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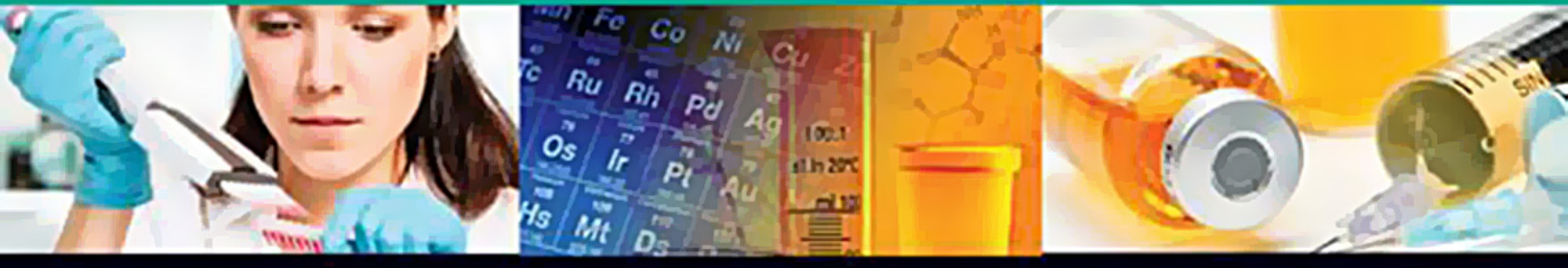
THE PRACTICE OF MEDICINAL CHEMISTRY



FOURTH EDITION

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THE PRACTICE OF MEDICINAL CHEMISTRY

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Foreword

The world's economy depends to a significant extent on our ability to deliver affordable and sustainable healthcare. As such, this new edition of *The Practice of Medicinal Chemistry* plays an important role in educating the next generation of scientists in the area as it goes beyond the simple delivery of new healing drugs to combat disease and illness. It enriches our knowledge and the very understanding of the lives of everyone on the planet. It is truly amazing how the different scientific disciplines can combine in this way to design and make such a wonderful array of functional molecules to serve some of our current needs. Nevertheless, the future presents enormous healthcare challenges that can only be met by appropriate investment and further fundamental scientific discovery. This new edition of *The Practice of Medicinal Chemistry* provides a unique scholarly compilation of the tools, techniques, and methods necessary to begin this journey of discovery, whether in industry or academia.

The book's practical overview differentiates this text from others. It provides a menu of topics that can be consulted individually, while also providing a holistic view covering the history of drug discovery through to the issues of today involving the consumption and production of pharmaceuticals. The process of drug discovery has become a highly complex operation requiring the medicinal chemist to acquire wide-ranging skills from areas such as biology, technology, modelling, delivery, physiochemistry, and synthesis. To pull this together in a single book is a heroic task that these authors have done magnificently.

As our science moves forward toward more biologicals, smaller volume products that are focused on patients, and more sustainable and flexible manufacturing in an ever more regulated environment, we will require new generations of creative individuals. They will need to be ever more innovative, using all the tools our modern society can offer. In particular, big-data mining, tissue sampling and genomic mapping, the "Internet of Things," and wearable health monitors will all be likely components in the armory of the next breed of medicinal chemist.

I regard this expanded textbook as essential reading for all those new to the field. It also provides a quality check for current practitioners in this rapidly evolving environment. The book also does not shy away from providing a future vision of the trends of the discipline. It is written by experts who elegantly convey their passion, experience, and insight for the benefit of all readers.

I therefore welcome this updated and expanded version of *The Practice of Medicinal Chemistry* and believe it provides—as did past editions—the bedrock of our discipline.

Steven V. Ley
Cambridge
http://www.leygroup.ch.cam.ac.uk/

Preface to the Fourth Edition

Bringing a new drug to patients is both a privilege and a challenge fraught with success and failure. A privilege because there can be no greater calling than to alleviate suffering to enable a healthier life.

> Without health, life is not life; it is only a state of languor and suffering—an image of death. ***Buddha***

A challenge fraught with success and failure as no drug makes it from idea to patients without experiencing success and failure.

> Success is not final; failure is not fatal. It is the courage to continue that counts. ***Winston Churchill***

Prof. Camille Wermuth recognized the need to capture in a single volume the essence of the disciplines needed by medicinal chemists, so as to enable those just entering the field or the seasoned professional to keep pace with the ever-changing nature of drug discovery and development. His vision became *The Practice of Medicinal Chemistry*, providing the medicinal chemistry community with access to experts from across the industry and academia who would share their knowledge to educate the community, thereby preparing the community to recognize and seize opportunities as they emerged.

> Fortune favors the prepared mind. ***Louis Pasteur***

The fourth edition has built off the previous editions. It is updated to reflect developments over the last seven years, including five new chapters on topics such as the evaluation of the biological activity of compounds and systems biology. More than seventy experts from ten countries have shared their insights and perspectives on the practice of medicinal chemistry.

The editorial work for the fourth edition has been shared by Camille Wermuth, Pierre Raboisson, Didier Rognan, and Dave Aldous. Odile Blin helped organize and shape how we initiated the fourth edition; we are indebted to her tireless professionalism. The editors wish to express their thanks to Molly M. McLaughlin and the Elsevier Academic Press, who have worked with us to keep this project moving forward.

I believe my final quote—from Jason Calacanis—captures the challenges medicinal chemists face every day.

> You have to have a big vision and take very small steps to get there. You have to be humble as you execute but visionary and gigantic in terms of your aspiration. In the Internet industry, it's not about grand innovation; it's about a lot of little innovations—every day, every week, every month—making something a little bit better. ***Jason Calacanis***

Medicinal chemistry is a highly collaborative and iterative process that has many paths. Being open, collaborative, and humble are qualities that will help you successfully navigate these paths from idea to patient.

Preface to the Third Edition

Like the preceding editions of this book, this third edition treats of the essential elements of medicinal chemistry in a unique volume. It provides a practical overview of the daily problems facing medicinal chemists, from the conception of new molecules through to the production of new drugs and their legal/economic implications. This edition has been updated, expanded and refocused to reflect developments in the past 5 years, including 11 new chapters on topics such as hit identification methodologies and cheminformatics. More than 50 experts in the field from eight different countries, who have benefited from years of practical experience, give personal accounts of both traditional methodologies and the newest discovery and development technologies, providing readers with an insight into medicinal chemistry.

A major change in comparison to the previous editions was the decision to alleviate my editorial burden in sharing it with seven section editors, each being responsible for one of the eight sections of the book. I highly appreciated their positive and efficacious collaboration and express them my warmest thanks (in the alphabetical order) to Michael Bowker, Hugo Kubinyi, John Proudfood, Bryan Reuben, Richard Silverman, David Triggle and Han van de Waterbeemd.

Another change was the decision taken by Elsevier/Academic Press to publish the book in full colors thus rendering it more pleasant and user-friendly. I take this occasion to thank Keri Witman, Pat Gonzales, Kirsten Funk and Renske van Dijk for having successively ensured the editorial development of the book. Taking into account that we had to work with a cohort of about 50 authors, each of them having his personality, his original approach and his main busy professional live, this was not an easy task. I am deeply indebted to my assistant Odile Blin for the way she had mastered, efficiently and with friendliness, all the secretarial work and particularly the contacts with the different authors and with the Elsevier development editors. As for the earlier editions, I also want to express my gratitude to my wife Renée and my daughters Delphine, Joëlle and Séverine for all their encouragements and for sacrificing many hours of family life in order to leave me enough free time to edit this new version of the "Medicinal Chemist's Bible."

My final thoughts go to the future readers of the book, and especially to the newcomers in Medicinal Chemistry having the curiosity to read the preface. I cannot resist giving them some advice for doing good science.

First of all, be open-minded and original. As Schopenhauer noted, the task of the creative mind is "not so much to see what no one has seen yet; but to think what nobody has thought yet, about what everyone sees." A wonderful illustration is found in Peter Hesse's cartoon below.

Second, always keep in mind that the object of Medicinal Chemistry is to synthetize new drugs useful for suffering patients. Like many scientists, medicinal chemists, have to navigate between two tempting reefs. On one side they should avoid doing "NAAR": non-applicable applied research, on the other side they may be attracted by "NFBR": non-fundamental basic search."

Third, convinced as they may be that the neighbors grass is always greener, they may be attracted to start their research in using as a hit a recently published competitor's product. In fact, the published compound may exhibit only a weak activity, therefore be very careful when starting a new program and never forget that the worst thing a medicinal chemist can do is to prepare a me-too of an inactive compound!

Camille G. Wermuth

Preface to the Second Edition

Like the first edition of *The Practice of Medicinal Chemistry* (nicknamed 'The Bible' by medicinal chemists) the second edition is intended primarily for organic chemists beginning a career in drug research. Furthermore, it is a valuable reference source for academic, as well as industrial, medicinal chemists. The general philosophy of the book is to complete the biological progress − Intellectualization at the level of function using the chemical progress Intellectualization at the level of structure (Professor Samuel J. Danishevsky, *Studies in the chemistry and biology of the epothilones and eleutherobins*, Conference given at the XXXIVémes Rencontres Internationales de Chimie Th6rapeutique, Facult6 de Pharmacie, Nantes, 8−10 July, 1998).

The recent results from genomic research have allowed for the identification of a great number of new targets, corresponding to hitherto unknown receptors or to new subtypes of already existing receptors. The massive use of combinatorial chemistry, associated with high throughput screening technologies, has identified thousands of hits for these targets. The present challenge is to develop these hits into usable and useful drug candidates. This book is, therefore, particularly timely as it covers abundantly the subject of drug optimization.

The new edition of the book has been updated, expanded and refocused to reflect developments over the nine years since the first edition was published. Experts in the field have provided personal accounts of both traditional methodologies, and the newest discovery and development technologies, giving us an insight into diverse aspects of medicinal chemistry, usually only gained from years of practical experience.

Like the previous edition, this edition includes a concise introduction covering the definition and history of medicinal chemistry, the measurement of biological activities and the three main phases of drug activity. This is followed by detailed discussions on the discovery of new lead compounds including automated, high throughput screening techniques, combinatorial chemistry and the use of the internet, all of which serve to reduce preclinical development times and, thus, the cost of drugs. Further chapters discuss the optimization of lead compounds in terms of potency, selectivity, and safety; the contribution of genomics; molecular biology and X-ray crystallization to drug discovery and development, including the design of peptidomimetic drugs; and the development of drug-delivery systems, including organ targeting and the preparation of pharmaceutically acceptable salts. The final section covers legal and economic aspects of drug discovery and production, including drug sources, good manufacturing practices, drug nomenclature, patent protection, social-economic implications and the future of the pharmaceutical industry.

I am deeply indebted to all co-authors for their cooperation, for the time they spent writing their respective contributions and for their patience during the editing process. I am very grateful to Didier Rognan, Paola Ciapetti, Bruno Giethlen, Annie Marcincal, Marie-Louise Jung, Jean-Marie Contreras and Patrick Bazzini for their helpful comments.

My thanks go also to the editorial staff of *Academic Press* in London, particularly to Margaret Macdonald and Jacqueline Read. Last but not least, I want to express my gratitude to my wife Renée for all her encouragements and for her comprehensiveness.

Camille G. Wermuth

Preface to the First Edition

The role of chemistry in the manufacture of new drugs, and also of cosmetics and agrochemicals, is essential. It is doubtful, however, whether chemists have been properly trained to design and synthesize new drugs or other bioactive compounds. The majority of medicinal chemists working in the pharmaceutical industry are organic synthetic chemists with little or no background in medicinal chemistry who have to acquire the specific aspects of medicinal chemistry during their early years in the pharmaceutical industry. This book is precisely aimed to be their 'bedside book' at the beginning of their career.

After a concise introduction covering background subject matter, such as the definition and history of medicinal chemistry, the measurement of biological activities and the three main phases of drug activity, the second part of the book discusses the most appropriate approach to *finding a new lead compound or an original working hypothesis*. This most uncertain stage in the development of a new drug is nowadays characterized by high-throughput screening methods, synthesis of combinatorial libraries, data base mining and a return to natural product screening. The core of the book (Parts III to V) considers the *optimization of the lead in terms of potency, selectivity, and safety*. In 'Primary Exploration of Structure-Activity Relationships', the most common operational stratagems are discussed, allowing identification of the portions of the molecule that are important for potency. 'Substituents and functions' deals with the rapid and systematic optimization of the lead compound. 'Spatial Organization, Receptor Mapping and Molecular Modelling' considers the three-dimensional aspects of drug-receptor interactions, giving particular emphasis to the design of peptidomimetic drugs and to the control of the agonist- antagonist transition. Parts VI and VII concentrate on the definition of satisfactory drug-delivery conditions, i.e. means to ensure that the molecule reaches its target organ. Pharmacokinetic properties are improved through adequate chemical modifications, notably prodrug design, obtaining suitable water solubility (of utmost importance in medical practice) and improving organoleptic properties (and thus rendering the drug administration acceptable to the patient). Part VIII, 'Development of New Drugs: Legal and Economic Aspects', constitutes an important area in which chemists are almost wholly self taught following their entry into industry.

This book fills a gap in the available bibliography of medicinal chemistry texts. There is not, to the author-editor's knowledge, any other current work in print which deals with the practical aspects of medicinal chemistry, from conception of molecules to their marketing. In this single volume, all the disparate bits of information which medicinal chemists gather over a career, and generally share by word-of-mouth with their colleagues, but which have never been organized and presented in coherent form in print, are brought together. Traditional approaches are not neglected and are illustrated by modern examples and, conversely, the most recent discovery and development technologies are presented and discussed by specialists. Therefore, *The Practice of Medicinal Chemistry* is exactly the type of book to be recommended as a text or as first reading to a synthetic chemist beginning a career in medicinal chemistry. And, even if primarily aimed at organic chemists entering into pharmaceutical research, all medicinal chemists will derive a great deal from reading the book.

The involvement of a large number of authors presents the risk of a certain lack of cohesiveness and of some overlaps, especially as each chapter is written as an autonomic piece of information. Such a situation was anticipated and accepted, especially for a first edition. It can be defended because each contributor is an expert in his/her field and many of them are 'heavyweights' in medicinal chemistry. In editing the book I have tried to ensure a balanced content and a more-or-less consistent style. However, the temptation to influence the personal views of the authors has been resisted. On the contrary, my objective was to combine a plurality of opinions, and to present and discuss a given topic from different angles. Such as it is, this first edition can still be improved and I am grateful in advance to all colleagues for comments and suggestions for future editions.

Special care has been taken to give complete references and, in general, each compound described has been identified by at least one reference. *For compounds for which no specific literature indication is given, the reader is referred to the Merck Index*.

The cover picture of the book is a reproduction of a copperplate engraving designed for me by the late Charles Gutknecht, who was my secondary school chemistry teacher in Mulhouse. It represents an extract of Brueghel's engraving *The alchemist ruining his family in pursuing his chimera*, surmounted by the aquarius symbol. Represented on the left-hand side is my lucky charm caster oil plant (*Ricinus communis L., Euphorbiaceae*), which was the starting point of the pyridazine chemistry in my laboratory. The historical cascade of events was as follows: cracking of caster oil produces n-heptanal and aldolization of n-heptanal – and, more generally, of any enolisable aldehyde or ketone – with pyruvic acid leads to a-hydroxy-γ-ketonic acids. Finally, the condensation of these keto acids with hydrazine yields pyrodazones. Thus, all our present research on pyridazine derivatives originates from my schoolboy chemistry, when I prepared in my home in Mulhouse n-heptanal and undecylenic acid by cracking caster oil!

Preparing this book was a collective adventure and I am most grateful to all authors for their cooperation and for the time and the effort they spent to write their respective contributions. I appreciate also their patience, especially as the editing process took much more time than initially expected.

I am very grateful to Brad Anderson (University of Utah, Salt Lake city), Jean-Jacques André (Marion Merrell Dow, Strasbourg), Richard Baker (Eli Lilly, Erl Wood, UK), Thomas C. Jones (Sandoz, Basle), Isabelle Morin (Servier, Paris), Bryan Reuben (London South Bank University) and John Topliss (University of Michigan, Ann Arbor) for their invaluable assistance, comments and contributions.

My thanks go also to the editorial staff of Academic Press in London, Particularly to Susan Lord, Nicola Linton and Fran Kingston, to the two copy editors Len Cegielka and Peter Cross, and finally, to the two secretaries of our laboratory, Franqois Herth and Marylse Wernert.

Last but not least, I want to thank my wife Renée for all her encouragement and for sacrificing evenings an Saturday family life over the past year and a half, to allow me to sit before my computer for about 2500 hours!

Camille G. Wermuth

SECTION ONE

General Aspects of Medicinal Chemistry

CHAPTER

1

Medicinal Chemistry: Definitions and Objectives, Drug Activity Phases, Drug Classification Systems

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OUTLINE

Medicinal chemistry remains a challenging science which provides profound satisfaction to its practitioners. It intrigues those of us who like to solve problems posed by nature. It verges increasingly on biochemistry and on all the physical, genetic and chemical riddles in animal physiology which bear on medicine. Medicinal chemists have a chance to participate in the fundamentals of prevention, therapy and understanding of diseases and thereby to contribute to a healthier and happier life. **A Burger** [1]

I. DEFINITIONS AND OBJECTIVES

A. Medicinal Chemistry and Related Disciplines and Terms

A definition of medicinal chemistry was given by a IUPAC specialized commission: "*Medicinal chemistry* concerns the discovery, the development, the identification and the interpretation of the mode of action of biologically active compounds at the molecular level. Emphasis is put on drugs, but the interests of the medicinal chemist are not restricted to drugs but include bioactive compounds in general. Medicinal chemistry is also concerned with the study, identification, and synthesis of the metabolic products of these drugs and related compounds" [2].

Drugs—natural and synthetic alike—are chemicals used for medicinal purposes. They interact with complex chemical systems of humans or animals. Medicinal chemistry is concerned with this interaction, focusing on the organic and biochemical reactions of drug substances with their targets. This is one aspect of drug chemistry.

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Other important aspects are the synthesis and the analysis of drug substances. The two latter aspects together are sometimes called *pharmaceutical chemistry*, but the synthesis of drugs is considered by some people—mainly chemists—to be part of medicinal chemistry, denoting analytical aspects as pharmaceutical chemistry. In German faculties of pharmacy, the literal translations of pharmaceutical and medicinal chemistry—Pharmazeutische and Medizinische Chemie—are used synonymously.

The general study of drugs is called *pharmacy* or *pharmacology*. A common narrower definition of pharmacology concentrates on the fate and effects of a drug in the body. *Clinical chemistry*, a different subject, is concerned with the determination of physiological and pathophysiological parameters in body fluids, such as enzyme activities and metabolites in blood and urine. The term *biopharmacy* has been reserved for the investigation and control of absorption, distribution, metabolism, excretion, and toxicology (ADMET) of drug substances.

Some further terms are more or less synonymous with medicinal chemistry: *(molecular) pharmacochemistry, drug design, selective toxicity*. The French equivalent to medicinal chemistry is chimie thérapeutique, and the German terms are Medizinische/Pharmazeutische Chemie and Arzneimittelforschung.

In academia, medicinal chemistry is a major subject in most pharmacy faculties—both for undergraduates and in research—and in many chemistry faculties. In the pharmaceutical industry, medicinal chemistry is at the heart of the search for new medicines.

The main activities of medicinal chemists are evident in the analysis of their most important scientific journals (e.g., *Journal of Medicinal Chemistry, European Journal of Medicinal Chemistry, Bioorganic and Medicinal Chemistry, ChemMedChem, Archiv der Pharmazie, Arzneimittelforschung, Chemical and Pharmaceutical Bulletin*).

The *objectives of medicinal chemistry* are as easily formulated as they are difficult to achieve: find, develop, and improve drug substances that cure or alleviate diseases (see below, Section I.C.) and understand the causative and accompanying chemical processes (see below, Section III.A).

Medicinal chemistry is an interdisciplinary science covering a particularly wide domain situated at the interface of organic chemistry with life sciences such as biochemistry, pharmacology, molecular biology, genetics, immunology, pharmacokinetics, and toxicology on one side, and chemistry-based disciplines such as physical chemistry, crystallography, spectroscopy, and computer-based techniques of simulation, data analysis, and data visualization on the other side.

B. Drugs and Drug Substances

Drugs are composed of *drug substances* (syn. *active pharmaceutical ingredients, APIs*) and *excipients* (syn. *ancillary substances*). The combination of both is the work of pharmaceutical technology (syn. *galenics*) and denoted a *formulation*.

In 2014, the World Drug Index contained over 80,000 marketed and development drug substances [3]. The United States *Orange Book* listed approx. 3,500 products in 2014, and the United States Pharmacopeia contains monographs of approx. 1,400 small-molecules Active Pharmaceutical Ingredients (APIs) and 160 biologic drug substances [4]. In 2013 in Germany, the "Rote Liste" contained approximately 6,000 drugs in 7,500 formulations representing approximately 2,000 APIs [5]. The WHO Essential Medicines List held approximately 350 drug substances in 2013 that WHO claims sufficient for the treatment of approx. 90 percent of all diseases where drugs are useful [6].

What makes a chemical "drug-like?" Because of the versatility of their molecular targets (see below), there can be no universal characteristic of drug substances. However, since the general structure of the target organisms is identical, generalizations as to drug substance structure are possible for biopharmacy [7,8]. For a chemical to be readily absorbed by the gut and distributed in the body, its size, hydrophilicity/lipophilicity ratio, stability toward acid media and hydrolytical enzymes, etc. have to meet defined physicochemical criteria. A careful analysis of reasons for drug attrition revealed that only 5 percent were caused by pharmacokinetic difficulties, whereas 46 percent were due to insufficient efficacy and 33 percent to adverse reactions in animals or humans [9]. Since both wanted and unwanted effects are due to the biological activity, 79 percent of drug candidates had unpredicted or wrongly predicted sum activities.

Predictions of toxicity from molecular features are still precarious [10–12]. Only rather general rules are for sure; such as avoidance of very reactive functional groups, for example, aldehyde because of oxidative instability and haptene nature; α,β-unsaturated carbonyl compounds and 2-halopyridines because of their unspecific reactivity as electrophiles. Torcetrapib is a typical example of toxicity—or adverse effects—challenges. It was an antiatherosclerotic drug candidate promising to become a blockbuster when in latter phase III of clinical trials, an increased risk of mortality led the company to discontinue its development. It was not clear whether the effects

were caused by the mechanism of action—inhibition of cholesteryl ester transfer protein—some other effect or an interaction with another drug. This is just one instance that "it isn't that simple [and] nothing's obvious and nothing's for certain" in rational drug development [13].

C. Stages of Drug Development

Most drugs were discovered rather than developed [14]. That is why a large number of drug substances are natural products or derivatives thereof. It is a matter of debate if ethnic medicines or nature still hold gems as yet undiscovered by pharmacy [15,16]. Synthetic substance collections ("libraries") have been created through (automated) organic chemistry. The very high number and diversity of natural and synthetic chemical entities is faced with an equally growing number of potential reaction partners (targets) from biochemical and pathophysiological research.

In virtual, biochemical and cell-based testing, compound selections are run against an isolated or physiologically embedded target that may be involved in the disease process [17]. Compounds that exceed a certain threshold value in binding to the target or modulation of some functional signal behind it, are called *hits*. If the identity and purity of the compound and the assay result are confirmed in a multipoint activity determination, the compound rises to the status of *validated hit*. From this one hopes to develop leads. A *lead* is a compound or series of compounds with proven activity and selectivity in a screen and fulfills some drug development criteria such as originality, patentability, and accessibility (by extraction or synthesis). Molecular variation hopefully tunes the physicochemical parameters so that it becomes suitable for ADME. An example of a small optimization algorithm is shown in Figure 1.1.

If the resulting *optimized lead* (*preclinical candidate*) displays no toxicity in cell and animal models, it becomes a *clinical candidate*. If this stands the tests of efficacy and safety in humans and overcomes marketing hurdles, a *new drug entity* will enter the treasure trove of pharmacy. Box 1.1 illustrates that activity is a necessary but not sufficient quality of medicines. There is, of course, no ideal drug in the real world, but one has to find a relative optimum. This often means developing a drug that has a different side-effect profile than drugs marketed for the same therapeutic indication so prescriptions can be tailored to the ways different patients react to a drug.

The role of medicinal chemistry is most prominent in steps one and two of drug development:

1. The discovery step, consisting of the choice of the therapeutic target (biochemical, cellular, or *in vivo* model; see below) and the identification or discovery and production of new active substances interacting with the selected target.
2. The optimization step that deals with the improvement of an active compound. The optimization process primarily takes into account the increase in potency, selectivity, and decrease in toxicity. Its characteristics are the establishment of structure–activity relationships, ideally based on an understanding of the molecular mode of action.

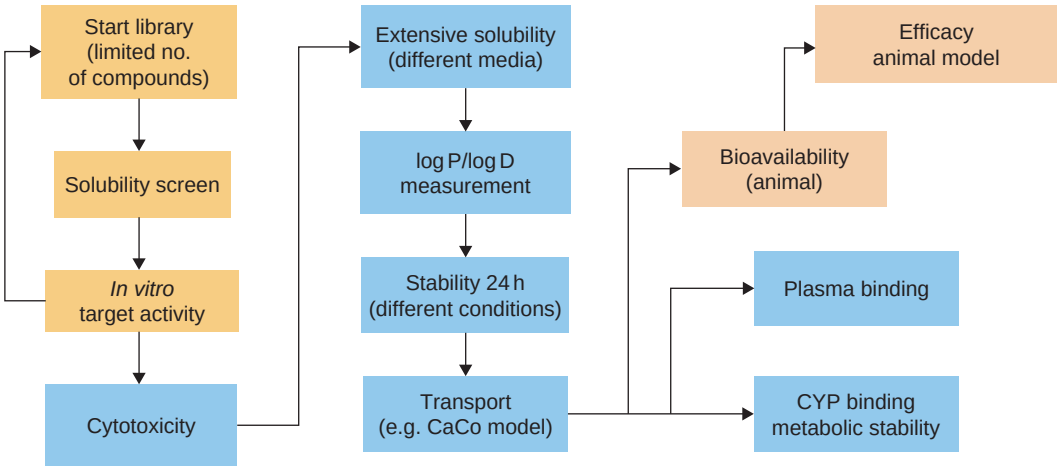

FIGURE 1.1 Example of an optimization algorithm. *Source: Adapted from a presentation by Dr. U. Heiser, Probiodrug AG, Halle, Germany, reproduced with permission.*

3. The formulation step, whose purpose is the continuation of the improvement of the pharmacokinetic properties and the fine-tuning of the pharmaceutic properties of active substances to render them suitable for clinical use. This can consist—to name just a few instances—of the preparation of better absorbed compounds, of sustained release formulations, and of water-soluble derivatives or in the elimination of properties related to the patient's compliance (irritation, painful injection, undesirable organoleptic properties). For an example, see Figure 1.2.

The main tasks of medicinal chemistry consist of the optimization of the following characteristics:

a. Higher affinity and target-intrinsic activation for better clinical activity so the dosage and nonspecific side effects will be as low as possible. There are no examples of drugs that are dosed below 10 mg/day that cause idiosyncratic adverse drug reactions. For drug substances that have to be given in higher doses—i.e., the majority—medicinal chemistry tries to find active derivatives that will be metabolized in a safe way [18]. This includes assaying for inhibition of or reaction with key enzymes of biotransformation, such as oxidases of the cytochrome type, some of which are highly demanded by food constituents and xenobiotics including drug substances [19]. Medicinal chemistry tries to prepare drugs that are not metabolized by bottleneck enzymic pathways [20].

b. Better selectivity, which may lead to a reduction of unwanted side effects. This sometimes entails the assaying of a very high number of other targets; for example, an antidepressive serotonin re-uptake inhibitor has to be tested against all subtypes of serotonin, adrenaline, and dopamine receptors, plus many other key receptors and enzymes.

BOX 1.1

THE IDEAL NEW DRUG SUBSTANCE

- New chemical entity for patentability and registration.
- Maximum four-step synthesis with, for example, no heavy metal catalysts and no environmentally problematic waste; no chromatographic purification steps; purity>99 percent.
- Stable up to 70°C even in humid air and light.
- Solid-state properties (crystalline, not polymorphous, not hygroscopic) that make it a perfect partner for (tablet) compaction.
- Solubility in water sufficient for the production of stable blood-isotonic solutions.
- Oral bioavailability .90 percent with no interindividual variation.
- Very high activity and pharmacokinetic profile enable once-a-day-dosage at 5–10 mg.

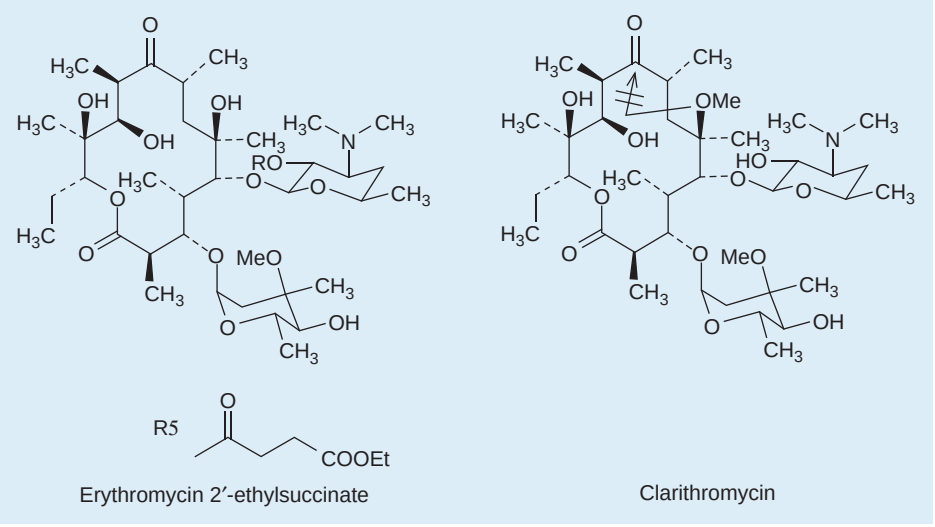

FIGURE 1.2 An example of fine-tuning of pharmacologically active chemicals: Erythromycin 29-ethylsuccinate and clarithromycin are semisynthetic derivatives of the macrolide antiinfective erythromycin. The small molecular change in the former leads to the elimination of bitterness which is important as this class of drugs is often used in pediatrics and administered as a syrup. In the latter, because hemiketal formation is no longer possible (arrow), clarithromycin is stable in the acidic milieu of the stomach (pH 2).

In spite of the high number of compounds, targets, and assays, the development pipeline of new chemical entities as drug substances has not got fuller in the past 20 years. For possible explanations, see the discussion of drug targets below and Ref. [9].

II. DRUG ACTIVITY PHASES

The progression of a drug into the body, to its target(s), and out again can be broken down into three mechanistically distinct phases, the second and third being partly simultaneous. During drug development, all three phases are investigated interdependently, because structural changes required for one phase must not abolish suitability in another phase.

A. The Pharmaceutical Phase

Drug substances are applied orally (preferred mode) or parenterally (e.g., by subcutaneous or intravenous injection, rectally, or through inhalation). A combination of the skills of medicinal chemists and pharmaceutical technologists has to provide the drug candidate in suitable formulations. For tablets, the drug substance needs to be crystalline and not have a low melting point. For injections, it should be water soluble (e.g., as a salt). The required structural features must be compatible with the pharmacological activity, of course.

B. The Pharmacokinetic Phase

For this phase, medicinal chemists and biopharmacists work together to design a compound that will have suitable ADME parameters. Sufficient solubility in an aqueous medium for absorption and blood transport has to be combined with sufficient lipophilicity for passage through cell membranes. If an active compound is too hydrophilic and at the same time contains a carboxylic acid group, for instance, conversion to a simple ester will facilitate absorption. Once in the blood, unspecific esterases will catalyze hydrolysis to the active carboxylic acid form. Such an ester is an instance of a *prodrug*.

Drug substances should remain active and in the body for a period of time that is neither too short nor too long. For many drugs, a metabolic and/or excretion rate that enables "once a day" dosage is sought. Sometimes this requires the identification of sites in the molecule that will be metabolized quickly with concomitant loss of activity. The vasodilator iloprost, for instance, was developed from the endogeneous mediator prostacyclin that has very short half-life both *in vivo* and on the shelf. Modification of several chemically and metabolically vulnerable positions yielded a stable and active derivative—a highly sophisticated product of synthetic medicinal chemistry (Figure 1.3) [21]. By contrast, sometimes functionality is introduced for the acceleration of biotransformation and excretion. Articaine is a local anesthetic of the anilide type. Systemically, it interferes with heart rate—an unwelcome side effect in dentistry. That is why articaine contains an additional ester group. Once in the blood stream, this will be hydrolyzed quickly to an inactive carboxylic acid (Figure 1.4) [22]. Medicinal chemistry here has come full circle, as anilide local anesthetics were developed from ester anesthetics like procain in order to prolong activity.

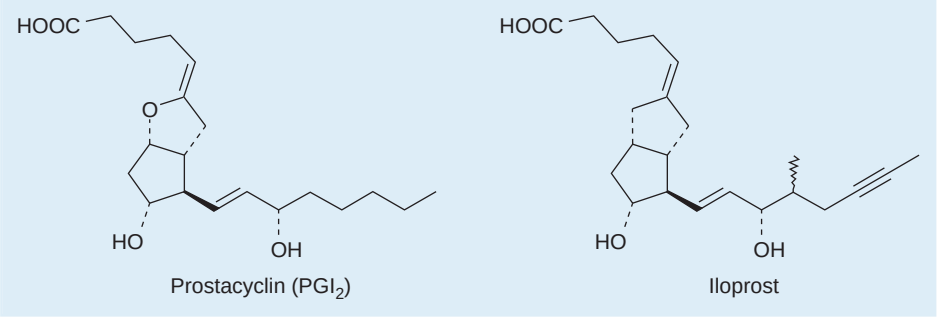

FIGURE 1.3 Prostacyclin and its synthetic analog, iloprost, that combines activity with sufficient *ex vivo* and *in vivo* stability.

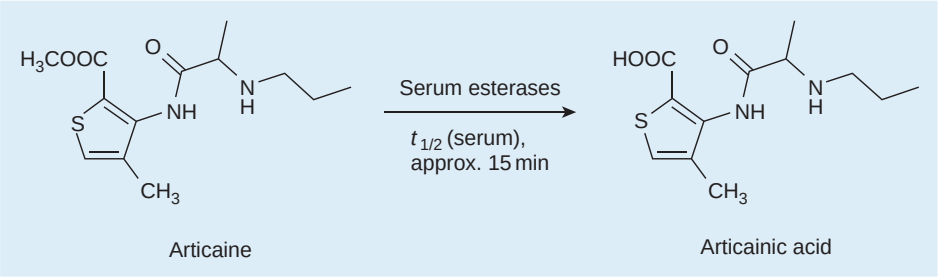

FIGURE 1.4 Articaine, a common local anesthetic dentists use, and its inactive metabolite that is formed off the scene of painful action. The value for $t_{1/2}$ is from the reference Oertel R, Ebert U, Rahn R, Kirch W. The effect of age on pharmacokinetics of the local anesthetic drug articaine. Reg Anesth Pain Med 1999;24:524–8.

C. The Pharmacodynamic Phase

While pharmacokinetics investigates what the body does to the drug, pharmacodynamics is concerned with what the drug does to the body. Most scientists who consider themselves medicinal chemists will be most comfortable with and interested in this phase. They will cooperate with biochemists and pharmacologists to elucidate mechanistic details of the interaction of the drug with its target(s), a topic we will treat in the Section III.

D. The Road to Successful Drug Development?

In the past years, many analyses have appeared that try to explain the dearth of new drug substances in the face of billions of dollars that have been spent, billions of assay data points that have been accumulated, and ten thousands of virtual and thousands of real hits that have been generated. By comparison, the Belgian medicinal chemist Paul Janssen and his relatively small group had tremendous success in the development of new drug entities and activities [23]. It was postulated that the individualization rather than integration of research guidelines into successive hypes (e.g., "as target subtype selective as possible"; "ADME rules have to be strictly adhered to"; "modeling programs automatically give a correct representation of molecules"; "the more combinatorial ligands, the more hits") is responsible for the disappointing state of drug discovery. What is needed is to keep what we already know about how successful drugs were actually discovered or invented [24], while providing an atmosphere of creativity in a team of scientists from various disciplines. Summarizing their long-lasting experiences in antibacterial research, an industrial team concluded that for this therapeutic area at least, synthesizing novel chemical structures that interact with and block *established* targets in new ways is a robust strategy [24,25].

So what does the increasing knowledge of targets mean for medicinal chemistry? This subject will be introduced in the following paragraphs and discussed in detail in later chapters.

III. DRUG CLASSIFICATION SYSTEMS

Classification systems help with understanding what a drug actually does at the molecular level (classification by target), and they are indispensable for categorizing the large number of drug substances (classification by clinical effect).

A. Classification by Target and Mechanism of Action

1. Targets

Targets are molecular structures, chemically definable by at least a molecular mass, that will undergo a specific interaction with chemicals that we call drugs because they are administered to treat or diagnose a disease [26]. To be meaningful, the interaction has to have a connection with the clinical effect(s). It is very challenging to prove that the interaction of a drug substance with a specific molecular target indeed triggers the clinical effect(s).

A clinically relevant target might consist not of a single biochemical entity but the simultaneous interference of a number of receptors. Only this multi-target interaction will give a net clinical effect that might be considered beneficial. It is only by chance that the current *in vitro* screening techniques will identify drugs that work through such targets.

The number of targets presently used is still open to discussion in medicinal chemistry, but various approaches concurred in finding several hundred. The number of potential targets, however, was estimated to be

several hundred thousand in view of the manifold protein complexes, splicing variants, and possible interventions with signaling pathways [26,27]. The problem with counting is two-fold: first, the identification of the reaction partners of drug substances in the body; and second, exactly what to define and count as the target. A target definition derived from the net effect rather than the direct chemical interaction will require input from systems biology, a research field that promises to affect the drug discovery process significantly [28]. At the other end of the scale of precision, we can define some targets very precisely on the molecular level. For example, we can say that dihydropyridines block the CaV1.2a splicing variant in heart muscle cells of L-type high-voltage activated calcium channels.

The actual depth of detail used to define the target is primarily dependent on the amount of knowledge available about the target and its interactions with a drug. Even if the target structure has already been determined, the molecular effect of the drug could still not be fully described by the interactions with one target protein alone. For example, antibacterial oxazolidinones interact with 23S-rRNA, tRNA, and two polypeptides, ultimately leading to inhibition of protein synthesis [29]. In this case, a description of the mechanism of action that only includes interactions with the 23S-rRNA target would be too narrowly defined. In particular, in situations in which the dynamic actions of the drug substance stimulate or inhibit a biological process, it is necessary to move away from the description of single proteins, receptors and other targets to view the entire signal chain as the target. and so on to view the entire signal chain as the target.

Lists that classify all marketed drug substances according to target, with references, were published. An excerpt is given in Table 1.1 [26].

2. *Mechanisms of Action*

An effective drug target comprises a biochemical system rather than a single molecule. Present target definitions are static. We know this to be insufficient, but techniques to observe the dynamics of drug–target interactions are just being created. Most importantly, we are not able to gauge the interaction of the biochemical "ripples" that follow the drug's initial molecular effect. The first molecular step of drug activity consists in mass-action governed drug–target recognition. For clinically observable activity, a series of biochemical steps need to follow that have to shift physiological equilibria in a transient way. Indeed, the gap between chain and circles of molecular events and clinical effects is still wide open, as reflected by the complementarity of target and phenotypic-oriented drug discovery approaches [30].

Although the term "mechanism of action" itself implies a classification according to the dynamics of drug substance effects at the molecular level, the dynamics of these interactions are only speculative models at present, and so mechanism of action can currently only be used to describe static targets, as discussed above.

All drugs somehow interfere with signal transduction, receptor signaling, and biochemical equilibria. For many drugs we know—and for most we suspect—that they interact with more than one target. So there will be simultaneous changes in several biochemical signals, and there will be feedback reactions of the pathways disturbed. In most cases, the net result will not be linearly deducible from single effects. For drug combinations, this is even more complicated. Awareness is also increasing of the nonlinear correlation of molecular interactions and clinical effects. For example, the importance of receptor–receptor interactions (receptor mosaics) was summarized for G-protein-coupled receptors (GPCRs), resulting in the hypothesis that cooperativity is important for the decoding of signals, including drug signals [31]. Table 1.2 lists examples of dynamic molecular mechanisms of drugs. Table 1.1 is the excerpt of an attempt at a complete list of drug targets. Notably, inhibitors and antagonists by far outnumber effectors, agonists, and substitutes. It appears that reconstitution of biochemical and pharmacological balances is more easily achieved by blocking excessive or complementary pathways rather than by substitution or repair of deficient or defective biochemical input.

Greater knowledge of how drugs interact with the body (e.g., mechanisms of action, drug–target interactions) has led to a reduction of established drug doses and inspired the development of newer, highly specific drug substances with a known mechanism of action. However, a preoccupation with the molecular details has resulted in a tendency to focus only on this one aspect of the drug effects. For example, cumulative evidence suggests that the proven influence of certain psychopharmaceuticals on neurotransmitter metabolism has little to do with the treatment of schizophrenia or the effectiveness of the drug for this indication [32]. With all our efforts to understand the molecular basis of drug action, we must not fall into the trap of reductionism. For antibacterial research, multitargeting is now considered to be essential [33]. More generally, in recent years the limits of the reductionist approach in drug discovery have become painfully clear. Nobel laureate Roald Hoffmann put it this way: "Chemistry reduced to its simplest terms, is not physics. Medicine is not chemistry … knowledge of the specific physiological and eventually molecular sequence of events does not help us understand what [a] poet has to say to us" [34]. The cartoon (Figure 1.5) illustrates this point. Although it is too early for systems biology

TABLE 1.1 The Main Drug Target Classes with Examples of Targets and Ligands. A Full List Can Be Found in Ref. [26]

Target class	Target subclass	Target example	Drug substance example (activity)
Enzymes	Oxidoreductases	Aldehyde dehydrogenase	Disulfiram (inhibitor)
	Transferases	Protein kinase C	Miltefosine (inhibitor)
	Hydrolases	Bacterial serine protease	β-Lactams (inhibitors)
	Lyases	DOPA decarboxylase	Carbidopa (inhibitor)
	Isomerases	Alanine racemase	D-cycloserine (inhibitor)
	Ligases (syn. synthases)	Dihydropteroate synthase	Sulphonamides (inhibitors)
Proteins	Growth factors	Vascular endothelial growth factor	Bevacizumab (antibody)
	Immunoglobulins	CD3	Muromonab-CD3 (antibody)
	Integrins	α4-Integrin subunit	Natalizumab (antibody)
	Tubulin	Human spindle	Vinca alkaloids (development inhibitors)
Substrates, metabolites	Substrate	Asparagine	Asparaginase (enhanced degradation)
	Metabolite	Urate	Rasburicase (enhanced degradation)
Receptors	Direct ligand-gated ion channel receptors	γ-Aminobutyric-acid (GABA)-	Barbiturates (allosteric agonists)
	G-protein-coupled receptors	Acetylcholine receptors	Pilocarpine (muscarinic receptor agonist)
		Opioid receptors	
		Prostanoid receptors	Buprenorphine (κ-opioid antagonist)
			Misoprostol (agonist)
	Cytokine receptors	TNFα receptors	Etanercept (receptor mimic)
	Integrin receptors	Glycoprotein IIb/IIIa receptor	Tirofiban (antagonist)
	Receptors associated with a tyrosine kinase	Insulin receptor	Insulin (agonist)
	Nuclear receptors, steroid hormone receptors	Mineralocorticoid receptor	Aldosterone (agonist)
	Nuclear receptors, other	Retinoic acid receptors	Isotretinoin (RARα agonist)
Ion channels	Voltage-gated Ca^{2+} channels	L-type channels	Dihydropyridines (inhibitors)
	K^+ channels	Epithelial K^+ channels	Diazoxide (opener)
	Na^+ channels	Voltage-gated Na^+ channels	Carbamazepine (inhibitor)
	Ryanodine-inositol 1,4,5-triphosphate receptor Ca^{2+} channel	Ryanodine receptors	Dantrolene (inhibitor)
	Transient receptor potential Ca^{2+} channel	TRPV1 receptors	Acetaminophen metabolite (inhibitor)
	Chloride channels	Mast cell chloride channels	Cromolyn sodium (inhibitor)
Transport proteins	Cation-chloride cotransporter family	Thiazide-sensitive NaCl symporter	Thiazide diuretics (inhibitors)
	Na^+/H^+ antiporters		Amiloride (inhibitor)
	Proton pumps	H^+/K^+ ATPase	Omeprazole (inhibitor)
	Eukaryotic (putative) sterol transporter (EST) family	Niemann-Pick C1 like 1 protein	Ezetimibe (inhibitor)
	Neurotransmitter/Na^+ symporter family	Serotonin/Na^+ symporter	Paroxetine (inhibitor)

(*Continued*)

TABLE 1.1 (Continued)

Target class	Target subclass	Target example	Drug substance example (activity)
DNA, RNA	Nucleic acids	Bacterial 16S-RNA	Aminoglycosides (protein synthesis inhibition)
	Ribosome	Bacterial 30S subunit	Tetracyclines (protein synthesis inhibition)
Physicochemical mechanism	Ion exchange	Hydroxide	Fluoride (enhanced acid stability of adamantine)
	Acid binding	In stomach	Hydrotalcite
	Adsorptive	In gut	Charcoal
	Surface-active	On oral mucosa	Chlorhexidine (disinfectant)
	Oxidative	On skin	Permanganate (disinfectant)
	Reductive	Disulphide bonds	*N*-acetylcysteine (mucolytic)
	Osmotically active	In gut	Lactulose (laxative)

TABLE 1.2 Examples of Dynamic (Process) Mechanisms of Drug Action

Dynamic mechanism	Example
Covalent modifications of the active center	Acylation of bacterial transpeptidases by β-lactam antibiotics
Drugs that require the receptor to adopt a certain conformation for binding and inhibition	Trapping of K^+ channels by methanesulphonanilide antiarrhythmic agents
Drugs that exert their effect indirectly and require a functional background	The catechol *O*-methyltransferase inhibitor entacapone, the effect of which is due to the accumulation of nonmetabolized dopamine
Anti-infectives that require the target organism to be in an active, growing state	β-Lactam antibacterials
Molecules requiring activation (prodrugs)	Enalaprilate, paracetamol
Modifications of a substrate or cofactor	Asparaginase, which depletes tumour cells of asparagine;[a] isoniazide, which is activated by mycobacteria leading to an inactive covalently modified NADH[b]
Simultaneous modulation of several signaling systems	GPCR receptor mosaics for the decoding of drug signals
Fluctuations of physiological signaling molecules	Dopamine fluctuations after administration of cocaine, followed by a gradual increase in steady state dopamine concentration[c]

[a]*Graham ML. Pegaspargase: a review of clinical studies. Adv Drug Deliv Rev 2003;55:1293–302.*
[b]*Larsen MH, Vilchèze C, Kremer L, Besra GS, Parsons L, Salfinger M, et al. Overexpression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in Mycobacterium smegmatis, M. bovis BCG, and M. tuberculosis. Mol Microbiol 2002;46:453–66.*
[c]*Heien ML, Khan AS, Ariansen JL, Cheer JF, Phillips PE, Wassum KM, et al. Real-time measurement of dopamine fluctuations after cocaine in the brain of behaving rats. Proc Natl Acad Sci USA 2005;102:10023–8.*

to provide clear-cut protocols for medicinal chemistry, translational medicine [35] and other integrative research efforts stress the functional as opposed to reductionist character of living systems, hopefully improving the success rate of drug research [36].

B. Other Classification Systems

From a pharmaceutical standpoint, many different criteria can be used to classify medications: type of formulation, the frequency with which it is prescribed or recommended, price, refundibility, prescription or nonprescription medication, etc. If a classification of the APIs is undertaken, numerous possibilities are revealed as well. At the end of the 19th century, drug substances were classified the same as other chemical entities: by nature of their primary elements, functional moieties, or organic substance class. Recently, the idea of classifying drug substances strictly according to their chemical constitution or structure has been revived. Databases attempt to gather

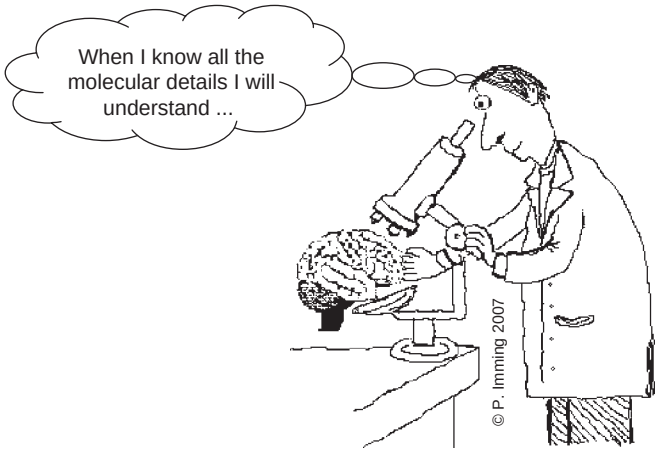

FIGURE 1.5 Searching for molecular mechanisms … "The meaning of the message will not be found in the chemistry of the ink." Sperry R. Brain circuits and functions of the mind. Cambridge: Cambridge University Press; 1990. *Source: Roger Sperry, neurophysiologist, Nobel Prize in Medicine, 1981.*

and organize information on existing or potential drug substances according to their chemical structure and diversity. The objective is to create—virtual or real—substance or fragment "libraries" that contain pertinent information about possible ligands for new targets (e.g., an enzyme or receptor) of clinical interest [37,38], and, more importantly, to understand the systematics of molecular recognition (ligand−receptor) [39,40].

The most commonly used classification system for drug substances is the *ATC system* [41]. It was introduced in 1976 by the Nordic Council on Medicines as a method for carrying out drug utilization studies throughout Scandinavia. In 1981, the World Health Organization recommended the use of the ATC classification for all global drug utilization studies, and in 1982 founded the WHO Collaborating Centre for Drugs Statistics Methodology in Oslo to establish and develop the method. The ATC system categorizes drug substances at five different levels according to (1) the organ or system on which they act (anatomy), (2) therapeutic and pharmacological properties, and (3) chemical properties. The first level comprises the main anatomical groups, while the second level contains the pharmacologically relevant therapeutic subgroup. The third level consists of the pharmacological subgroup, and the fourth the chemical subgroup. The fifth level represents the chemical substance (the actual drug entity). Drugs with multiple effects and different target organs can be found more than once within the system. The anti-inflammatory agent diclofenac, for instance, has three ATC numbers, one of them being M01AB05. This key breaks down to: M01 (musculo-skeletal system; anti-inflammatory and antirheumatic agents, nonsteroids); M01AB (acetic acid derivatives and related substances); and 05 (diclofenac in M01AB). The two other keys classify diclofenac as a topical agent and its use for inflammation of sensory organs.

While ATC is better suited if the emphasis is on therapeutic use, the TCAT system [26,42] puts the target chemistry first, particularly suiting the medicinal chemical approach.

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CHAPTER

2

Evaluation of the Biological Activity of Compounds: Techniques and Mechanism of Action Studies

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OUTLINE

I. INTRODUCTION

Drugs are molecules that are designed to perturb biological systems (cells, isolated tissues, whole animals, and ultimately patients). The responses observed are, in most cases, the result of the drug interacting with proteins, which have the capacity to convert chemical information into biological information. These proteins include plasma membrane bound receptors such as G protein coupled receptors (GPCRs) and tyrosine kinase receptors, ion channels (both ligand gated and voltage operated), enzymes, transporters, and transcription factors such as the nuclear hormone receptors (NHRs), which bind to specific consensus sequences of DNA and modulate gene transcription. Many of these target classes have been very successfully exploited to produce pharmacological agents designed to treat human (and animal) diseases (Figure 2.1). However, a significant number of potentially therapeutically useful drug targets have proven to be less tractable to small molecule approaches. In many cases this is because the interactions in question are protein–protein in nature and therefore difficult to modulate with conventional drugs. This realization has driven significant efforts in the field of biologicals. These large molecular weight agents, which include monoclonal antibodies, oligonucleotides, and small interfering RNAs (siRNA),

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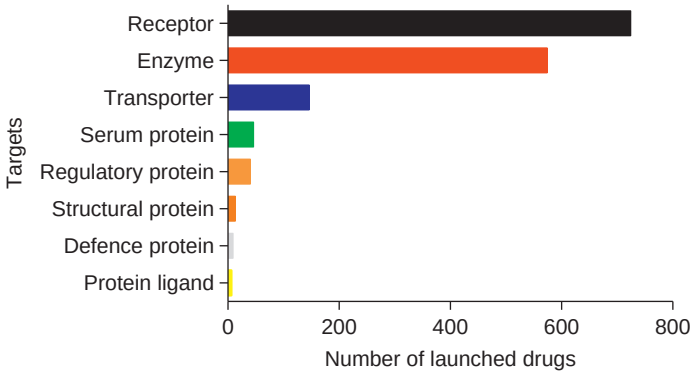


FIGURE 2.1 Number of Launched Small Molecule Drugs by Protein Target Class. As of May 2014, according to Thomson Reuters Cortellis.

have significantly increased the armamentarium of researchers allowing them to probe the role of previously intractable targets in human disease (see Chapter 3). However, to date, with the exception of monoclonal antibodies (such as the anti-TNFs) very few of these biological agents have made it to the market. In addition, the higher cost of large molecules means that small molecule drug programs remain an attractive proposition, even for chemically challenging targets.

The focus of this chapter is the description of how small molecules synthesized by medicinal chemists are assessed for biological activity (although many of the principles are equally applicable to large molecules). Such agents have been the mainstay of pharmacological treatment of human disease for decades and remain a very important class of drugs in the continuing search for new medicines to address unmet clinical needs. Historically, medicinal chemists used the naturally occurring ligands or substrates (for enzymes) of target proteins as starting points for small molecule-based research programs, although nowadays "hits" from high throughput screens (HTS), fragment screens, or *in silico* screens are more likely to act as initial "leads." Irrespective of the chemical basis of the program, the assays employed, the data generated, and their subsequent analysis form the basis of screening cascades that are designed ultimately to identify and progress molecules with appropriate properties for clinical testing. If the target is novel, and in the absence of definitive data linking it to human disease (e.g., genetic association data such as the link between the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) dysfunction and cystic fibrosis (CF)), such clinical testing provides the ultimate validation (or invalidation) of the target.

II. DRUG DISCOVERY APPROACHES AND SCREENING CASCADES

A. Target Based Screening

Modern day drug discovery programs largely center on target based screening, i.e., they aim to identify compounds that modulate the activity of a target that is potentially implicated in a human disease. To this end, compounds are typically tested in a range of *in vitro* biological assays designed to measure primary activities (potency, intrinsic activity, and/or efficacy), selectivity (activity versus related and unrelated targets), cellular toxicity, and physiologically relevant activity. The primary assay sits at the top of the screening cascade and data derived from it drive understanding of structure-activity relationships (SAR), allowing compound optimization. Criteria are set at each level of the cascade for compound progression to the next assay. Compounds with suitable robust properties progress to animal model testing with the aim of showing activity in a "disease relevant" setting as a prelude to picking a candidate drug (CD) for clinical trials. As drug discovery programs progress from early (Hit and Lead Identification) to late (Lead Optimisation, Candidate Selection) phases, the screening cascade evolves to become increasingly complex. Figure 2.2A illustrates a typical example of a screening cascade, although it is important to realize that the cascade used will be target dependent.

B. Phenotypic Screening

Although target based screening has proven to be very successful in the discovery of new medicines, a major disadvantage of the approach is that the evidence linking the target to the disease is often relatively weak, and the hypothesis is only proven (or disproven) after considerable investment of time, effort, and money.

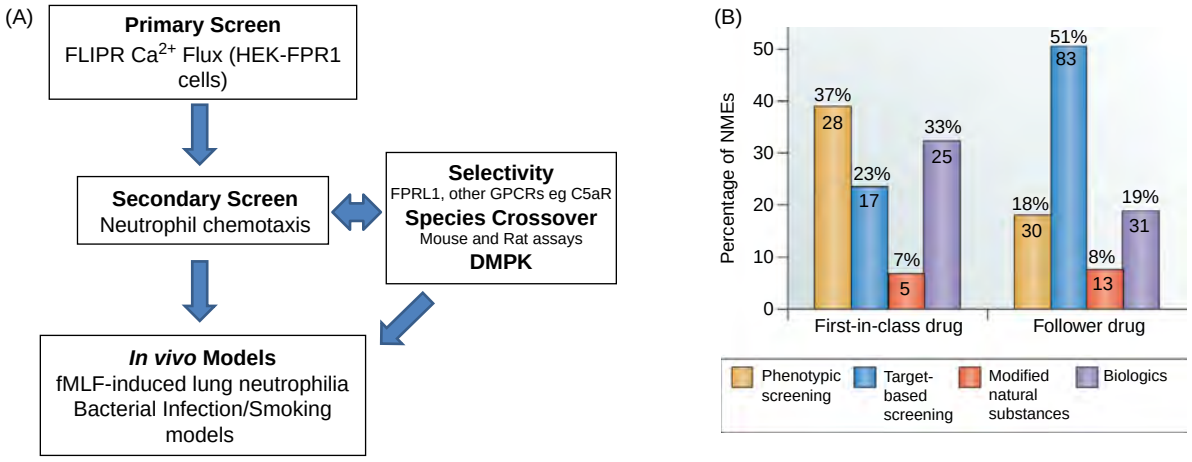


FIGURE 2.2 **Screening Cascades and Drug Discovery Approaches.** (A) A typical screening cascade for an early discovery program. In this instance the aim was to discover antagonists of the formyl peptide receptor, FPR1, as potential treatments for COPD. The receptor mediates a number of effects on a range of cell types but is perhaps best known as an activator of neutrophils. Hence the focus of the cascade is on neutrophil biology. As the project proceeded, a number of other assays were introduced to the cascade including neutrophil and macrophage superoxide generation and airway smooth muscle contraction. (B) The distribution of new drugs discovered between 1999 and 2008, according to the discovery strategy. *Reprinted by permission from Swinney & Anthony, 2011 [1].*

Phenotypic screening, on the other hand, starts from the premise that the assay readout has high disease relevance, and therefore active compounds are more likely to be clinically efficacious. The cell-based assays that are typically employed also have the advantage that "hits" by definition have cellular activity: in target based approaches using isolated proteins, this property often has to be built in later. The disadvantage of this approach is that the mechanism of action (MOA) of the compounds in the assay is usually unknown, so subsequent optimization of "hits" involves significant de-convolution activities. Nevertheless, phenotypic screens are becoming more popular in the drug industry, driven by the increased availability of novel higher throughput technologies and the success of this approach in identifying new "first in class" molecules [1] (Figure 2.2B). Examples of areas in which the approach has proven fruitful are neglected parasitic diseases such as human African trypanosomiasis and cystic fibrosis. In the former example, compounds can be screened for trypanocidal activity against the whole parasite [2]. In the case of CF, the well-understood genetic basis of the disease has encouraged screening for compounds that improve the functional activity of the defective protein (CFTR). This latter example is somewhat of a "halfway house" between a target-based screen and a phenotypic screen: the target is clearly known, but improved function of CFTR can result from compound interaction with CFTR itself or with proteins involved in its processing, trafficking and ion channel function. The recent approval of the CFTR potentiator, Kalydeco™ [3,4] is testament to the power of this approach.

III. *IN VITRO* ASSAYS

As outlined above, the initial phases of a target based screening cascade typically employ a range of *in vitro* assays. The exact system(s) used will be target and mechanism dependent. For example, a project targeting antagonists may use a binding assay as the primary screen, whereas one targeting agonists is more likely to use a functional assay. The following sections give a basic introduction to some of the more commonly used types of primary assays outlining their advantages and disadvantages. The measurements made in these assays that are typically reported to medicinal chemists, the properties of various different classes of drugs, and the principles underlying their analysis are also described.

A. Primary Assays

Glossary

A glossary of commonly used biochemical/pharmacological parameters is presented in Table 2.1 to assist the reader's understanding of the following sections.

TABLE 2.1 Glossary of Key Pharmacological/Biochemical Terms

Pharmacological/ biochemical term	Definition
K_A (pK_A)	Standard pharmacologic convention for the equilibrium dissociation constant of an agonist receptor complex with units of M. It is a measure of affinity. ($pK_A = -\log_{10}K_A$)
K_B (pK_B)	Convention for the equilibrium dissociation constant of an antagonist receptor complex determined in a functional assay. It has units of M and is a measure of affinity. ($pK_B = -\log_{10}K_B$)
K_D (pK_D)	Convention for the equilibrium dissociation constant of a ligand receptor complex measured in a binding assay. It has units of M. ($pK_D = -\log_{10}K_D$)
K_i (pK_i)	The K_B for an antagonist (or inhibitor) but measured in a binding study or enzyme assay. It has units of M. ($pK_i = -\log_{10}K_i$)
K_m	The Michaelis constant K_m is the substrate concentration at which the reaction rate is half of V_{max}. It has units of M and is measure of the substrate's affinity for the enzyme.
$[A]_{50}$ or EC_{50} (pA_{50} or pEC_{50})	The effective concentration of an agonist producing 50 percent maximal response to that particular drug (not necessarily 50 percent of the maximal response of the system). It has units of M and is a measure of agonist potency. (pA_{50} and $pEC_{50} = -\log_{10}A_{50}$ and $-\log_{10}EC_{50}$).
IC_{50} (pIC_{50})	The concentration (usually molar) of an inhibitor (receptor, enzyme antagonist) that blocks a given predefined stimulus by 50 percent. It is a measure of inhibitor potency but is an empirical value in that its magnitude can vary with the strength of the stimulus to be blocked. ($pIC_{50} = -\log_{10}IC_{50}$).
pA_2	The negative logarithm of the molar concentration of an antagonist that produces a 2-fold shift to the right of an agonist concentration-effect curve. It is a measure of antagonist potency.
Intrinsic Activity	A measure of agonist activity, it is the fractional response of an agonist (positive or inverse) relative to a standard full agonist. It is unit-less and ranges from 0 for antagonists to 1.0 for full agonists.
Efficacy (e or τ)	A measure of the capacity of an agonist to produce a physiological response. It is unit-less but can have both positive and negative values (for inverse agonists).
ED_{50}	The *in vivo* counterpart of EC_{50} referring to the dose (D) of agonist that produces 50 percent maximal effect

1. *Binding assays*

The aim of binding experiments is to determine the affinity (the strength with which a compound binds to the target site) of the compound for the biological target. They are the simplest and most robust assays. Today, binding assays are commonly run using recombinantly generated human protein or mammalian cell lines (such as human embryonic kidney 293 (HEK293) or Chinese hamster ovary (CHO) cells) engineered to express the human version of the target protein. Isolated protein, membrane preparations from cells, or whole cells can all be used to measure the affinity of test compounds. Isolated proteins are often employed for enzyme targets whereas membrane and cell preparations have been widely used in programs aimed at finding drugs that target GPCRs and ion channels. As it is impractical routinely to label test compounds, typically the measurements made are indirect, in that the ability of the test compound to inhibit binding of a standard labeled compound is assessed. Such assays depend of course on the availability of a suitably affine and selective labeled compound. Historically the label has been radioactive, but more recently fluorescently labeled compounds have also been employed. Increasingly, label free technologies such as that developed by Biacore [5], which use surface plasmon resonance to measure binding events as changes in molecular mass, are being used in drug discovery programs. This technique has the advantage of allowing real time measurements to be made. Thus, affinity, kinetics, and thermodynamics are easily studied (see Section IIIA (2)). It is also a very sensitive technique and therefore can detect the low affinity interactions that are typical of low molecular weight fragments (<250 Da). The disadvantages of this technique are that the development of successful protein target immobilization can take considerable time and effort, and its application is mainly with solubilised proteins like kinases rather than integral membrane receptors like GPCRs.

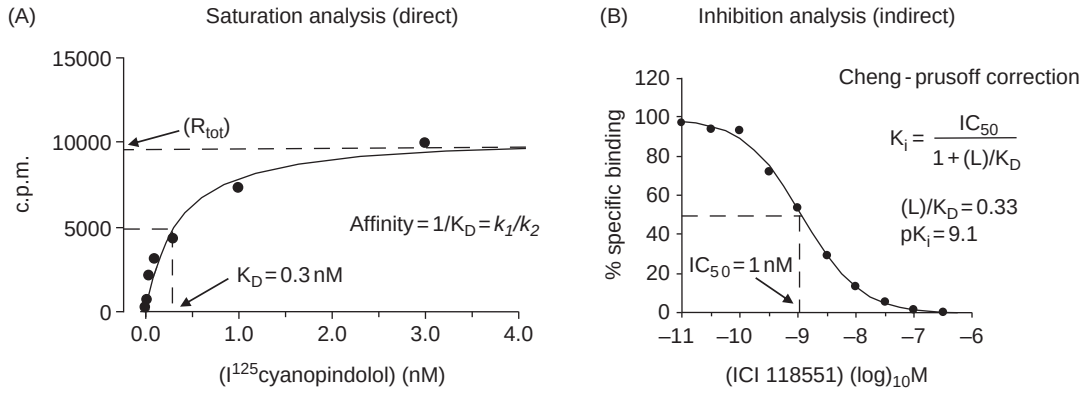


FIGURE 2.3 **Binding Assays: Direct and Indirect Measurements.** (A) A direct binding assay using I^{125} labeled cyanopindolol as a β_2-adrenoceptor ligand. The curve describes a rectangular hyperbola which saturates at high ligand concentration. The ligand dissociation constant (K_D) was estimated as 0.3 nM and is a measure of the ligand affinity. (B) A typical inhibition analysis using membranes expressing the human β_2-adrenoceptor and employing 0.1 nM I^{125} cyanopindolol as the labeled ligand. The displacing ligand, the selective β_2-adrenoceptor antagonist ICI 118551, produces complete inhibition of the specific binding yielding an IC_{50} of 1 nM. In this instance $[L]/K_D$ is <1.0 so the IC_{50} is a good estimate of the K_i as calculated by the Cheng-Prusoff equation. *Unpublished data.*

2. *Binding Studies: Principles and Analysis*

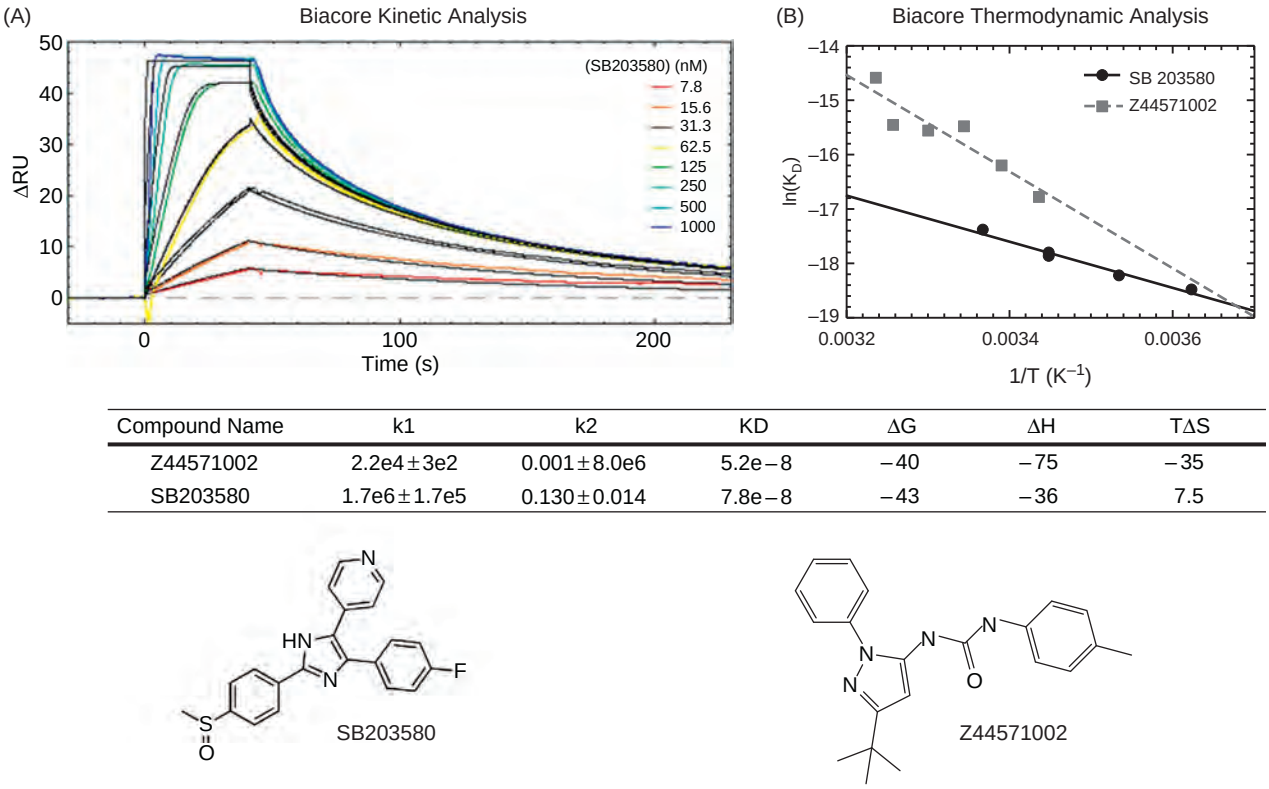
The simplest model of drug-receptor (or more generally protein) interaction is the Law of Mass Action in which the drug binds reversibly to the protein at a single site. Under such conditions, regardless of the assay employed, ligand (L) binding to its receptor (R) at equilibrium is described by the following equation:

$$[LR] = \frac{[R_{tot}][L]}{[L] + K_D} \tag{2.1}$$

where [LR] represents the concentration of ligand occupied receptors, $[R_{tot}]$ the total receptor pool and K_D is the ligand equilibrium dissociation constant (offset rate constant (k_2) divided by the onset rate constant (k_1)), a measure of the affinity of the ligand for its receptor. Eq. (2.1) describes a saturable curve with all the receptors being occupied at high ligand concentrations. K_D represents the concentration of ligand that occupies 50 percent of $[R_{tot}]$. Thus, in principle the direct binding of a labeled compound to the target can be simply measured and the K_D estimated from the midpoint of the saturation curve (Figure 2.3A). However, as it is clearly not practical to label all test compounds, the affinity of these is measured indirectly by assessing the displacement of a labeled ligand. In such experiments, the ability of the test compound to inhibit a single concentration (usually at approximately the K_D or below) of labeled ligand is measured. They typically yield a sigmoidal curve (when the drug concentration is expressed in log form) from which the IC_{50} (concentration of the test compound that produces 50 percent inhibition of the specific binding of the labeled compound) can be measured (see Figure 2.3B). Curve parameter estimates are usually derived from direct fitting of the experimental data to simple mathematical equations as described in Section IIIA (6). Assuming that the interaction between the labeled compound and the test compound is competitive, the dissociation constant (K_i) of the test compound can be calculated from the Cheng-Prusoff [6] equation:

$$K_i = \frac{IC_{50}}{1 + [L]/K_D} \tag{2.2}$$

Proof of the assumption that the interaction is competitive requires further experimentation, such as studying the inhibition by the test compound with different concentrations of the labeled ligand. As is evident from Eq. (2.2), at concentrations of L in excess of K_D the IC_{50} estimate will increase proportionately (i.e., higher concentrations of L will require higher concentrations of test compound to displace it). It is therefore imperative that when IC_{50} values are used to compare compound activities, that this is done under identical conditions (i.e., $[L]/K_D$ should be constant).



Compound Name	k1	k2	KD	ΔG	ΔH	TΔS
Z44571002	2.2e4±3e2	0.001±8.0e6	5.2e−8	−40	−75	−35
SB203580	1.7e6±1.7e5	0.130±0.014	7.8e−8	−43	−36	7.5

FIGURE 2.4 **Binding Assays: Kinetic and Thermodynamic Measurements.** Direct binding assay using the Biacore label free technique. The sensorgram (A) illustrates the time course of SB203580 binding to immobilized mitogen activated kinase p38α. The y-axis shows the mass change resulting from compound binding to p38α. At t = 0 a range of SB203580 concentrations were passed across the immobilized p38α to measure net association, and then at t = 50 s compound is replaced with buffer to initiate dissociation. The table shows the association (k_1 ($M^{-1}s^{-1}$)) and dissociation (k_2 (s^{-1})) rate constants as well as the equilibrium dissociation constants (K_D (M)) for two compounds. (B) Thermodynamic analysis of two p38α inhibitors using Biacore. Enthalpy and entropy components of binding derived from the Van't Hoff analysis are detailed in the attached table. ΔG, ΔH and TΔS values are in kJ/mol. *Unpublished data.*

Since IC_{50} values do not infer a particular MOA, they are routinely used to compare the activities of compounds in binding assays. Other modes of compound binding beyond simple competition are discussed in the sections below on enzymes and functional studies.

The kinetics of drug binding are also most easily studied in simple binding assays rather than functional assays where association and dissociation rates are more likely to be affected by diffusion barriers. Label free techniques such as Biacore are being increasingly utilized for this purpose as illustrated in Figure 2.4A for inhibitors of p38α. Interestingly the compounds in this example have similar affinities (52 and 78 nM), but markedly different kinetics (e.g., k_1 values 2.2×10^4 and 1.7×10^6 $M\,s^{-1}$). Slow kinetics and hence longer residence times are potentially advantageous compound characteristics [7,8], therefore such measurements are becoming increasingly important in drug discovery programs. An interesting example of a clinically used drug with unusual kinetics is the muscarinic antagonist, Tiotropium. It binds M_2 and M_3 receptor subtypes nonselectively (K_i values of 0.1 to 0.2 nM), but the compound has a much slower off rate (>10-fold) at the M_3 subtype, enough to make it a physiologically selective M_3 antagonist [9].

Finally, thermodynamic studies can be employed to determine the relative contributions of enthalpy and entropy to a compound's binding energy. The Gibbs free energy of binding (ΔG) is made up of enthalpic and entropic contributions and for reversible binding events can described as:

$$\Delta G = \Delta H - T\Delta S \tag{2.3}$$

where ΔH defines enthalpy and ΔS, entropy at temperature T.

The relationship between binding affinity (K_D) and temperature (T) is defined by the Van't Hoff equation:

$$\ln(K_D) = (\Delta H/R)(1/T) - \Delta S/R \tag{2.4}$$

TABLE 2.2 Enzyme Assay Techniques

Enzyme class	Product	Principle	Type	Detection examples
Protein kinases	Phosphoprotein	Labeled antibody	Homogeneous	AlphaLisa
	Phosphoprotein	Labeled antibody	Multi-step	ELISA
	ADP	Coupling enzyme	Multi-step	Luciferase (luminescent product)
Metabolic enzymes	Small molecule	Radioactivity	Multi-step	Radiometric
	• no chromophore	Radioactivity/Proximity	Homogeneous	Radiometric (SPA)
	• no antibody detection	Mass/charge	Homogeneous	LC-MS (RapidFire)
		Lipophilicity/charge	Multi-step	HPLC
		Coupling enzyme	Multi-step	Variety (secondary product)
Methyltransferases	Methylhistone	Labeled antibody	Homogeneous	AlphaLisa
	Methylhistone	Labeled antibody	Multi-step	ELISA
	H_2O_2	Coupling enzyme	Multi-step	Peroxidase (Light-based product)
	Formate	Coupling enzyme	Multi-step	Variety (secondary product)

where R is the Gas Constant. Thus by performing binding kinetics at different temperatures and then plotting ln (K_D) data as a function of 1/T, the relative enthalpy and entropy contributions to the compound's binding energy can be determined (Figure 2.4B). Such measurements allow chemistry to be steered towards optimization of either component or both. Generally in drug design, effort is focused on making optimal interactions (electrostatic, H-bonding etc.) with the target (enthalpic), whilst rigidifying the compound to reduce conformational (entropic) contributions (i.e., reduction in rotational freedom on binding).

3. *Enzyme Assays*

Enzymes are highly specific biological catalysts evolved to perform a broad range of biochemical transformations under physiological conditions. They operate in multiple locations (e.g., cytoplasmic, lysosomal, and extracellular) and under different spatial constraints (e.g., membrane bound, soluble, multimer). The reactions catalyzed by enzymes range in complexity from simple one-step chemical oxidations (e.g., alcohol dehydrogenase) to targeted, subtle protein modifications (e.g., kinases, methylases, etc.). The nature of an enzyme's activity is driven by precise substrate recognition at the active site (where the catalysis takes place) and through other regulatory sites.

Nearly all enzyme-targeted drugs are inhibitors, so most enzyme assays are designed to detect inhibitors by measuring the blockade of product production − substrate depletion is generally not used as the high starting background makes it technically more difficult. In order to develop an enzyme assay, one needs active and pure enzyme, substrates (e.g., protein, lipid, sugar, metabolite etc.), and a way to measure product formation and a good understanding of the optimal conditions for enzyme activity. Sufficient enzyme needs to be produced in a functionally active state at high purity (>95 percent) and in large amounts (>10 mg). To achieve this, enzymes are expressed recombinantly at high levels in various cell systems with tags (e.g., histidines) attached to aid purification. These tags are genetically encoded such that they are expressed at the C or N termini of the protein, distal from the active site and so less likely to affect the enzyme's activity. Once enzyme overexpression has been achieved, the enzyme is purified from the lysed cells by affinity chromatography using the attached tag (e.g., nickel column for his tag), followed by size exclusion (gel filtration) or pI (ion exchange). Enzyme production is not always straightforward and can require considerable optimization to reproducibly deliver a highly pure and active product. Substrates are usually commercially available, but if proteins, they may have to be made in the same way as the enzyme target. There are many different ways to measure product formation (Table 2.2), but the guiding principles are summarized below:

- Most enzyme assay readouts are now:
  - Light-based (e.g., fluorescence, luminescence, absorbance, fluorescence polarisation, HTRF, etc.)
  - Homogeneous (i.e., no separation steps, e.g., AlphaScreen)
  - Scalable (amenable to 384-well plates and HTS)
- Rarely:
  - ELISA (multiple wash steps)
  - Radiometric or HPLC (usually for metabolite, small molecule products)

The overall enzyme catalyzed reaction process is summarized below:

$$E + S \leftrightarrow ES \leftrightarrow EP \rightarrow E + P$$

where E (enzyme), S (substrate), ES (enzyme:substrate complexes), EP (enzyme:product complexes) and P (product). Enzyme reactions are generally studied under steady state conditions in which [S] is in excess of [E] and the reaction rate is linear—for most enzymes, [E] is nM and [S] is μM or mM. Initially upon addition of substrate to enzyme, only a very small percentage of total substrate is turned over and the rate is linear, but as more substrate is consumed it becomes rate-limiting and the enzyme velocity slows, usually when >10 percent substrate is used. The relationship between [S] and initial enzyme rate (v) was initially described by Briggs and Haldane [10]:

$$v = \frac{V_{max}[S]}{[S] + K_m} \tag{2.5}$$

Where V_{max} is the maximal rate (when $[S] \gg [E]$) and K_m is the Michaelis constant ([S] at which v is $V_{max/2}$).

In essence, enzyme assays are designed to optimally measure product formation and its inhibition by test compounds (I). It is important to show that the rate of product formation is proportional to [E] over the time course of the assay (i.e., steady state), so that a decrease in product rate by [I] relates directly to a reduction in active [E] due to inhibitor occupancy or indirect reduction in the number of substrate accessible active sites (i.e., formation of [EI]). Enzyme inhibitor mechanisms are discussed later on in Section IIIA (4). To minimize insolubility issues during dilution, compounds are usually dissolved in DMSO (anhydrous) to 10 mM and subsequently diluted in DMSO in half-logarithmic steps (usually 7) to produce a range of concentrations. These compound DMSO solutions are then diluted in assay media (large dilution, e.g., 25-fold) and then into the assay (small dilution, e.g., 4-fold) such that the final assay [DMSO] is tolerated (typically <1 percent (v/v)). Visual inspection for insolubility can be easily monitored during this process and fed back to the project team.

As with receptor functional and binding assays, compound potency is usually measured using an IC_{50} value (or pIC_{50} ($-\log_{10}IC_{50}$)) and typically determined from an eight point concentration inhibition curve using a four parameter logistic fit:

$$\%I = I_{min} + \frac{(I_{max} - I_{min})[I]^n}{[I]^n + [IC_{50}]^n} \tag{2.6}$$

where I_{max} is the maximal inhibition (usually ~100 percent), I_{min} the minimal inhibition (~0 percent) and n the slope of the curve.

Robert Copeland's book, *Evaluation of Enzyme Inhibitors in Drug Discovery* is highly recommended further reading [11].

4. *Types of Enzyme Inhibition and Their Analysis*

A. REVERSIBLE INHIBITORS

The majority of enzyme inhibitor drugs are reversible in that removal of the inhibitor (e.g., by dialysis) fully restores the enzymatic activity. Such inhibitors bind to their target enzyme through a combination of noncovalent interactions, such as hydrogen bonding or ionic, hydrophobic, and Van der Waals interactions, and don't generally undergo any chemical transformation while enzyme bound. Their behavior is described by the following equation:

$$E + I \rightleftharpoons EI$$

where E represents the active enzyme, I the reversible inhibitor, and EI the inactive inhibitor-bound enzyme. Examples of drugs that are reversible enzyme inhibitors and their mechanisms of action are shown in Table 2.3.

Testing for reversible inhibition relies on separation of the inhibitor from the inhibitor bound enzyme, which can be achieved using differences in enzyme and inhibitor mass (i.e., enzyme: >30 000 Da, inhibitor: ~400 Da) using a variety of techniques (e.g., dialysis, gel filtration, ultracentrifugation, etc.). By reducing free [I], EI complex dissociates leading to the recovery of enzyme activity. It is important during the pre-incubation of inhibitor with enzyme prior to reversibility that substrate is included to ensure the enzyme turns over and the inhibitor is exposed to all enzyme states during its catalytic cycle. Two common techniques to demonstrate reversible enzyme inhibition are jump dilution and immobilization. In the former, enzyme is incubated with inhibitor at $10 \times IC_{50}$ to give ~90 percent inhibition, and then, after sufficient time to allow EI formation, the mixture is rapidly ("jump") diluted 100-fold in assay buffer so that the final [I] is 10-fold below the IC_{50} such that if fully