth. In the following years, thousands of knockout mouse models have been developed to study a wide range of disease states. The p53 knockout mouse, for example, has been an important model in the study of cancer progression and therapy. The absence of functional p53 tumor suppressors, encoded by the TP53 gene, provides a mouse strain that mimics Li-Fraumeni syndrome. The resulting mice are far more susceptible to tumor formation.[37] Additional examples include the Fmr1 knockout mouse that serves as a model for Fragile X-related mental retardation,[38] the nescient helix loop helix 2 (Nhlh2) knockout mouse that decreases the levels of α-melanocyte-stimulating hormone and thyrotropin-releasing hormone, providing a model for the study of obesity,[39] and the ApoE knockout mouse in which the expression of Apolipoprotein E is suppressed, leading to the formation of vascular plaques similar to those found in humans suffering

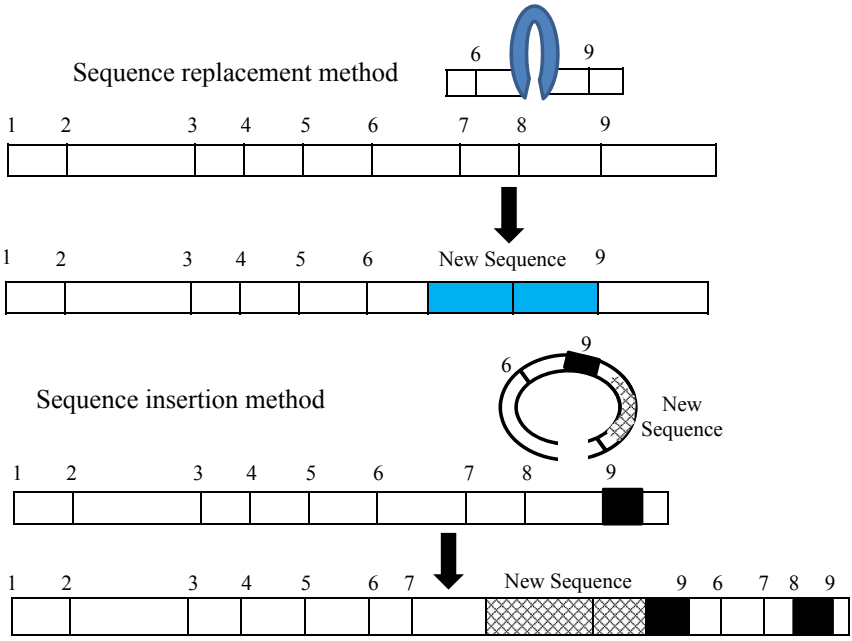

FIGURE 2.11 In the sequence replacement method of producing knockout animal models, gene disruption is accomplished by replacing a portion of the target DNA with a new sequence. An alternative method of producing knockout animals, the sequence insertion method, inserts a new sequence of DNA that is a repeat of a portion of the original DNA sequence. In both instances, the DNA is no longer capable of producing the gene product. Genetic screening of offspring animals followed by selective breeding can then be used to establish the germ line.

from hypercholsteroemia.[40] Since the introduction of knockout technology, thousands of knockout mice have been created in an effort to better understand gene function and disease progression. The importance of this technology was recognized in 2007 with the awarding of the Nobel Prize to Capecchi, Evans, and Smithies for their pioneering work in this area.[41]

Milestones in Molecular Science

While advances in animal models were providing more and more information into the physiological outcomes of potential therapies, they provided little, if any, knowledge as to the molecular interaction required for biological activity. Elucidating the mechanistic aspects of drug action or disease progression at a molecular level requires the ability to prepare molecules suitable for testing, an understanding of the structure of the target (e.g., enzymes, receptors, etc. See Chapter 3), and the ability to screen for biological activity in isolated systems (e.g., *in vitro* screening. See Chapter 4). In the intervening time between Paul Ehrlich's pioneering

efforts and the present day, substantial progress has been made in preparing novel compounds through advanced organic synthesis, elucidating the molecular structure of biological targets, understanding the interaction of the aforementioned targets with biologically relevant molecules, and increasing the pace at which the science is explored through the application of robotics, automation, and computer technology. Advances in one of these overlapping fields often provided support for new discoveries or technological advancements in related areas. The growth of X-ray crystallographic knowledge, for example, had a tremendous impact on the science of molecular modeling and computational chemistry, and both of these fields relied heavily on advances in computer technology, an area totally outside of drug discovery, to increase capabilities and capacity. While it is not possible to describe the complete history of the development of the full range of tools employed to understand the molecular basis of disease processes and drug action, an examination of the history of some of the key technologies developed for this purpose provides a wealth of insight into how modern drug discovery systems developed over the course of the last century.

X-ray Crystallography

Understanding the molecular structure of biological targets and associated ligands is a critical aspect of modern drug discovery. At the beginning of the twentieth century, however, modern analytical methods were just beginning to be developed. The field of X-ray crystallography was still in its infancy when Paul Ehrlich launched the research that eventually led to the identification of Salvarsan. In fact, the existence of X-rays themselves had only recently been discovered by Wilhelm Conrad Röntgen in 1895,[42] and the concept that crystalline materials could diffract an X-ray beam and the resulting scattering pattern was related to the molecular structure of the material was still a novel one at the turn of the twentieth century.[43]

The first successful application of this technology to an organic compound was reported in 1923 by Raymond and Dickinson, who elucidated the structure of hexamethylenetetramine,[44] but it was Dorothy Crowfoot Hodgkin[45] who propelled the field into the world of biomolecules and drug discovery. She was among the first to realize the potential for the application of X-ray crystallographic techniques to organic compounds and biomolecules. If Paul Ehrlich is the father of drug discovery, then Dorothy Crowfoot Hodgkin is the mother of protein crystallography. Her accomplishments include the first diffraction pattern of a crystalline protein, pepsin,[46] as well as diffraction pattern images of a host of important proteins including lactoglobulin[47] and insulin.[48] In 1969, 34 years after Hodgkin took her first X-ray diffraction photographs of insulin, she

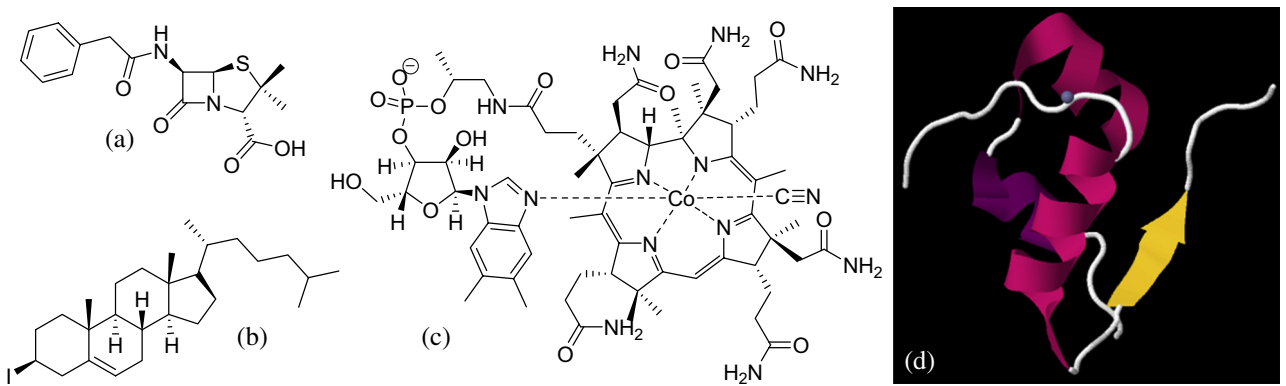

FIGURE 2.12 Dorothy Crowfoot Hodgkin (1910–1994), a graduate of the University of Cambridge, was an earlier pioneer in the field of X-ray crystallography, especially with respect to its application to biomolecules. She is credited with providing definitive structures for a variety of important molecules including (a) benzylpenicillin, (b) cholesteryl iodide, (c) vitamin B_{12}, and (d) insulin (RCSB 4INS).

and her colleagues reported the crystal structure of rhombohedral 2 zinc insulin at 2.8 Å resolution, providing an atomic model for the protein.[49] In the intervening years, she revolutionized the field of X-ray crystallography by solving numerous atomic structures of compounds such as cholesteryl iodide,[50] establishing for the first time the relative stereochemistry of steroids, benzylpenicillin salts,[51] identifying the β-lactam substructure for the first time, and vitamin B_{12},[52] the first naturally occurring organometallic compound with biological significance (Figure 2.12). In 1964, Hodgkin received the Nobel Prize in chemistry for her contributions to the field.[53]

The remarkable work of Dorothy Crowfoot Hodgkin and the scientists that followed in her footsteps provided the scientific community with their first clear pictures of structures of biomolecules. Thousands of protein structures, both in the presence and absence of a ligand have been reported, and the information embedded within these structures has provided a detailed understanding of how drugs interact with their target proteins. The Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), first established in 1971 with 7 structures, contains over 82,000 protein structures as of 2012.[54] Nucleic acid X-ray structures, the most famous of which is the Watson and Crick DNA structure introduced in 1953,[55] have also been exceptionally valuable tools in determining the molecular interaction required for normal, pathological, and drug-mediated biology. The Nucleic Acid Database (http://ndbserver.rutgers.edu/index.html), a more recently created publicly available database, was established in 1992 to provide the scientific community with access to three dimensional structures of nucleic acids, and contains over 6300 solved structures as of 2012.[56] Finally, The Cambridge Structural Database (http://www.ccdc.cam.ac.uk/products/csd/), founded in 1965,[57] focuses on small molecule crystal structures, and contains structural information on nearly 600,000 small molecules as of 2012.[58]

Molecular Modeling and Computational Chemistry

Although Heisenberg's 1925 paper on quantum mechanics[59] is widely considered to be the first publication in the field of computational chemistry and molecular modeling, it would take an additional 36 years for the concept of using computers to calculate and predict chemical properties and interactions to arrive. In 1961, James Hendrickson calculated the conformational energies of cycloheptanes using an IBM 709 computer (Figure 2.13) that was capable of "8000 additions/subtractions, 4000 multiplications/divisions, or 500 complex functions per second."[60] In essence, he launched the field of molecular modeling with a computer that had fewer capabilities and less capacity than most cellular telephones.

FIGURE 2.13 The IBM 709 computer, introduced in 1958, had less computer power than modern cellular phones. *Source: IBM 709 front panel at the Computer History Museum by Arnold Reinhold* http://en.wikipedia.org/wiki/IBM_709#mediaviewer/File:IBM_709_front_panel_at_CHM.agr.jpg.

A few years later (1966), Cyrus Levinthal described his efforts to combine computer simulations with molecular graphics to visualize and study the structures of proteins and nucleic acids,[61] marking the dawn of computer-aided drug design.

The impact of molecular modeling and computational chemistry grew as the computer industry became more and more sophisticated, but the overall premise of the field remained the same. Computers and software could be used to understand the relationship between structural features and physical/chemical properties, including those that were critical to drug function. In addition, knowledge of these relationships could be used to alter or improve the physical and chemical properties of compounds, such as biological activity, solubility, and metabolic stability. By the late 1970s, independent commercial ventures based on computer-assisted modeling were beginning to appear. Molecular Design Limited and Tripos (Figure 2.14) were the first of many organizations built to exploit the ever-growing understanding of molecular interaction with the goal of designing better molecules *in silico*. In 1984, computing capabilities and molecular modeling capabilities had grown to the point where protein simulation was possible and BioDesign launched the first commercial program designed for this purpose. Continued growth in computer power and changes in the drug discovery industry led to the development of additional software tools between 1984 and the present day. Tools designed to assess molecular diversity, design compound libraries, create screening sets based on molecular similarity, and automate the docking

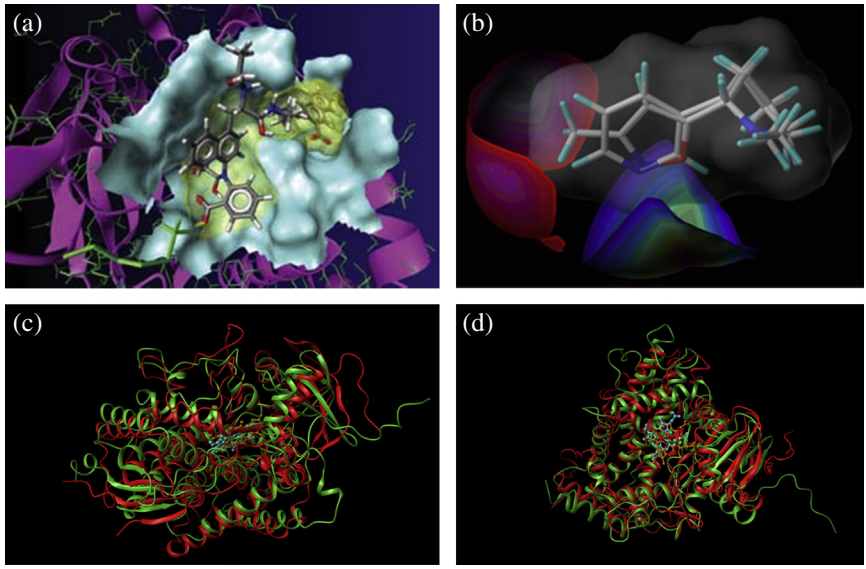

FIGURE 2.14 Tripos, the first independent company focused on the use of computer aided drug design, was founded in 1979. One of its products, Benchware 3D Explorer, provides drug discovery scientists with the ability to visualize and manipulate protein-ligand structures on a desktop computer. Ligands can be modified within the context of a protein in order to gain insight into the impact of structural changes on the potential binding energy of a new proposed ligand. Image (a) shows the PDB structure of protein tyrosine phosphatase 1B (RCSB 1NNY) with a potent inhibitor, which can be readily visualized and manipulated by non-experts in molecular modeling using the Tripos Benchware 3D Explorer software. Important aspects of binding, such as hydrogen bonding, hydrophobic interactions, and structural compatibility between the ligand and protein are readily identified. The surface of the binding site, highlighted in light blue (Connolly surfaces), enables the user to see the shape complementarity of the ligand and the protein. The Sybyl-X software system, also a product of Tripos, offers more advanced capabilities, such as virtual high throughput screening in which potentially millions of compounds are docked into a target protein's binding site and scored to provide an estimate of their relative binding energy at the target of interest. Pharmacophore-based virtual high throughput screening, a method of overlaying and comparing a compound of interest with potentially millions of compounds to determine their similarity, and therefore, potential for binding at a macromolecular target, is also possible with Sybyl-X. Image (b) shows an overlay of nicotine and an oxazole derivative, comparing their overall molecular architecture. The grey, translucent surface provides visualization of the molecular volume of the aligned molecules, the red area represents significant differences in hydrophobic surfaces between the two compounds, and the blue/green surface indicates a high degree of electrostatic potential overlap in the two structures. Comparisons of this type can be automated, scored, and sorted in order to facilitate the identification of potentially interesting molecules based on their similarity to known compounds of interest using Sybyl-X. Comparison of macromolecular structures is also facilitated with Sybyl-X. Panels (c) and (d) provide different views of an overlay of steroid 17-alpha-monooxygenase (Cyp17A1, RCSB 3RUK), a key enzyme in steroidogenesis, and cholesterol 7-alpha-monooxygenase (CYP7a1, RCSB 3DAX), the rate limiting enzyme in the synthesis of bile acid from cholesterol. Key differences in the binding sites in the two related enzymes can be exploited by drug discovery scientists to create compounds that are highly selective for one enzyme over the other.

of large compound libraries into biological targets are now commonplace in the pharmaceutical industry.[62] Computer-driven predictions of chemical and physical properties are also commonplace, as are homology models[63] designed to provide a better understanding of molecular interactions when X-ray crystal structures are not available. The ability to employ computer-aided design will continue to grow as computer science advances and additional structural details become available.

High Throughput Technology: Chemical Synthesis and Screening Science

While advances in animal models, X-ray crystallography, and molecular modeling had a substantial impact on the course of drug discovery, they did not address the two key bottlenecks in the process, chemical synthesis and screening science. In fact, for the majority of the twentieth century, these issues remained unresolved. Prior to the development of high throughput technologies, drugs were discovered primarily using endogenous ligands, natural products, or marketed drugs as starting points in an animal model. Chemical modifications to improve efficacy was followed by additional *in vivo* screening to chart a path forward.[64] By the 1980s, most pharmaceutical companies' compound collections consisted of only a few thousand compounds acquired through historical projects and screening programs remained primarily a manual process, heavily dependent on low throughput assays and animal models.[65] The situation changed, however, over the last two decades of the twentieth century with the creation of the fields of high throughput chemistry and high throughput screening. Although it is not clear when the concepts for each field were developed, there were significant technological hurdles to overcome in order to accomplish the end goal of increased efficiency in both chemical synthesis and biological screening.

In the case of high throughput chemistry, also referred to as combinatorial chemistry or parallel synthesis, the groundwork that provides the basis for much of the modern methods can be traced back to earlier synthetic efforts that were not originally geared towards increasing efficiency. The preparation of small, druglike compounds on polymer-based material, for example, was first reported by Robert B. Merrifield in 1963 when he described the synthesis of a short peptide sequence on a polystyrene resin (also known as solid phase peptide synthesis).[66] Shortly thereafter, Merrifield reported the preparation of the biologically active peptides bradykinin,[67] bovine insulin,[68] and deaminooxytocin,[69] thereby validating the approach. As interesting as these efforts may have been at the time, the utility of preparing compounds on solid support was met with some degree of skepticism, as indicated by Rappaport and Crowley's 1976 publication entitled "Solid Phase Organic Synthesis: Novelty or

Fundamental Concept?" The article focused on the "ambiguous limitations of non-peptide solid-phase chemistry whose resolution is required if the process is to mature from publishable novelty to fundamental methodology."[70] By the mid-1980s, however, advances in polymer science, automation, and chemical synthesis paved the way for explosive growth in the field of high throughput synthesis, beginning with the independent work of Richard Houghten[71] and H. Mario Geysen.[72] Houghten and Geysen separately described methods for the synthesis of large arrays of small peptides using solid support and successfully applied them to identify biologically active peptides. The practice of high throughput chemistry transitioned out of peptides and into druglike space by the early 1990s with the nearly simultaneous disclosure of the synthesis of arrays of functionalized 1,4-benzodiazepines on solid support by Jonathan A. Ellman[73] and S. Hobbs DeWitt (Figure 2.15).[74]

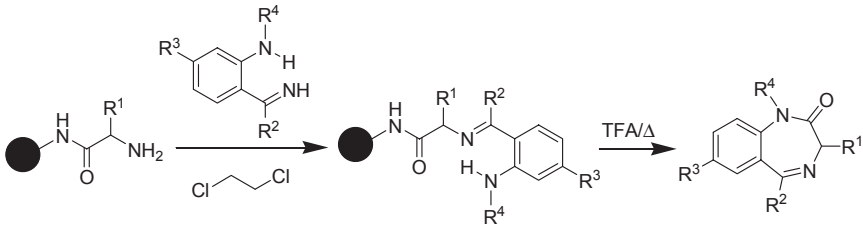

FIGURE 2.15 In 1992, professor Jonathan Ellman and his colleagues demonstrated that 1,4-benzodiazepine derivatives could be prepared on solid support. The application of solid phase chemistry to produce analogs of market drugs such as Valium® (diazepam), Ativan® (lorazepam), and Rivotril® (clonazepam) demonstrated that drug-like compounds could be prepared in this manner.

After these seminal reports, pharmaceutical companies began to incorporate the concepts and practices of solid phase synthesis and high throughput chemistry into their research programs. Resin-bound synthesis continued to progress into the small molecule arena,[75] but at the same time, older techniques were reexamined and new technologies were developed with the goal of increasing the synthetic output of medicinal chemists. Multicomponent reactions designed to incorporate multiple elements of diversity in a single step, such as the Ugi reaction,[76] the Biginelli reaction,[77] and the Passerini reaction[78] were revisited and employed to generate libraries of druglike compounds (Figure 2.16). New equipment dedicated to the rapid synthesis of hundreds, if not thousands, of compounds was developed, along with the technology necessary to purify, store, and retrieve hundreds of thousands of compounds. By the end of the twentieth century, compound collections at most major pharmaceutical companies had eclipsed 500,000 compounds,[65] and by 2013, the number of commercially available screening compounds exceeded 21 million.[79]

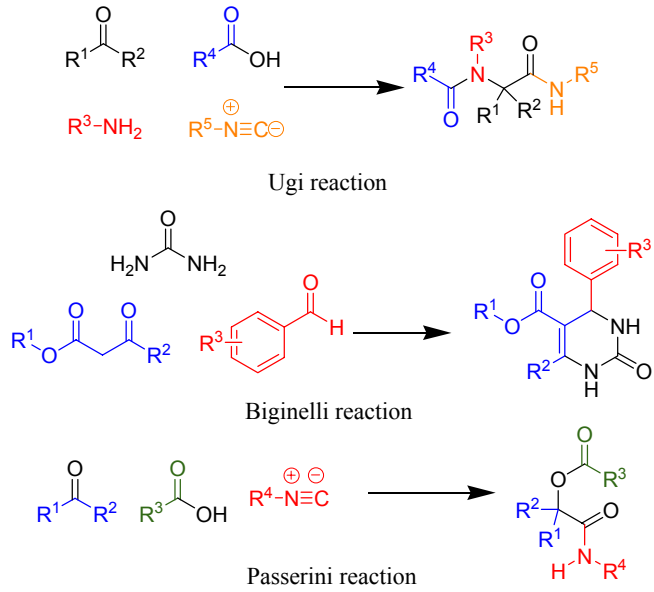

FIGURE 2.16 The Ugi reaction was discovered in 1959 by Karl Ugi, the Biginelli reaction was reported in 1891 by Pietro Biginelli, and the Passerini reaction was discovered in 1921 by Mario Passerini. These reactions have been repurposed for the preparation of large compound libraries suitable for HTS screening.

The development of high throughput screening occurred almost in parallel with high throughput chemistry, although a different set of technological advances were required. Through the 1950s, 1960s, and the 1970s, the pharmaceutical industry moved more and more towards a paradigm of screening compounds in cellular assays and isolated enzyme assays prior to animal testing in an effort to decrease costs and increase efficiency. An increasing understanding of the biochemical basis of disease provided the foundation for new biochemical assays, but the capacity to screen natural product extracts and compound collections was limited by the technology of the time. Prior to the mid-1970s and earlier 1980s, conventional methods of protein isolation and purification severely limited the amount of protein available for any given screen, thus driving up the costs. In addition, cellular assays were limited to using naturally occurring cell lines that could be grown in a reliable fashion.

The biotechnology revolution and the rise of robotics and automation, however, profoundly altered the landscape of compound screening. By the mid-1980s, major advances in biochemistry and molecular biology opened new pathways to the production of large quantities of proteins and "designer" cell lines. Technological breakthroughs, such as recombinant DNA, transfection science, polymerase chain reaction (PCR), and

cloning made it possible for scientists to generate cell lines that overexpressed targeted biomolecules, essentially eliminating the supply limitations of the past. Recombinant proteins could be harvested from cellular factories, providing ample quantities of target proteins. Alternatively, custom cell lines could be designed to incorporate biomolecular targets to support cellular screening assays. At the same time, advances in the fields of computer science, robotics, and automation led to the development of robotic platforms capable of performing repetitive motion tasks previously handled by humans, increasing accuracy and efficiency of any number of tasks in multiple fields (Figure 2.17).

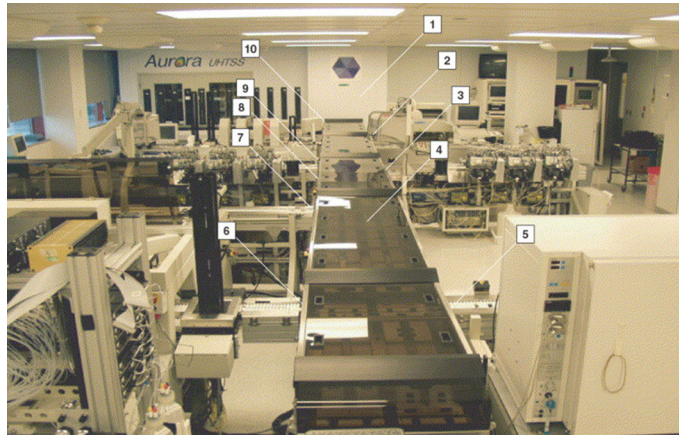

FIGURE 2.17 The automated uHTS system at Bristol-Myers Squibb. Integral components and subsystems are shown; (1) Compound store, (2) Hit-picking robot, (3) 3456 reagent dispensing robot, (4) Transport, (5) Incubators, (6) Piezo-electric distribution robot, (7) Topology compensating plate reader, (8) 1536 reagent dispensing robot, (9) Automated plate replicating system, (10) High-capacity stacking system. *Source: Reprinted from Cacace, A.; Banks, M.; Spicer, T.; Civoli, F.; Watson, J. An ultra-HTS process for the identification of small molecule modulators of orphan G-protein-coupled receptors. Drug Discovery Today, 8 (17), 785–792, copyright 2003, with permission from Elsevier.*

While it is not clear exactly when and where automation technology merged with the field of drug discovery, it is clear that by the end of the twentieth century nearly all pharmaceutical companies had transitioned to high throughput screening methods. Initially, screening assays were performed in 96-well microplates (standardized by the Society for Biomolecular Screening and the American National Standards Institute), but the drive for increased efficiency and lower costs eventually lead to the development of 384-, 1536-, and even 3456-well plate technology (Figure 2.18). The miniaturization of screening technologies also spawned advances in micro-fluidics and signal detection methods, as the increased plate density required decreased solution volumes and smaller signal windows. A standard 96-deep well plate could hold up to 1.0 mL of fluid per well, while the

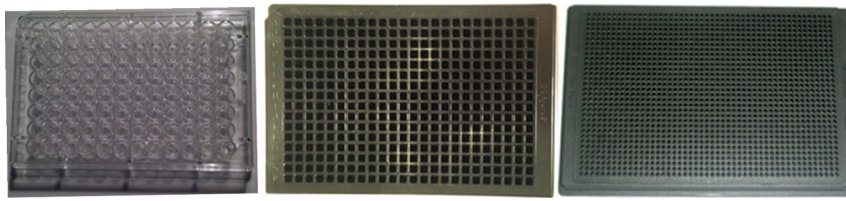

FIGURE 2.18 The typical *in vitro* screening assay employs 96 (left), 384 (middle), or 1536 (right) well plates. As the plates increase in well number (density), the well volume decreases and reagent requirements drop accordingly. The cost saving associated with higher density plates can be substantial.

corresponding 3456-well plate would be limited to a much smaller fluid volume per well. In addition, the density of signals from a 3456-well plate is much higher than that of a 96-well plate (a 3456-well plate contains the same number of wells as 36 96-well plates in the same space), requiring the development of more sophisticated data acquisition tools. By the end of the twentieth century, compound libraries containing hundreds of thousands of compounds could be screened for activity against multiple targets in a matter of days, a feat that would be impossible if attempted manually.

The combination of high throughput chemistry and high throughput screening, however, led to a massive increase in the amount of data produced in any given research program. It quickly became apparent that the bottlenecks of chemical synthesis and biological screening had been replaced by a new bottleneck, data analysis. If, for example, a single enzyme target was screened against a compound library containing 500,000 compounds at a single concentration in triplicate to ensure accuracy, this would produce 1.5 million data points that would need to be associated with the compound library. If one assumes that the hit rate for this hypothetical library of compounds is 0.2%, then 1000 compounds would be identified for follow-up screening to determine their potency (i.e., their IC_{50}). In addition, the majority of drug discovery programs have multiple screening targets for the purposes of determining selectivity, so millions of more data points would become available on compounds of interests across multiple biological targets. The addition of high throughput screening assays to determine physical properties, such as solubility, and druglike properties, such as microsomal stability and permeability, add even more data for analysis and correlation.

Clearly, the level of data available rapidly exceeded the human capacity to evaluate in the absence of computer-driven support. Efforts to address this growing issue led to the development of complex database software systems designed to capture data from a variety of sources (i.e., robotic screening platforms), link the data to a specific chemical structure within the database, and convert the data to a human readable form. Advances in molecular modeling and computational chemistry were also leveraged to increase efficiency, leading to the incorporation of structural data into

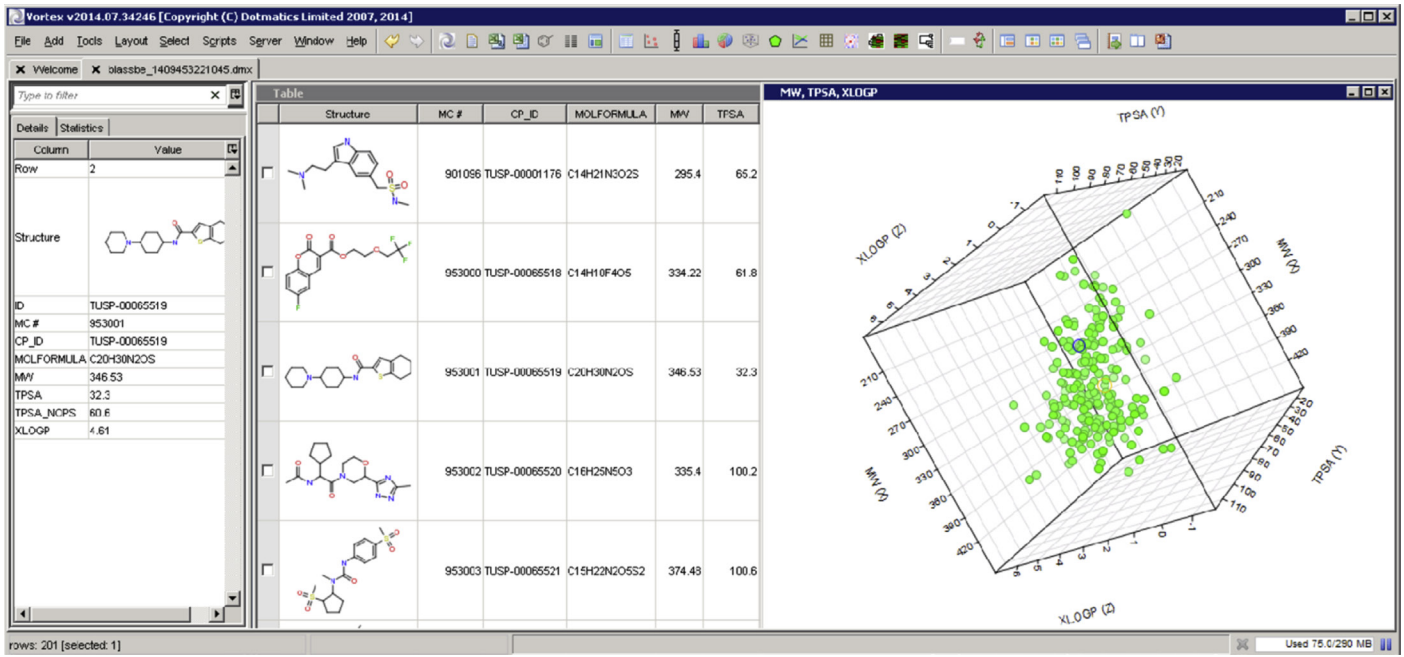

FIGURE 2.19 Cheminformatics software platforms provide scientist with the ability to link compound structures to physicochemical properties (e.g., molecular formula, molecular weight, Topological Polar Surface Area (TPSA), solubility, etc.) and screening data from multiple sources in a searchable database. Groups of structurally related compounds can be identified using sub-structure searching tools, and multidimensional analysis of compound associated data can be used to design next generation compounds with properties consistent with program goals. In this example, a series of compounds are analyzed using the Dotmatics software suite and three dimensional plot has been created to compare changes in molecular weight, TPSA, and cLogP.

modern database software and the birth of the field of cheminformatics. Originally defined by F.K. Brown in 1998,[80] cheminformatics has been applied in drug discovery to store, index, and search information related to individual compounds or groups of compounds. Specialized software provided by companies such as Chemaxon, Core Informatics, Tripos, and Dotmatics is now common place in drug discovery, and allows scientists to evaluate millions of data points with the click of a mouse (Figure 2.19).

Milestones in Biotechnology

Although there were many remarkable discoveries made in the first 70 years of the twentieth century, such as penicillin antibiotics,[81] benzodiazepine central nervous system (CNS) drugs,[82] and macrolide antibiotics,[83] drug discovery scientists of this age were limited in their ability to identify and interrogate targets of interest. The generation of new animal models was restricted to selective breeding of naturally occurring mutations (e.g., the nude mouse) and protein production was limited by the expression levels of proteins in naturally occurring cells. Similarly, the development of cellular assays was dependent on naturally occurring cell lines. The dawn of the age of biotechnology, however, ushered in a new era of drug discovery and disease understanding. Beginning in the 1970s, the restrictions imposed by natural evolution and selection were lifted as scientists began to develop technologies that allowed them to manipulate the DNA of living organisms. Initial experiments in the early 1970s designed to demonstrate that non-native DNA could be prepared (recombinant DNA) and transferred into living cells (transfection technology) were quickly followed by the application of similar technology to generate animals with non-native DNA (transgenic and knockout animal models). In 1975, monoclonal antibodies were introduced, adding further fuel to the biotech fire, and by 1980 companies such as Genentech and Amgen were founded to harness the new techniques for therapeutic purposes. Continued scientific advances, such as polymerase chain reaction (PCR) technology, successful macromolecular therapeutics (recombinant proteins, monoclonal antibodies, and receptor construct/fusion proteins), and the Human Genome Project, further expanded the reach of biotechnology. By the end of the twentieth century, less than 30 years after the initial experiment that launched the field, biotechnology had transformed the process of drug discovery and created a multi-billion dollar industry of its own. In early 2009, Genentech was purchased by Roche for over $46 billion,[84] and as of the end of 2013, Amgen had grown into a $90 billion company.[85] These examples clearly demonstrate the importance of the biotechnology revolution and the profound impact it had on the pharmaceutical industry.

Recombinant DNA and Transfection Technology

Watson and Crick's 1953 discovery of the three dimensional structure of DNA provided an understanding of its physical structure, but this knowledge did not provide the tools necessary to manipulate DNA. It would take another 20 years to develop this technology. The first step in this process was the identification of the enzymes involved in DNA production, modification, and degradation. Significant progress was achieved in the 1950s and 1960s. In 1956, DNA polymerase I, an enzyme capable of copying DNA template strands, was identified by Arthur Kornberg.[86] This was the first of many enzymes identified as acting on polynucleotide sequences that would lay a critical foundation for the experiments that led to the development of the technology necessary to not only manipulate the DNA of a species, but also to transfer functional DNA between species. The time period between 1956 and 1975 witnessed the identification of DNA active enzymes such as the DNA ligases, the enzymes responsible for joining DNA strands end to end,[87] exonucleases, which remove nucleotides from DNA chains,[88] and terminal transferases (also known as terminal deoxynucleotidyl transferases), enzymes capable of adding nucleotides to the 3′ end of DNA.[89] Reverse transcriptases,[90] enzymes capable of converting RNA into DNA, were also identified in this time period. The identification of the restriction enzymes (also known as restriction endonucleases), however, was the key to unlocking the puzzle. This class of enzymes, capable of creating two incisions across a double stranded DNA chain, provided DNA duplex segments with complimentary single stranded ends (also referred to as "sticky ends" or "cohesive ends").[91] Essentially, this provided scientists with the ability to carve out specific segments of duplex DNA strands, the nature of which are dictated by the selectivity of the particular restriction enzymes employed. DNA strands with complimentary "stick ends" could then be stitched together with the appropriate enzymes, thereby creating synthetic DNA, also referred to as recombinant DNA (Figure 2.20).

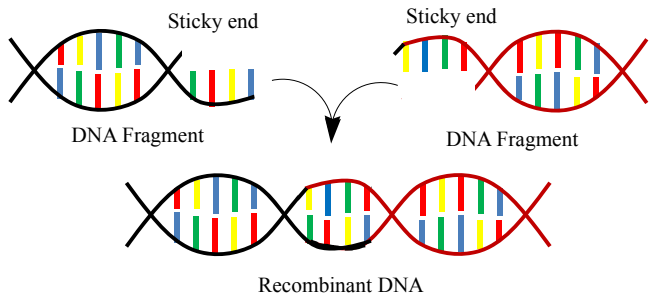

FIGURE 2.20 The identification of enzymes responsible for building, degrading and modifying DNA changes was critical to the development of recombinant DNA technology. Once these enzymes became available, DNA chains with complementary "sticky ends" could be stitched together to form "designer" DNA strands.

While the molecular biology of nucleic acid synthesis was being unraveled, scientists were also developing an understanding of virus form and function. The concept of infectious agents smaller then bacteria originated with French microbiologist Charles Chamberland in 1884. His studies of the infectious agent using filtration methods designed to remove bacterial organisms clearly demonstrated that a non-bacterial agent (eventually identified as the tobacco mosaic virus) was responsible for an infection present in tobacco plants. Over the course of the next several decades, methods to grow, isolate, and examine viruses evolved. Bacteriophages, viruses that infect bacteria which eventually became powerful tools in the study of DNA transfer, activation, and inactivation, were identified through the separate work of Frederick Twort[92] and Félix d'Herelle[93] at the turn of the twentieth century. In 1931, the cultivation and isolation of influenza and a number of other viruses using fertilized chicken eggs was reported by Ernest William Goodpasture,[94] opening the doorway to mass production of virus particles for scientific study. Further improvements in methods for the production and study of viruses continued between 1930 and 1970, setting in place another piece of the puzzle that eventually became recombinant DNA technology. The knowledge developed through the study of virus biology would eventually be utilized to develop the delivery vehicles necessary to move the science of recombinant DNA forward.

By the end of the 1960s, all of the tools necessary for the manipulation of genetic material in living organisms were in place, and in 1969, Peter Lobban, a graduate student working in the Biochemistry Department at Stanford University Medical School took the first steps down the path. His Ph.D. project proposal, presented to his research review committee as part of his progress towards his degree, suggested the merging of DNA modification technology and viral biology to provide a method of artificially transferring genetic material from one species to another.[95] His theories were quickly validated with the first publication of this new technology appearing in 1972 in which David Jackson et al. described methods of inserting new DNA into simian virus 40 (SV40).[96] By 1973, scientists at Stanford University had published methods for the end to end joining of DNA molecules[97] and the construction of biologically functional bacterial plasmids.[98]

Then, in 1974, the labs of Stanley N. Cohen and Herbert W. Boyer at Stanford University fundamentally altered the landscape of the pharmaceutical industry with a patent application (serial number 520,961) that described methods

> for genetically transforming microorganisms, particularly bacteria, to provide diverse genotypical capability and producing recombinant plasmids… which is used to transform a susceptible and compatible microorganism…. The newly functionalized microorganism may then be used to carry out their new function; for example, by producing proteins which are the desired end products, or metabolites of enzymatic conversions or be lysed and the desired nucleic acids or proteins recovered.[99]

The work of Cohen and Boyer provided methods to produce proteins and other cellular products by simply creating a stable microorganism that could be tailor made to produce the material and used as a factory (Figure 2.21). Cell lines that overexpressed cell surface receptors were also

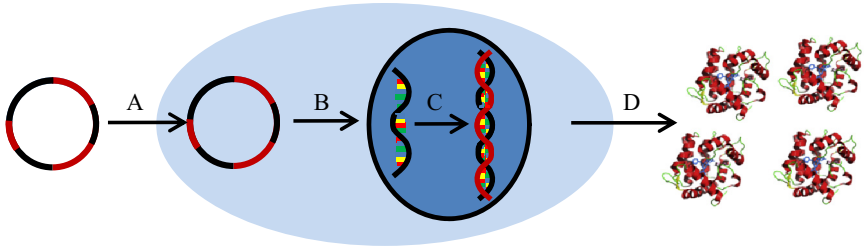

FIGURE 2.21 Stable cell lines capable of producing large amounts of a desired protein such as insulin can be prepared by (A) introducing a gene of interest into a cell (B) that subsequently enters the nucleus where it is (C) incorporated into the cell's chromosomal DNA. A stable cell line can be grown that will (D) express the desired protein that can be harvested from the growth media.

eventually developed as a result of this work, enabling the detailed study of a host of cellular targets that were previously difficult to examine due to low levels of expression. Inserting the proper DNA sequences into a suitable cell line would provide cells that overexpressed the desired target or protein (an overexpressing cell line), greatly amplifying the presence of the biological target of interest. Transfection technology is nearly omnipresent in the modern drug discovery lab. In the decades that followed Cohen and Boyer's initial patent, thousands of new cell lines have been developed using recombinant DNA and transfection technology. This seminal work also became one of the major underpinnings for the biotechnology industry. Recombinant human insulin, developed at Genentech and licensed to Eli Lilly,[100] was the first recombinant protein to gain market approval and many others have followed. As of 2012, less than 45 years after the initial suggestions of Peter Lobban, the biotechnology industry has grown from a series of lab experiments into a $300 billion industry[101] and has become an integral part of the modern drug discovery process.

Polymerase Chain Reaction (PCR) Technology

One of the early limitations of the biotechnology industry was the ability to prepare and analyze DNA. Although the tools to manipulate and analyze DNA (i.e., enzymes acting upon DNA) had been identified in between 1950 and 1970, the process was slow and manual. The utility of the science itself had been clearly demonstrated by the early

1980s, but the ability to generate large quantities of DNA remained lacking. The first attempt to replicate DNA using the enzymatic tools developed in the previous decades was reported by Kleppe and his coworkers in 1971,[102] but the process was far from optimal. DNA replication required that the double stranded helix be separated into the two parent strands, which can be accomplished by heating the sample to a high enough temperature (DNA melting). Upon cooling and in the presence of complimentary DNA primers (oligonucleotide starting points for DNA synthesis), nucleotide building blocks, and a suitable DNA polymerase, the DNA is replicated, providing copies of the original for study. Repeating the cycle provides additional copies of identical DNA with an exponential growth rate (Figure 2.22). The key limitation prior to the advent of modern PCR

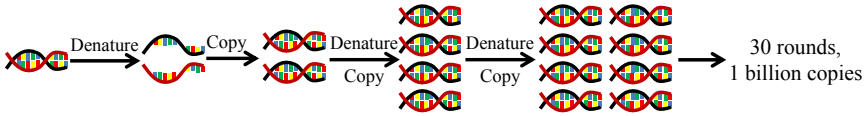

FIGURE 2.22 In the polymerase chain reaction process, each cycle of the denaturing and copying process doubles the number of copies of DNA present. Three copies cycles provides eight copies of the DNA chain. After 30 rounds of the sequence, the DNA amplification exceeds one billion copies.

technology, however, was the melting step. Like most enzymes, most DNA polymerases denature at the higher temperatures required for separation of duplex DNA, so each cycle of heating and cooling required the addition of fresh DNA polymerases, making the process of DNA replication both time consuming and expensive.

The landscape changed in 1976 with the discovery of Taq polymerase. This particular variant of DNA polymerase was isolated from *Thermus aquaticus*, a member of a family of unusual bacteria, thermophilic bacteria, which can survive in temperatures of up to 80 °C (175 °F). Prior to the discovery of these microbes in the geysers of the Yellowstone National Park,[103] it was generally believed that life could not be sustained above 55 °C, but clearly these organisms and other like them disagreed. In order to survive the harsh conditions of the geysers, *T. aquaticus* had developed biological systems that did not break down at the elevated temperatures of its environment. This included a variation of DNA polymerase, Taq polymerase,[104] which could function at elevated temperatures without denaturing. This paved the way for automation of DNA amplification. With the addition of this tool to the growing biotechnological toolbox, DNA amplification could be accomplished without adding additional DNA polymerase at the end of each cycle, greatly simplifying the process. In 1983, Kerry Mullis and

his colleagues at Cetus Corporation, an early player in the biotechnology field, were the first to harness these tools to create automated PCR equipment based on thermocycling, an alternating series of heating and cooling steps.[105]

With the advent of this technology, it became possible to generate millions of copies of a DNA strand using an efficient, automated process. This, in turn, facilitated rapid advances in numerous areas including genetic sequencing of organisms, cloning, diagnosis of hereditary diseases, and the detection of infectious agents. Genetic finger printing as a means to determine paternity and the use of genetic material in forensic sciences also developed as a result of the development of PCR technology. Multiple variations of PCR technology have been developed since the original reports, and much like recombinant DNA technology, PCR has become common place in modern drug discovery. The importance of this work was recognized with the awarding of the Nobel Prize in chemistry to Kerry Mullis in 1993. (The prize was shared with Michael Smith, who focused on site-directed mutagenesis.)

Monoclonal Antibody and Hybridoma Technology

While the development of monoclonal antibody technology is certainly a product of the biotechnological revolution of the twentieth century, it is clear that the importance of antibodies in general was recognized decades earlier. Paul Ehrlich was the first to suggest the term antibodies in 1891, and in 1897, he introduced the "side chain theory" of antibody/antigen interaction, which suggested that receptors on the surface of a cell could bind to antigens and stimulate the production of antibodies.[106] At the time, Ehrlich did not have the tools necessary to definitively test his theory, and it was almost 50 years later that Astrid Fagreaus determined that B-cells were the source of Ehrlich's antibodies.[107] The concept of monoclonal antibodies is also significantly older than the technology itself, as it was originally suggested by F. M. Burnet and his colleagues in the 1950s.[108] In brief, Burnet's theory, which proved to be correct, stated that upon full differentiation, antibody-producing B-cells (and their progeny) produce only a single type of antibody that would bind to only one target molecule. He further suggested that the polyclonal nature of the immune response observed in animals upon exposure to an antigen was the result of multiple lines of B-cells producing different antibodies targeting the same antigen, but through different structural features of the antigen (antigenic determinants). Although Burnet's theories clearly pointed to the concept that monoclonal antibodies could be produced from a uniform B-cell line, the technology to create a stable, antibody-producing cell line was not available at the time.

In a somewhat ironic twist of nature and science, the solution to the problem of generating stable cell lines capable of producing monoclonal

antibodies evolved from the disease that is the target of numerous monoclonal antibody-based therapies. Cancer, specifically multiple myeloma, was the key to unlocking this puzzle. By the early 1970s, multiple myeloma had been recognized as a malignant disorder of antibody-producing cells, and in 1973 Jerrold Schwaber and Edward Cohen reported the fusion of antibody-secreting mouse myeloma cells with human peripheral blood lymphocytes. The resulting hybrid cell line could be grown continuously and more importantly, produced human antibodies along with the mouse antibodies.[109] These milestone experiments were followed by the 1975 report of Georges Köhler and César Milstein[110] of stable cell lines generated by the fusion of mouse myeloma cells with antibody producing mouse B-cells. Each fusion cell, more commonly referred to as a hybridoma cell, produced a single antibody and, upon isolation and cloning, provided access to a stable cell line that produced a single antibody (Figure 2.23). This new process opened the

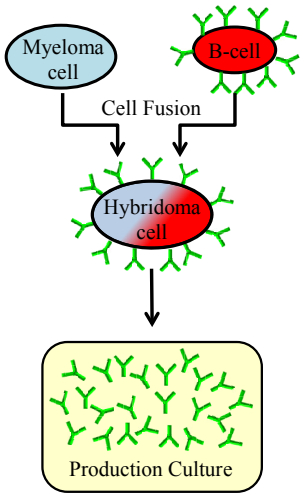

FIGURE 2.23 Monoclonal antibody producing cells can be prepared by fusing an antibody producing b-cell with a myeloma cell. The resulting hybridoma cell can be isolated, and cloned to provide a stable cell line capable of producing a single antibody (a monoclonal antibody).

doorway to generating large amounts of tailor-made, highly specific, monoclonal antibodies, creating immense opportunities for the fledgling biotechnology industry. In recognition of these groundbreaking achievements, Georges Köhler and César Milstein shared the 1984 Nobel Prize in Physiology or Medicine with Niels Kaj Jerne, a Danish immunologist, "for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies."

The ability to prepare significant quantities of monoclonal antibodies targeting virtually any macromolecular target had a profound impact on the drug discovery industry. It was quickly realized that these new tools

could be used to enhance screening technology, study cell surface proteins in more detail, purify proteins, and perhaps most importantly, they had the potential to be exquisitely specific therapies. For example, a monoclonal antibody could be designed to target a specific cell type, such as cancer cells, through an antigen unique to the target, such as a cell surface protein. Binding of the monoclonal antibody to the target would then prompt an immune system response targeting the cells for destruction.

The allure of treating patients with such highly specific drugs led to an immense level of research in the area. By the late 1980s, the humanization of monoclonal antibodies[111] had been achieved and in 1986, the first monoclonal antibody therapy, Orthoclone OKT3® (Muromonab-CD3), was approved by the FDA for the prevention of transplant rejection.[112] By the beginning of the twenty-first century, monoclonal antibody therapy had established itself as a significant player in the pharmaceuticals industry. Drugs such as Herceptin® (Trastuzumab, breast cancer) and Remicade® (Infliximab, arthritis) had achieved blockbuster status, with multibillion dollar annual sales, and every major pharmaceutical company was investing in this technology.

THE RISE OF BIOLOGICS AND MACROMOLECULAR THERAPEUTICS

The importance of the biotechnological revolution that began in the 1970s cannot be understated. Modern drug discovery would be a far more difficult task if it were not for the ground breaking technologies that were developed during this time period. High throughput screening, for example, depends on the production of large quantities of proteins and antibodies, neither of which would be available without recombinant DNA, PCR, and hybridomas. The identification of novel, druggable targets would be far more difficult, and programs designed to fully map the genome of a given species, such as the Human Genome Project,[113] would be all but impossible. Transgenic and knockout animal models that have provided significant insights into disease mechanisms and drug therapy would not be available if not for the pioneering efforts of the scientists that drove biotechnology forward.

The most important impact of this era, however, has been the development of groundbreaking therapeutic agents. Recombinant human insulin is probably the most well-known, but dozens of other important therapeutic agents have changed the lives of patients around the world. Recombinant human proteins such as Activase® (Alteplase tissue plasminogen activator, Genentech),[114] Epogen® (erythropoietin, Amgen)[115] and many others provide treatments for conditions that might otherwise be impossible to treat. Monoclonal antibodies such as Remicade® (Infliximab, Janssen

Biotech)[116] and Herceptin® (Trastuzumab, Genentech)[117] have revolutionized the treatment of arthritis and cancer, respectively, providing therapeutic relief to millions.

Hybrid technologies have also emerged. Receptor construct fusion proteins, for example, have emerged as a new tool that employs both antibody and protein technology. Therapies such as Orencia® (Abatacept, arthritis),[118] AmeviveE® (Alefacept, psoriasis),[119] and Eylea® (Aflibercept, wet macular degeneration)[120] are each composed of a protein receptor segment and an immunoglobulin structure. The protein receptor portion provides selectivity for the target of interest, while the immunoglobulin structure provides metabolic stability.

Major companies such as Genentech and Amgen emerged as key early player in the commercialization of biotechnology, and in the modern world of drug discovery, every major pharmaceutical company is vying for a piece of the biotechnology pie. While it is clear that the therapeutic utility of macromolecules is of vital importance to the drug industry, it is well beyond the scope of this text to describe the methods used to discover them. Those interested in the specific details of the discovery of each class of biotherapeutics are encouraged to consult texts that are focused on those particular areas. Sections of this text covering aspects of drug discovery such as pharmacokinetics, clinical trials, patent law, animal models, and translation medicine are, however, just as relevant to biotherapeutics as they are to small molecule therapies.

SOCIETAL AND GOVERNMENTAL IMPACTS

While scientific advances certainly played a major role in defining the drug discovery process that has evolved over time, it is abundantly clear that societal forces also helped shape the modern drug discovery process. It is well established that humanity has been searching for treatments to alleviate suffering and disease for hundreds, if not thousands, of years, and in many cases, the identification of new therapeutic agents can be directly linked to a major public health need of a particular time period. Agostino Salumbrino's observations that eventually led to the discovery of quinine,[8] for example, were driven by societal needs to deal with malaria, a major public health issue. In the same sense, but in a more modern setting, the development of modern cardiovascular drugs was driven by the realization that cardiovascular disease is a major mortality risk. Similarly, advances in antiviral drug technology were primarily driven by the societal impact of the AIDS epidemic.

Of course, many drugs have been brought to market by pharmaceutical companies in the absence of social pressures, but the appearance of the drugs on the market led to societal demand for additional

treatments. It is hard to argue, for example, that the development of Rogaine® (Minoxidil)[121] for the treatment and prevention of hair loss was prompted by a major health issue surrounding baldness. However, this drug was originally developed as an antihypertensive agent. The company that developed it, Upjohn Corporation, simply capitalized on an interesting side-effect observed in patients being treated for high blood pressure to create a new market for one of its products. Since the introduction of Rogaine, many companies have spent millions of dollars to break into the hair growth market. The social pressures of vanity and an aging population, as well as a corporate desire for increased profits, clearly influenced the choice to pursue Rogaine and drugs with similar utility.

Apart from influencing which disease states and conditions would be addressed over time, it is also clear that societal forces, often through governmental intervention and regulation, have played an important role in determining how drugs are developed. The modern drug discovery process is a highly regulated path to market that must be adhered to in order to successfully bring a drug to market, but this was not always the case. In fact, prior to the twentieth century, there were few, if any, laws or guidelines in place that specified what could or could not be sold as a medicine. Similarly, there were essentially no guidelines or requirements in place for determining if materials used as drugs were effective or even safe to use. While it is well beyond the scope of this text to provide a detailed accounting of the history of governmental and societal actions that contributed to the evolution of the modern drug discovery process, an examination of some of the major milestones is instructive.

The Pure Food and Drug Act of 1906[122]

At the beginning of the twentieth century, the preparation and sale of medication was virtually unregulated. In the absence of restriction by government regulation, virtually anything could be sold as a "drug" and, in many cases, chemicals that are now known to be harmful were marketed as "medicines." Syrups marketed to soothe the crying of infants and children were often laced with opiates, and addictive drugs such as cocaine and heroin were routinely part of "patent medicines." The simple requirement of an accurate listing of ingredients was not in place, and secret ingredients in medicine were part of the back drop against which the Pure Food and Drug Act of 1906 came into being. Samuel Hopkins Adams' series of articles entitled "The Great American Fraud," in which he detailed the abuses of the pharmaceutical industry of the time, is often credited with igniting the fire that lead to the passage of the first law designed to regulate the drug industry.[123]

While limited in its scope, this first attempt to ensure the safety of drugs had far reaching consequences for the pharmaceuticals industry, as it established the foundation upon which the Food and Drug Administration (FDA) would eventually be built. "Dangerous" drugs such as cocaine, heroin, alcohol, and morphine could no longer be used as secret ingredients in medicines, although they could still be included as long as they were accurately labeled. In addition, the U.S. Pharmacopeia[124] and the National Formulary[125] were put in place as the authorities for drug composition and formulation. More importantly, however, the new law provided the Bureau of Chemistry in the U.S. Department of Agriculture, the authority to establish a group of federal inspectors to enforce the laws. These inspectors were empowered to seize and destroy material found to be in violation of the new law (at the company's expense) and provide publication of all violations that occurred. Although direct financial penalties were modest, the prospect of negative publicity and physical loss of manufactured materials became a major tool in enforcing drug regulations.

The Elixir of Sulfanilamide Disaster of 1937[126]

Although the Pure Food and Drug Act of 1906 set the stage for further improvements in the regulation of the pharmaceuticals industry, the law was far from adequate. The law provided no guidance or requirements for the safety of drugs brought to market. As is often the case, a disaster of some type would be required before this would change. Thus, in 1937, the S. E. Massengill Company began marketing a new formulation for the antibiotic sulfanilamide under the name Elixir of Sulfanilamide. Sulfanilamide had been successfully employed for the treatment of streptococcal infections when provided in the form of a tablet or powder, but a liquid formulation was not available. After receiving requests from field sale agents, the company's head of chemistry and pharmacy, Harold Watkins, created a new formulation in response to this request. The new product contained three key ingredients, sulfanilamide, raspberry flavoring, and diethylene glycol (Figure 2.24). After tests for flavor, appearance, and fragrance were deemed acceptable, the new product

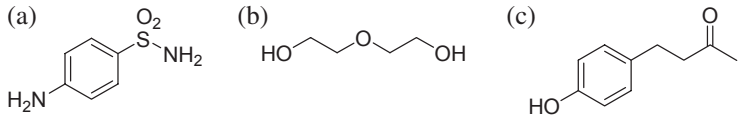

FIGURE 2.24 Elixir of Sulfanilamide marketed by S. E. Massengill contained (a) sulfanilamide, (b) diethylene glycol, and (c) Raspberry flavoring.

was prepared in bulk and distributed nationwide in September of 1937. No safety studies of any kind were performed, as none were legally required at the time.

By October of 1937, the consequence of not studying the safety of a potential new therapy became abundantly clear. In Tulsa, Oklahoma, the American Medical Association received reports from physicians that Elixir of Sulfanilamide had been linked to a number of deaths. Their laboratory quickly recognized that diethylene glycol, a compound often used as antifreeze and a deadly poison, was the cause of the deaths. The federal government was notified on October 14th, and began the process of recalling the material. Of the 240 gallons prepared and distributed for sale, 234 gallons were retrieved and destroyed, but the damage had already been done. At least 107 deaths were attributed to Elixir of Sulfanilamide poisoning. The victims, many of them children suffering from simple sore throats, died as a result of kidney failure brought on by ingestion of diethylene glycol.

Although a simple animal safety study would have quickly revealed the toxicity of diethylene glycol, there were no legal requirements for animal safety studies at the time, and none were performed. Even a brief review of the scientific literature at the time would have been sufficient to uncover the deadly nature of diethylene glycol, but even this simple precaution was not taken, and the public paid a heavy price. In fact, in 1937, there were no laws barring the sale of dangerous, untested, or even poisonous drugs. Legal authority to confiscate and destroy the Elixir of Sulfanilamide was based on misbranding the material as an "elixir" when there was no alcohol in the product, rather than the deaths that it caused, as there was no other legal basis for the recovery in 1937. The rather trivial charge of "misbranding" was the only charge that could legally be applied against the company, even though it was clearly responsible for marketing a known poison as a medicine. When pressed to admit some level of accountability for this tragedy, the company's owner, Dr. Samuel Evans Massengill, denied any responsibility for the tragedy, stating "My chemists and I deeply regret the fatal results, but there was no error in the manufacture of the product. We have been supplying a legitimate professional demand and not once could have foreseen the unlooked-for results. I do not feel that there was any responsibility on our part."

The Elixir of Sulfanilamide disaster was not the first time a dangerous drug had been brought to market, but it is widely viewed as a watershed event in the history of drug regulations. In response to this disaster, congress passed the Food, Drug, and Cosmetic Act of 1938. Under this new law, pharmaceutical companies were required to prove the safety of their new products through animal safety studies prior to receiving marketing approval. In addition, manufacturers would be required to submit an application for marketing approval to the FDA before new products

could be brought to market. The New Drug Application (NDA) process had been born.

The new laws were not without flaws, however, as applications for marketing approval would be automatically approved if the FDA did not act within a set period of time. In addition, companies were not required under the new law to demonstrate that their products were effective. These issues would be addressed in later legislative and regulatory proceedings, but for all intents and purposes, the Food, Drug, and Cosmetic Act of 1938 established the framework of the modern drug approval system.[127]

The Thalidomide Story[128]

The commercialization and subsequent withdrawal from market of Thalidomide is perhaps one of the most compelling and tragic events in the history of drug discovery and development. Originally prepared in 1954 by scientists at Chemie Grünenthal GmbH, a German pharmaceutical company, Thalidomide was studied clinically soon after it was patented. By July of 1956, safety studies on animals had demonstrated that it was nearly impossible to achieve a lethal dose of the drug, so it was licensed for sale as an over-the-counter sleep aid in Germany and most of Europe. Use among pregnant woman increased significantly when it was discovered that Thalidomide was also useful as an antiemetic for the suppression of morning sickness, and the drug was marketed under as many as 37 different names worldwide.

Unfortunately, Thalidomide turned out to be far more dangerous than expected. While animal safety studies did indicate a lack of acute toxicity, other safety issues were not studied, especially those related to the effects of a drug on a developing fetus. The prevailing theory on fetal development during the 1950s was that the placenta provided perfect protection to a developing fetus, protecting it from any drugs or toxic material ingested by the mother. As such, few, if any, studies were performed to determine the safety of new drugs during pregnancy. If such testing had been done, Thalidomide would probably have never made it out of the labs at Chemie Grünenthal GmbH. In the absence of such testing, Thalidomide was used by thousands of pregnant women across the globe, but by 1959 questions began to arise about the true safety of the drug. In 1960, reports of peripheral neuropathy after long term usage began to appear in England, although the manufacturer continued to insist that the drug was safe. Frances Oldham Kelsey, an FDA physician assigned to review the Thalidomide New Drug Application, refused to provide marketing approval, however, insisting that additional safety studies were necessary before Thalidomide could be approved in the United States.

By 1961, it had become abundantly clear that Dr. Kelsey's decision to insist on more safety studies was justified. Peripheral neuropathy was just the tip of the iceberg in terms of safety issues. Less than five years after its launch as a safe drug for the treatment of morning sickness, over 10,000 children had been born with severe birth defects linked to the drug. "Thalidomide babies" were often born with misshapen or missing limbs (Figure 2.25). William McBride, an Australian obstetrician, and Widukind Lenz, a German pediatrician, independently suggested the link between Thalidomide and the birth defects that eventually lead to the revocation of marketing rights in most global markets by 1962.

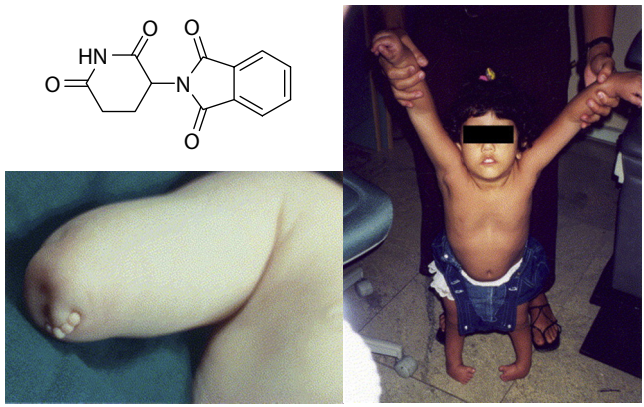

FIGURE 2.25 Thalidomide was brought to market in 1956 by Chemie Grünenthal GmbH as an over the counter treatment for morning sickness in pregnant women. By 1962, it had been withdrawn from markets across the globe as it had been definitively linked to severe birth defect in the children of woman that used it during their pregnancies. *Source: Lower left: Reprinted with permission from Davies, D. P.; Evans, D. J. R. Clinical dysmorphology: understanding congenital abnormalities. Curr. Paediatrics, 13 (4), 288–297, copyright 2003, with permission from Elsevier. Right: Reprinted from Miller, M. T.; Strömland, K.; Ventura, L.; Johansson, M.; Bandim, J. M.; Gillberg, C. Autism associated with conditions characterized by developmental errors in early embryogenesis: a mini review. Int. J. Dev. Neurosci., 23 (2-3), 201–219, copyright 2005, with permission from Elsevier.*

In response to the tragedy that had unfolded, regulatory standards for safety and efficacy testing of new drug candidates were significantly improved with the passage of the Kefauver Harris Amendment of 1962. It is also worth noting that theories on the protection provided by the placenta to an unborn child were substantially revised. The marked increase in birth defects caused by Thalidomide left little doubt that prenatal exposure was a serious safety issue that had to be addressed. The placenta was not the perfect protection it was thought to be. Also, the importance of drug chirality was brought forward on two different levels. First, it was eventually determined that (S)-isomer of Thalidomide is a major groove binder responsible for the teratogenic nature of the drug,

but the (R)-isomer does not share this liability. Prior to this realization, the possibility that single enantiomers might have different biological effects had not been widely considered, and racemic drugs were routinely studied. After the thalidomide tragedy, however, scientists began to focus more heavily on single enantiomers. In the modern era, racemic drug candidates are very rare.

In related findings, an understanding of chiral stability also began to take shape as a result of the tragedy that unfolded around Thalidomide. While it is true that the two enantiomers of Thalidomide have different biological properties, the (R)-isomer still represents a significant hazard for pregnant women. This is the result of the chiral instability of the (R)-isomer in an *in vivo* setting. At physiological pH, the (R)-isomer of Thalidomide undergoes racemization to a mixture of the two isomers (Figure 2.26),

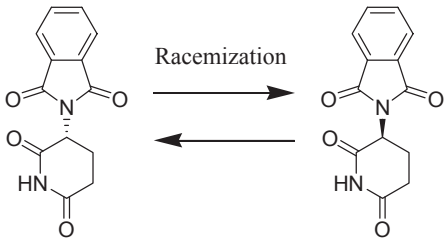

FIGURE 2.26 Thalidomide is not chirally stable *in vivo*. The (R) isomer (left) is readily converted to the (S) isomer (right). As a result, the pure (R) isomer is no safer than the originally marketed racemic material.

so even if a patient is provided only the safer (R)-isomer, the more dangerous (S)-isomer will be generated *in vivo*. In the modern drug discovery process, it is common place to check the chiral stability of possible drug candidates as a result of these findings.

REGULATORY MILESTONES

While major events such as the Thalidomide tragedy made it abundantly clear that new laws needed to be put in place to regulate the growing pharmaceutical industry, many other legislative events occurred in the absence of the substantial attention generated by a drug failure. In fact, over the course of the twentieth century, the growth of the pharmaceuticals industry has been tracked by a parallel growth in regulatory bodies responsible for oversight of the industry. The growth of the regulatory agencies was often a step behind the industry itself, as the laws granting them authority over the industry were most often reactionary in nature (i.e., developed and implemented in response to a perceived problem in the system). Over the course of the twentieth century, however, regulatory bodies such as the FDA, the European Medicines Agency (EMA), and many others across the globe have been created through legislative action with the goal of ensuring the safety of marketed drugs. Although it is well

beyond the scope of this text to describe the historical context and impact of all of the laws governing the drug industry (The FDA as it exists in 2013, for example, is the result of over 200 laws passed since 1906), there are some key milestones that should be examined.

Durham–Humphrey Amendment of 1951[129]

As discussed earlier, the Pure Food and Drug Act of 1906 and the Food, Drug, and Cosmetic Act of 1938 established the authority of the FDA to act in the interest of consumers and protect the populace from dangerous drugs. In fact, the FDA used the authority granted to it in 1906 and 1938 to declare that some drugs were not safe for use in the absence of individualized medical supervision. By 1941, more than 20 drugs and drug classes, including sulfa antibiotics, barbiturates, and amphetamines, required a prescription from either a physician or dentist. Neither of these two laws, however, provided clear definitions for prescription versus nonprescription drugs, and there was no specification as to who was responsible for labeling drugs as belonging to one class or the other. In addition, there were no clear guidelines regarding refilling of prescriptions. This lack of clarity led to a number of legal battles between the FDA, the drug industry, and professional pharmacy organizations over the distribution of prescription drugs.

The Durham–Humphrey Amendment of 1951 addressed this hole in the law by firmly establishing two classes of drugs, those that required a prescription, legend drugs, and those that did not, over-the-counter (OTC) drugs. In short, under this amendment, drugs that have been proven to be safe, effective, and require little, if any, medical oversight in their use (e.g., aspirin) could be sold as OTC products. On the other hand, drugs with addictive properties (e.g., morphine) or that required medical monitoring to ensure safety (such as monitoring of liver functions required with the use of statin cholesterol-lowering drugs) could only be distributed with the consent or under the direction of a physician via prescription. The new law also codified the role of the pharmacist in ensuring that prescription drugs were provided only with a properly documented prescription and the conditions under which refills would be available.

Kefauver–Harris Amendment of 1962[130]

The tragic events that unfolded around the failure of Thalidomide led to substantial public outrage and pressure to enact stricter laws and regulations designed to ensure that public safety and well-being were at the forefront of the drug discovery process. The Kefauver–Harris Amendment of 1962 significantly broadened the FDA's regulatory authority over

the pharmaceuticals industry with this goal in mind. The law required demonstration of both efficacy and safety of potential new drug candidates prior to granting marketing approval, effectively abolishing the automatic approval clause of the 1938 Food, Drug, and Cosmetic Act. The law also required that all drugs launched between 1938 and 1962 had to be proven effective in order to maintain their place in the pharmacy. The National Academy of Sciences and the FDA collaboratively studied this set of drugs, and discovered that nearly 40% were not effective. They were subsequently removed from the list of approved drugs.

Clinical trials, manufacturing processes, and even advertising of prescription drugs were also placed within the jurisdiction of the FDA. Clinical trial design had to be approved by the FDA, informed consent of study participants was required, and known side effects had to be disclosed to the public under the new law. Good manufacturing practices (GMP) and FDA access to company control and production records were also required in order to promote quality assurance. Finally, advertising for prescription drugs was placed under strict regulation. Marketing of generic drugs as new breakthrough medications was barred, and accurate disclosures of efficacy and side effects associated with drug treatment were required in all advertisements for prescription medications. In summary, the Kefauver-Harris Amendment of 1962 gave the FDA virtually complete authority over drug approval and marketing.

Hatch–Waxman Act of 1984[131]

Although the Drug Price Competition and Patent Term Restoration Act of 1984, also known as the Hatch–Waxman Act, did not have a direct impact on the drug discovery process itself, it did fundamentally alter the pharmaceutical landscape. Unlike the previously discussed laws which were focused on safety issues, the Hatch–Waxman Act was designed to encourage the growth of the generic drug industry, thereby decreasing the costs of prescription medication. Prior to the enactment of this legislation, generic drugs represented approximately 10% of the prescription market, despite the fact that many major medicines were no longer under patent protection. By 2008, the market share held by generic drugs had risen to nearly 70% of the market, providing a clear indication that the Hatch–Waxman Act was successfully increasing competition in the prescription drug market. The positive impact on health care costs in the form of cheaper, generic prescription drugs was also viewed as a positive sign to those interested in containing the rising cost of health care.

The success of the Hatch–Waxman Act was driven by a few key changes in the drug approval process and patent laws that simplified market entry for generic drugs. First, the approval process itself was simplified through

the creation of the Abbreviated New Drug Applications (ANDAs). Prior to 1984, companies interested in marketing a generic version of a prescription drug were required to provide the same level of studies as the original manufacturer of the medicine in question, including animal safety studies, bioavailability studies, and human clinical trials. At the same time, generic manufacturer would not have the benefit of patent protection afforded to the "innovator" companies, making it more difficult for generic companies to recoup the substantial investments required to bring a drug to market. Under the provisions of the Hatch–Waxman Act, generic drugs could be approved for marketing based on the "innovator's" clinical and safety data. Bioequivalence studies designed to demonstrate that the generic drug provided the same bioavailability as the marketed equivalent replaced the time-consuming and expensive efficacy and safety trials, significantly lowering the cost of market entry.

Changes to the patent law and market exclusivity rules were also put into place in order to support both the "innovator" manufacturers and generic manufacturers in an effort to provide a balanced playing field for both. A "safe harbor" clause was included in the legislation that allowed generic drug companies to manufacture and study patented drugs as part of an effort to generate data necessary for an ANDA submission. In the absence of this "safe harbor" clause, generic companies could have been sued for patent infringement if their efforts to generate a generic copy of a drug occurred during the lifetime of a patent. This change coupled with the law's allowance for lawsuits by generic companies seeking to invalidate drug patents, created significant openings in the prescription drug market that have been exploited by generic drug companies. Those interested in the details of generic market entry based on lawsuits to invalidate drug patent are encourage to consult paragraph four of 35 U.S.C. 271 for additional information.

Measures to protect the "innovator" companies were also put into place, as it had become widely realized that a significant portion of a drug's patent life was being consumed by clinical trials and the FDA approval process. These processes were dramatically shortening the useful patent life of potential new drugs, thus increasing their overall cost. The ANDA provisions of the Hatch–Waxman Act threatened to further erode the useful patent life of new therapeutic agents if enacted alone. In recognition of this possible negative outcome for "innovator" companies, provisions were put in place for the extension of patent terms to compensate for time lost in both clinical trials and the approval process. In general, the length of patent protection for any drug was increased by 50% of the time spent in clinical trials and 100% of the time spent in the NDA approval process. The law also created a new class of drugs, those directed towards "orphan indications" or diseases with fewer than 200,000 patients. Companies

that provide new therapies for orphan indications were granted market exclusivity for 7 years, irrespective of the patent status. This provision was designed to provide an incentive for targeting rare and neglected diseases.

Overall, the laws created under the Hatch–Waxman Act of 1984 were generally regarded as successful, although there continues to be both ongoing litigation and debate regarding when and how generic drugs may be brought to market. "Innovator" companies and generic drug manufacturers will likely continue to battle each other over the rights to either maintain or eliminate patent protection for new drugs in an attempt to maintain their profit margins, much to the delight of patent lawyers around the world.

Biologics Price Competition and Innovation Act of 2009[132]

When the Hatch–Waxman Act was being considered and eventually passed as a law, the biotechnological revolution had only just begun. Politicians concerned with the high cost of medication and health care were not aware of and did not account for the complexities of antibody therapeutics, recombinant proteins, or other macromolecular therapeutics. Their main concern at the time was the price of small molecule therapeutics and the creation of a more robust generic drug market that would lower overall health care costs. As such, generic biologics were not covered in the Hatch–Waxman Act, leaving generic drug companies without a regulatory pathway to gain approval of a generic equivalent of a macromolecular therapeutic. The high price of biologics, however, made it clear very quickly that this oversight needed to be addressed. A single year's treatment with Herceptin, an antibody used in the treatment of breast cancer, for example, can cost over $70,000.00.[133]

The substantial differences between small molecule and macromolecular therapeutics made it impractical to simply apply the rules for one to the other. While it would be a relatively simple matter, for example, to ensure that the identity of a small molecule is the same in a "branded" version versus the generic version, the same cannot be said of macromolecules. Under the guidelines for small molecules, a "generic" antibody possessing a 99.9% overlap in structure with the "branded" version, sharing the same function and mechanism of action of the original, and with the same safety features would not be allowed on the market under the provisions of the Hatch–Waxman Act. The Biologics Price Competition and Innovation Act of 2009 addresses these, as well as other issues that prevented generic drug manufacturers from marketing cheaper version of biological medicines. New rules set forth in this law removed the requirement that generic macromolecules had to be identical with their branded counterpart. Biosimilarity replaced the identity requirement with a "highly

similar" requirement, which dictated that generic macromolecules could have minor differences in the inactive components, so long as there were no clinically meaningful differences between the "branded" and "generic" versions in terms of safety, purity, and potency. Clinically, the "branded" and "generic" versions were also required to be interchangeable clinically without the aid of a health care provider and with no added safety risk upon switching between the two.

This legislation and similar laws in place across the globe will likely have a major impact on the pharmaceuticals industry. Most of the major pharmaceutical companies have shifted significant resources away from small molecules and into biologics in an attempt to maintain the high profit margins expected from drug companies, but generic companies are also entering the market. In July of 2010, the FDA approved its first generic biologic drug, a biosimilar to Lovenox® (enoxaparin sodium), the blockbuster blood-thinning drug originally brought to market by Sanofi-Aventis. It is likely additional biosimilars will be brought to market as the patent estates created in the biotech revolution come to the end of their enforcement period.

FUTURE DEVELOPMENTS IN DRUG DISCOVERY

The drug discovery process has changed significantly since the first experiments of Paul Ehrlich launched the science at the beginning of the twentieth century. Growth of the field has marched forward with advances in scientific understanding in the areas of biology, pharmacology, chemistry, and computer sciences, and it is likely that this will continue. The pace of innovation will likely quicken over time, as the technological tools currently in place allow for a far more rapid acquisition of scientific data than ever before. Where this will lead, however, is somewhat unpredictable, as the number of unanswered questions in the field of drug discovery is enormous. There is, however, some degree of certainty that the regulatory aspects of drug discovery will continue to grow with the field, although slightly out of phase with the field itself. It is, after all, difficult to create regulatory guidelines for new therapies that have as yet to be discovered.

QUESTIONS

1. Paul Ehrlich is known as the father of modern drug discovery. Between 1872 and 1874 he noted the selective affinity of dyes Trypan red, Trypan blue, and Methylene blue for biological tissues. What hypothesis did he develop based on these observations?
2. What is the significance of the Wistar rat?
3. What are SCID mice, why are they important, and how are they different from nude mice?

4. In 1974, Rudolf Jaenisch produced the first transgenic mouse. What is a transgenic animal model?
5. What is a knockout animal model?
6. Define high throughput chemistry.
7. What is recombinant DNA?
8. What technology enables the transfer of genetic material and can be used to prepare over-expression cell lines?
9. What is the general process of polymerase chain reaction (PCR) technology and why was the discovery of Taq polymerase important to the advancement of this technology?
10. Hybridoma technology was introduced by C Milstein and G. J. F. Köhler in 1975. What two cell types are fused to form a hybridoma cell line and what do the resulting cell lines produce?
11. What is a receptor construct/fusion protein and what is the function of the two components of the same?
12. What was the Elixir sulfanilamide disaster of 1937? This event led to the passage of the Food, Drug, and Cosmetic Act in 1938. What new requirements were put in place for the pharmaceutical industry?
13. First launched in 1957 as a treatment for birth defects, thalidomide was removed from the market in 1961 after 10,000 children were born with birth defects. Provide two key learnings from this event.
14. The Kefauver–Harris Amendment of 1962 placed additional requirements on new drugs entering the market. Describe them.

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