

The Process of Drug Discovery

One doesn't discover new lands without consenting to lose sight of the shore for a very long time.

— André Gide (1869–1951)

The real voyage of discovery consists not in seeking new landscapes but in having new eyes.

— Marcel Proust (1871–1922)

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8.1 PHARMACOLOGY IN DRUG DISCOVERY

The drug discovery process can be envisioned in four interconnected phases (see [Figure 8.1](#)). Generally, these are the acquisition of chemicals to be tested for biological activity, the determination of the activity of those chemicals on biological systems (pharmacodynamics), the formulation of the most active of these for therapeutic testing in humans (pharmaceutics), and the determination of adequate delivery of the active drug to diseased tissues (pharmacokinetics). Each phase of this collection of processes is interconnected with the others, and failure in any one of them can halt the development process. It is worth considering each process separately as well as the relationships between them.

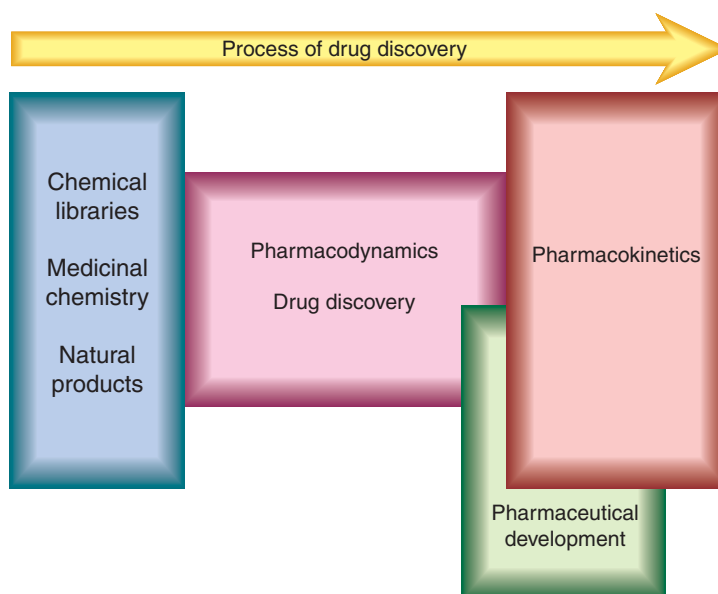
8.2 CHEMICAL SOURCES FOR POTENTIAL DRUGS

A starting point to this process is the definition of what the therapeutic end point of the drug discovery process will be; namely, a drug. There are certain properties that molecules must have to qualify as therapeutically useful chemicals. While, in theory, any molecule possessing activity that can be introduced into the body compartment

containing the therapeutic target could be a possible drug, in practice, therapeutically useful molecules must be absorbed into the body (usually by the oral route), distribute to the biological target in the body, be stable for a period of time in the body, be reversible with time (excreted or degraded in the body after a reasonable amount of time), and be nontoxic. Ideally, drugs must be low molecular weight bioavailable molecules. Collectively, these desired properties of molecules are often referred to as “druglike” properties. A useful set of four rules for such molecules has been proposed by Lipinski and coworkers [1]. Molecules that fulfill these criteria generally can be considered possible therapeutically useful drugs, providing they possess target activity and few toxic side effects. Specifically, these rules state that “druglike” molecules should have less than five hydrogen-bond donor atoms, a molecular mass of <500 Da, and high lipophilicity (Clog P >5), and that the sum of the nitrogen and oxygen atoms should be <10. Therefore, when estimating the potential therapeutic drug targets, these properties must be taken into consideration. This will be discussed in more detail in the next chapter.

There are numerous chemical starting points for drugs. Historically, natural products have been a rich source of molecules. The *Ebers Papyrus*, one of the earliest documents recording ancient medicine, describes 700 drugs, most from plants. Similarly, the Chinese *Materia Medica*

FIGURE 8.1 Schematic diagram of four interactive but also separate stages of drug discovery and development.



(100 B.C.), the *Shennong Herbal* (100 B.C.), the *Tang Herbal* (659 A.D.), the Indian *Ayurvedic* system (1000 B.C.), and books of Tibetan medicine *Gyu-zhi* (800 A.D.) all document herbal remedies for illness. Some medicinal substances have their origins in geographical exploration. For example, tribes indigenous to the Amazon River had long been known to use the bark of the *Cinchona officinalis* to treat fever. In 1820, Caventou and Pelletier extracted the active antimalarial quinine from the bark, which provided the starting point for the synthetic antimalarials chloroquin and mefloquine. Traditional Chinese herbal medicine has yielded compounds such as artemisinin and derivatives for the treatment of fever from the *Artemisia annua*. The anticancer vinca alkaloids were isolated from the Madagascar periwinkle *Catharanthus roseus*. Opium is an ancient medicinal substance described by Theophrastus in the third century B.C., used for many years by Arabian physicians for the treatment of dysentery and “relief of suffering” (as described by Sydenham in 1680) in the Middle Ages. Known to be a mixture of alkaloids, opium furnished therapeutically useful pure alkaloids when Serturner isolated morphine in 1806, Robiquet isolated codeine in 1832, and Merck isolated papaverine in 1848. At present, only 5–15% of the 25,000 species of higher plants have been studied for possible therapeutic activity. Of prescriptions in the United States written between 1959 and 1980, 25% contained plant extracts or active principals.

Marine life can also be a rich source of medicinal material. For example, C-nucleosides spongouridine and spongothymidine isolated from the Caribbean sponge *Cryptotheca crypta* possess antiviral activity. Synthetic analogues led to the development of cytosine arabinoside, a useful anticancer drug. Microbes also provide extremely useful medicines, the most famous case being penicillin

from *Penicillium chrysogenum*. Other extremely useful bacteria-derived products include the fungal metabolites, the cephalosporins (from *Cephalosporium cryptosporium*), aminoglycosides and tetracyclines from *Actinomycetales*, immunosuppressives such as the cyclosporins and rapamycin (from *Streptomyces*), cholesterol-lowering agents mevastatin and lovastatin (from *Penicillium*), and antihelmintics and antiparasitics such as the ivermectins (from *Streptomyces*). As with plants, less than 1% of bacterial and less than 5% of fungal sources have been explored for medicinal value. In general, the World Health Organization estimates that 80% of the world’s population relies on traditional medicine with natural products.

From this perspective, natural products appear to be a great future source of drugs. However, teleologically, there may be evolutionary pressure against biological activity of natural products. Thus, while millions of years of selective pressure has evolved molecules that specifically interact with physiological receptors (i.e., neurotransmitters, hormones) with little “cross talk” to other targets, it can be argued that those same years exerted a selective evolutionary pressure to evolve receptors that interact only with those molecules and not the myriad of natural products to which the organism has been exposed. In practical terms, natural products as drugs or starting points for drugs have certain inherent disadvantages as well. Specifically, these tend to be expensive, not chemically tractable (structurally complex and difficult to derivatize), and involve difficult and expensive scale-up procedures (active species tend to be minor components of samples). Natural products also often contain a larger number of ring structures and more chiral centers and have sp^3 hybridization bridgehead atoms present. Natural products are often high in stereo complexity and, containing few nitrogen, halogen, and sulfur atoms and being oxygen

rich with many hydrogen donors, natural products often are very prone to enzymatic reactions. In addition, a practical problem in utilizing such pharmacophores is the unpredictable novelty and intellectual property that may result. In spite of these shortcomings, between the years 1981 and 2002, of the 67% of 877 synthetic new chemical entities, 16.4% utilized pharmacophores derived directly from natural products.

Another approach to the discovery of drugs is “rational design.” The basis for this strategy is the belief that detailed structural knowledge of the active site binding the drug will yield corresponding information to guide the design of molecules to interact with that active site. One of the best-known examples, yielding rich dividends, is the synthesis of the angiotensin converting enzyme (ACE) inhibitor captopril from a detailed analysis of the enzyme active site. Similar design of small molecules to fit specific binding loci of enzymes was accomplished for HIV protease (nelfinavir) and Relenza for the prevention of influenza. Other rational design approaches utilize dual pharmacophores from other active drugs to combine useful therapeutic activities. This approach offers the advantage that the dual biological activity will be absorbed, metabolized, and excreted in a uniform manner, that is, the activity profile of the drug will not change with

varying ratios of two simultaneously dosed drugs. This also gives medicinal chemists a place to start. For example, ICS 205-903, a novel and potent antagonist of some neural effects of serotonin in migraine, was made by utilizing the structure of cocaine, a substance known to have seriously debilitating central effects but also known to block some of the neural effects of serotonin with the serotonin structure. The result was a selective serotonin antagonist devoid of the disadvantages of cocaine (Figure 8.2A). Similarly, a beta-adrenoceptor blocker with vasodilating properties has been made by combining the structure of the beta-blocker propranolol with that of a vasodilator (Figure 8.2B). The idea of introducing dual or multitarget activities in molecules is discussed further in Section 10.5.

There are numerous natural substances that have useful therapeutic properties as well as other undesirable properties. From these starting points, medicinal chemists have improved on nature. For example, while extremely useful in the treatment of infection, penicillin is not available by the oral route; this shortcoming is overcome in the analogue ampicillin (Figure 8.3A). Similarly, the obvious deleterious effects of cocaine have been eliminated in the local anesthetic procaine (Figure 8.3B). The short activity and weak steroid progesterone is converted to a stronger

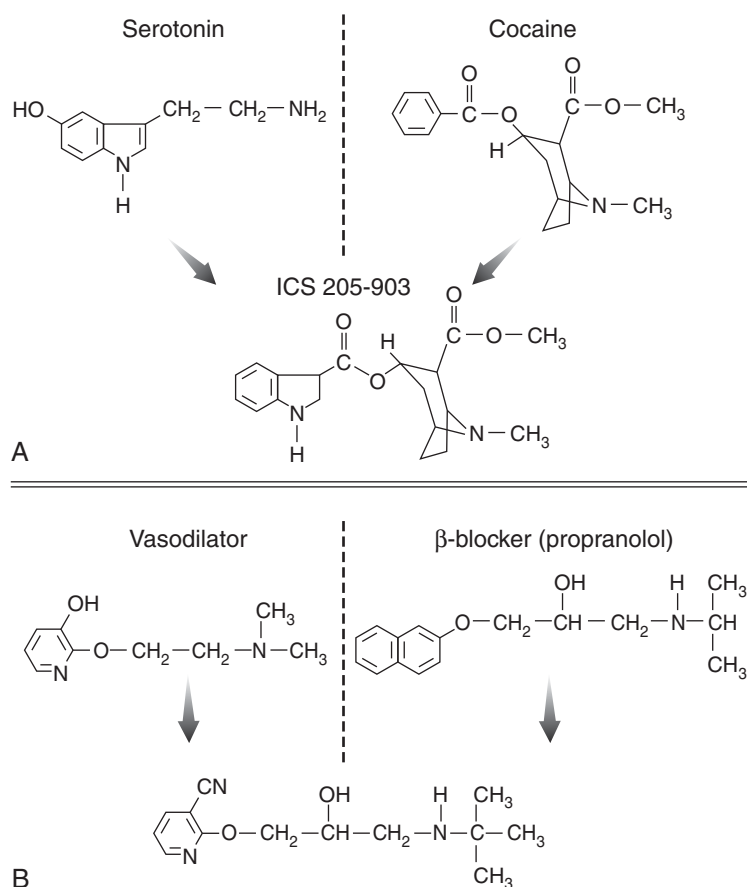
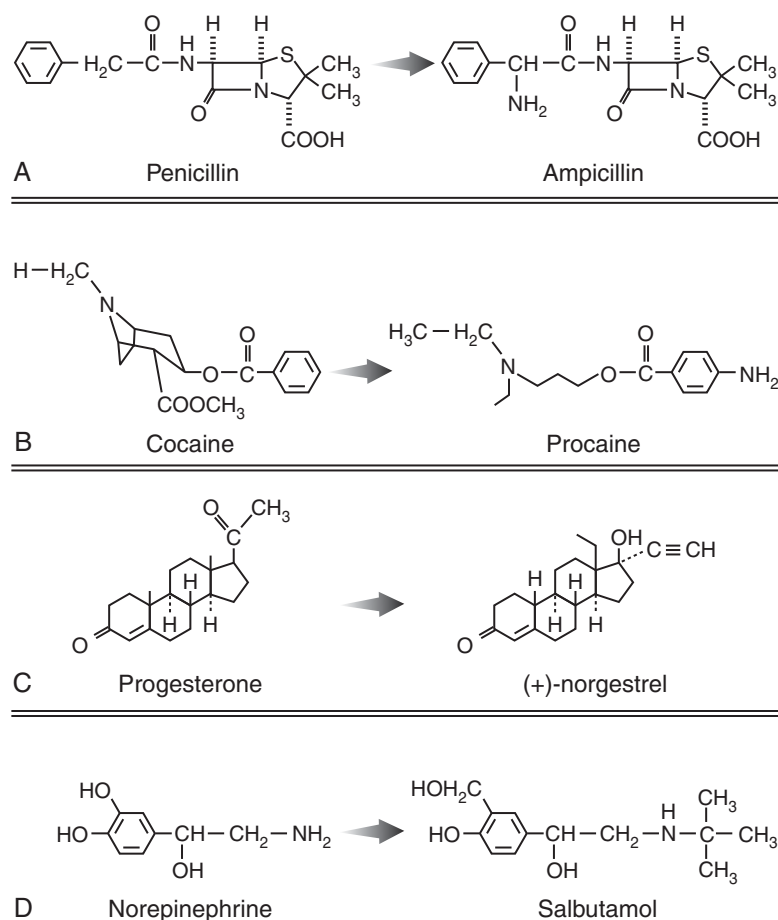


FIGURE 8.2 Examples of drug design through hybridization: combination of two structural types to produce a unique chemical entity. (A) Design of ICS 205-903 [2]. (B) Compound with vasodilating and beta-blocking properties [3].

FIGURE 8.3 Examples of chemical modification of active drugs that have either unwanted effects (cocaine, norepinephrine) or suboptimal effects (penicillin, progesterone) to molecules with useful therapeutic profiles.



long-acting analogue (+)-norgestrel through synthetic modification (Figure 8.3C). Catecholamines are extremely important to sustaining life and have a myriad of biological activities. For example, norepinephrine produces a useful bronchodilation that has utility in the treatment of asthma. However, it also has a short duration of action, is a chemically unstable catechol, and produces debilitating tachycardia, vasoconstriction, and digital tremor. Synthetic modification to salbutamol eliminated all but the tremorogenic side effects to produce a very useful bronchodilator for the treatment of asthma (Figure 8.3D).

It can be argued that drugs themselves can be extremely valuable starting points for other drugs in that, by virtue of the fact that they are tolerated in humans, they allow the observation of their other effects. Some of those effects (“side effects”) may lead to useful therapeutic indications. For example, the observed antiedematous effects of the antibacterial sulfanilamide in patients with congestive heart failure led to the discovery of its carbonic anhydrase inhibitor activity and the subsequent development of the diuretic furosemide (Figure 8.4A). Similarly, the antidiabetic effects of the antibiotic carbutamide led to the development of the antidiabetic tolbutamide (Figure 8.4B). Some of the early antihistamines were found to exert

antidepressant and antipsychotic properties; these led to modern psychopharmaceuticals. The immunosuppressant activity of the fungal agent cyclosporine also was exploited for therapeutic utility.

Endogenous substances such as serotonin, amino acids, purines, and pyrimidines all have biological activity and also are tolerated in the human body. Therefore, these can be used in some cases as starting points for synthetic drugs. For example, the amino acid tryptophan and neurotransmitter serotonin were used to produce selective ligands for 5-HT_{5A} receptors and a selective somatostatin₃ antagonist, adenosine A_{2b} receptor antagonists from adenine, and a selective adenosine 2A receptor agonist from adenosine itself (Figure 8.5).

Major pharmaceutical efforts revolve around the testing of large chemical libraries for biological activity. Assuming that most drugs must have a molecular weight of less than 600 (due to desired pharmacokinetic properties, as discussed later), there are wide ranges in the estimates of the number of molecules that exist in “chemical space,” that is, how many different molecules can be made within this size limit? The estimates range from 10⁴⁰ to 10¹⁰⁰ molecules, although the need for activated carbon centers for the construction of carbon–carbon bonds in

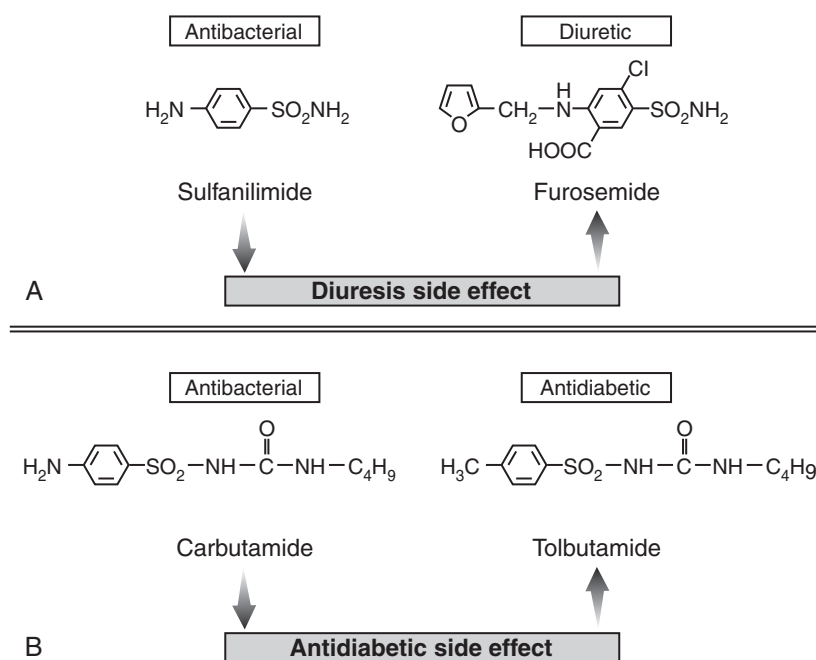


FIGURE 8.4 Examples of case where the side effects of drugs used for another indication led to the discovery and development of a new therapeutic entity for another disease.

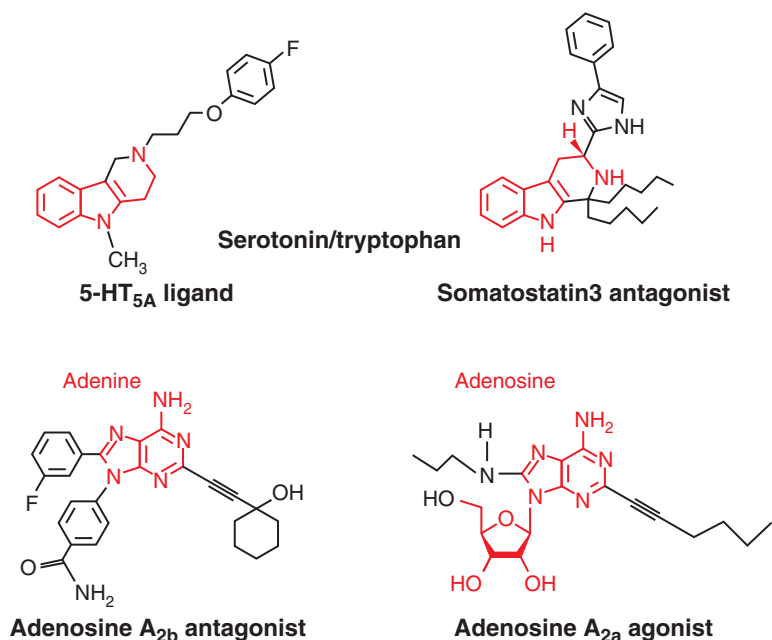


FIGURE 8.5 Examples of natural substances (shown in red) that have been chemically modified to yield therapeutically useful selective drugs.

synthetic procedures reduces the possible candidates for synthetic congeners. In spite of this fact, the number of possibilities is staggering. For example, in the placement of 150 substituents on mono to 14-substituted hexanes there are 10^{29} possible derivatives. Considering a median value of 10^{64} possible structures in chemical space clearly indicates that the number of possible structures available is far too large for complete coverage by chemical synthesis and biological screening. It has been estimated that a library of 24 million compounds would be required to furnish a randomly screened molecule with biological activity in the

nanomolar potency range. While combinatorial libraries have greatly increased the productivity of medicinal chemists (i.e., a single chemist might have produced 50 novel chemical structures in a year 10 years ago, but with the availability of solid and liquid phase synthesis and other combinatorial techniques, a single chemist can produce thousands of compounds in a single month at a fraction of the cost of previous techniques), 24 million compounds per lead is still considerably larger than the practical capability of industry.

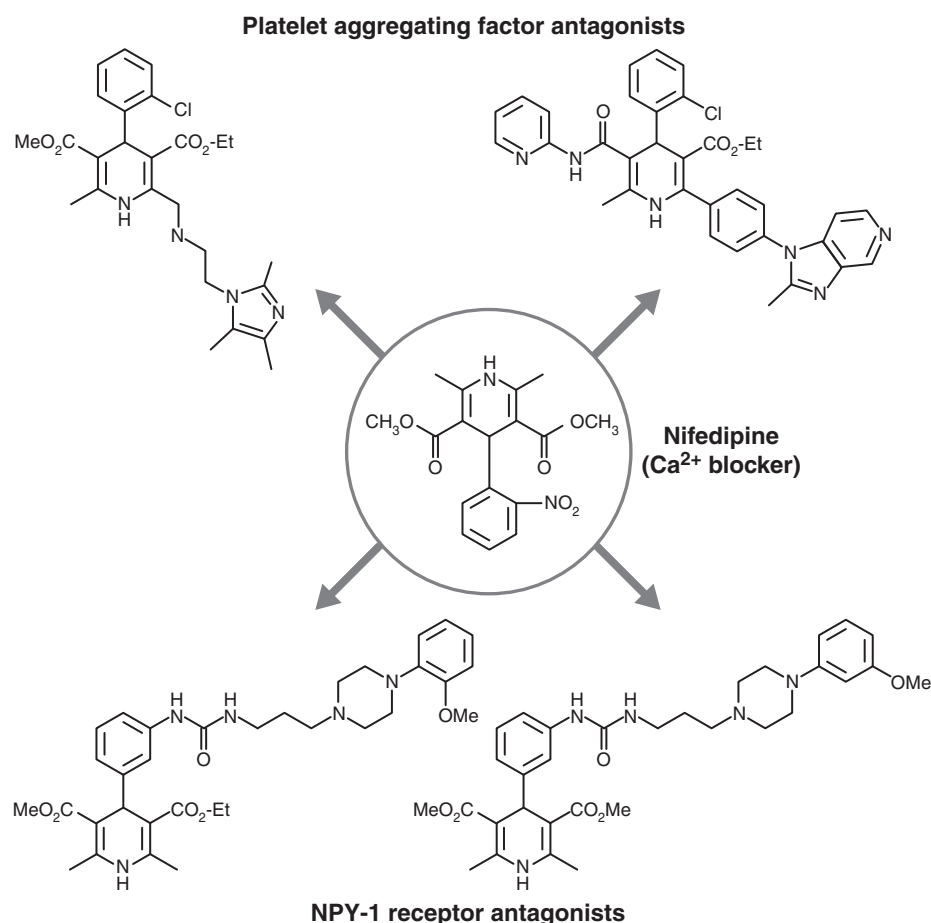
One proposed reason for the failure of many high-throughput screening campaigns is the lack of attention

to “druglike” (namely, the ability to be absorbed into the human body and having a lack of toxicity) properties in the chemical library. The non-druglike properties of molecules leads to biological activity that cannot be exploited therapeutically. This is leading to improved drug design in chemical libraries incorporating features to improve “druglike properties.” One difficulty with this approach is the multifaceted nature of the molecular properties of druglike molecules, that is, while druglike chemical space is more simple than biological target space, the screens for druglike activity are multimechanism based and difficult to predict. Thus, incorporating favorable druglike properties into chemical libraries can be problematic. Also, different approaches can be counter-intuitive to the incorporation of druglike properties. Thus, rational design of drugs tends to increase molecular weight and lead to molecules with high hydrogen bonding and unchanged lipophilicity; this generally can lead to reduced permeability. A target permeability for druglike molecules (which should have aqueous solubility minimum of $>52 \mu\text{g/ml}$) should achieve oral absorption from a dose of $>1 \text{ mg/kg}$. High-throughput screening approaches tend to increase molecular weight, leave hydrogen bonding unchanged from the initial hit, and increase lipophilicity; this can lead to decreases in

aqueous solubility with concomitant decrease in druglike properties.

The assumption made in estimations of the number of molecules that would be required to yield biologically active molecules is that potential drugs are randomly and uniformly distributed throughout chemical space. Analysis of known drugs and biologically active structures indicates that this latter assumption probably is not valid. Instead, drugs tend to cluster in chemical space, that is, there may be as little as 10,000 druglike compounds in pharmacological space [4]. The clustering of druglike molecules in chemical space has led to the concept of “privileged structures” from which medicinal chemists may choose for starting points for new drugs. A *privileged structure* is defined as a molecular scaffold with a range of binding properties that yields potent and selective ligands for a range of targets through modification of functional groups. Privileged structures can be a part of already known drugs such as the dihydropyridines (known as *calcium channel blockers*). In this case, inhibitors of platelet aggregation (PAF inhibitors) and neuropeptide Y type 1 receptor ligands have been made from the dihydropyridine backbone (Figure 8.6). Privileged structures also can simply be recurring chemical motifs such as the indole motif

FIGURE 8.6 Example of a preferred structure, in this case the dihydropyridine scaffold.



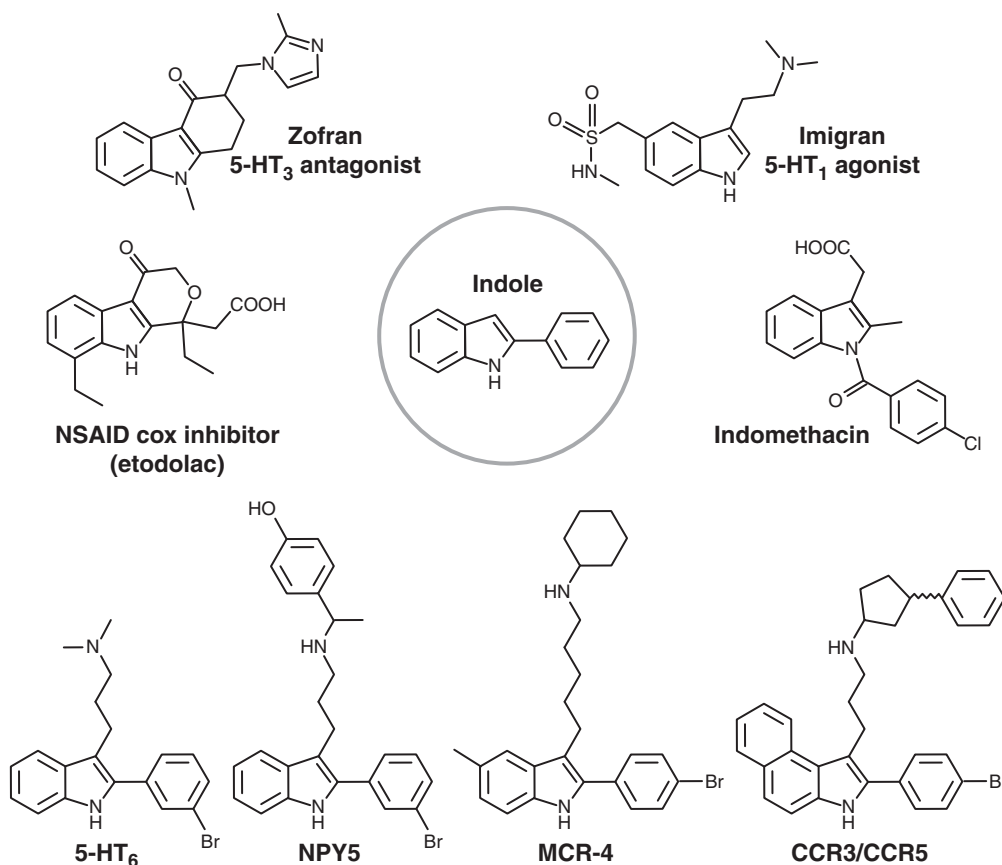


FIGURE 8.7 The preferred indole structure forms the basis of a number of selective ligands for receptors.

shown in Figure 8.7 and shared by marketed drugs and investigational ligands. Similarly, the 2-tetrazole-biphenyl motif is found in the angiotensin2 receptor antagonist losartan and GHS receptor ligand L-692,429 (Figure 8.8A), and a wide range of biologically active structures is based in spiropiperidines (Figure 8.8B).

8.3 PHARMACODYNAMICS AND HIGH-THROUGHPUT SCREENING

The history of medicine and pharmacology abound with anecdotes of serendipitous drug discovery. Perhaps the most famous example of this is the discovery of penicillin by Fleming in 1928. This led to the systematic screening of hundreds of microorganisms for antibiotics. However, even in those early discovery efforts, the value of screening was appreciated. For example, though Ehrlich's invention of salvarsan for syphilis has many serendipitous elements, it was nevertheless the result of a limited screening of 600 synthetic compounds.

Without prior knowledge of which chemical structure will be active on a particular target, a wide sampling of chemical space (i.e., diverse choice of chemical structures) must be made to detect biological activity. This is done

through so-called *high-throughput screening* (HTS), whereby a robust biological assay is used to test as large as possible a sample of chemical compounds. Usually robotic automation is employed in this process. Presently, sophisticated liquid-handling devices, extremely sensitive detection devices, and automated assay platforms allow testing of multiple thousands of compounds in very small volumes (<10 μ L). The ideal HTS is generic (i.e., can be used for a wide range of targets utilizing formats in which any receptor can be transfected and subsequently expressed), robust (insensitive to assumptions), relatively low cost with a low volume (does not require large quantities of substance), amenable to automation (has a simple assay protocol), ideally nonradioactive, and has a high tolerance to solvents such as DMSO. Some requirements for functional screening assays are given in Table 8.1.

One of the most negative aspects of drug screening is that basically it is a one-way experiment. The single direction stems from the fact that, while activity guides structure activity relationships, much less use can be made of lack of activity. This is because of the numerous reasons why a compound may not show activity, that is, there are more defined reasons why a molecule is active on a biological target than reasons why it lacks activity [4]. For example, lack of aqueous solubility accounts for a

FIGURE 8.8 Examples of preferred structures (2-tetrazole-biphenyls, panel A; and spiropiperidines, panel B) yielding selective ligands for receptors.

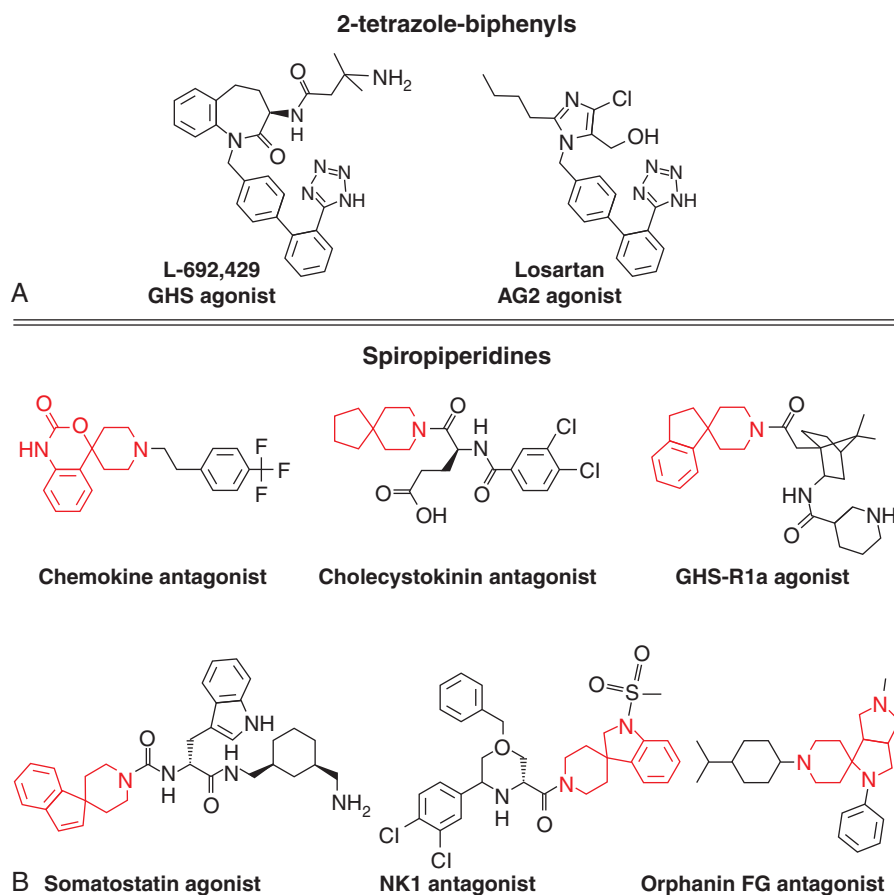


TABLE 8.1 Requirements for a Functional Screening Assay

Minimal

1. Cell line with appropriate receptor is available.
2. There is some means of detecting when there is a ligand-receptor interaction taking place.
3. Agonist and selective antagonist are available.
4. Agonist is reversible.

Optimal

1. There is a commercial cell line available.
2. Response should be sustained, not transient.
3. Response should be rapid.

substantial number of potentially false negatives in the screening process.

A major consideration in screening is the detection capability of the screen for both false negatives (lack of detection of an active drug) and propensity to find false positives (detection of a response to the compound not due to therapeutic activity of interest). Ostensibly, false

positives might not be considered a serious problem in that secondary testing will detect these and they do not normally interfere with the drug discovery process. However, this can be a serious practical problem if the hit rate of a given HTS is abnormally high due to false positives and the major resource for decoding (following up initial hits) becomes limiting. In this regard, binding assays generally have a lower false positive rate than do functional assays. Also, the false positive rate in functional assays where the exposure time of the assay to the compounds is short (i.e., such as calcium transient studies) is lower than in assays such as reporter assays where the time of exposure is on the order of 24 hr. On the other hand, binding studies require confirmation of primary activity in a functional assay to identify therapeutic activity.

A more serious problem is the one of false negatives, since there is no way of knowing which compounds are active but not detected by the assay. In this regard, binding assays have the shortcoming of detecting only compounds that interfere with the binding of the tracer probe. Within this scenario, allosteric compounds that affect the physiological function of the target but otherwise do not interfere with binding of the tracer are not detected. Since allosterism is probe dependent (i.e., not all molecules are equally

affected by an allosteric ligand; see Chapter 7), the endogenous agonist should be used for screening to detect physiologically relevant activity. For example, the allosteric ligand for muscarinic receptors, alcuronium, produces a 10-fold change in the affinity of the receptor for the natural endogenous agonist acetylcholine but only a 1.7-fold change is observed for the synthetic muscarinic agonist arecoline [5]. Therefore, screening with arecoline may not have detected a physiologically relevant (for acetylcholine, the natural agonist) activity of alcuronium.

There are instances where the screen for biologically active molecules cannot be the ideal and appropriate biological test. For example, the screening process for drugs that block against HIV infection theoretically should involve live HIV. However, there are obvious limitations and constraints with using virus that can cause AIDS; specifically, the containment required with such virulent species is not compatible with HTS. Therefore, a surrogate screen must be done. In this case, a receptor screen of the protein recognition site for HIV, namely the chemokine receptor CCR5, can be used to screen for drugs that block HIV infection. What then is required is a secondary assay to ensure that the ligands that block CCR5 also block HIV infection.

The complex protein–protein interactions involved in HIV entry strongly suggest that the blockade of these effects by a small molecule require an allosteric mechanism, that is, a specific orthosteric hindrance of a portion of the protein interfaces will not be adequate to block HIV infection. Therefore, the surrogate screen for HIV blockers would be a surrogate allosteric screen. As noted in Chapter 7 and discussed previously, allosteric effects are notoriously probe dependent and therefore there is the possibility that the HTS will detect molecules devoid of the therapeutically relevant activity, that is, block the binding of the probe for screening but not HIV. This also means that the screen may miss therapeutically relevant molecules by using a therapeutically irrelevant allosteric probe. Figure 8.9 shows how usage of a surrogate probe for biological testing can deviate from therapeutic relevance. Initially, a molecule with potent blocking effects on the surrogate probe (radioactive chemokine binding) was shown to also be a potent antagonist of HIV infection (ordinate scale as the IC_{95} for inhibition of HIV infection; see data point for compound A in Figure 8.9). In efforts to optimize this activity through modification of the initial chemical structure, it was found that chemokine-blocking potency could be retained while HIV activity was lost (see data point for compound B in Figure 8.9). In this case, alteration of the chemical structure caused a 2-fold decrease in chemokine antagonist potency and a disproportionate 3020-fold decrease in HIV antagonist potency. These compounds clearly show the independence of chemokine binding and HIV binding effects with this molecular series.

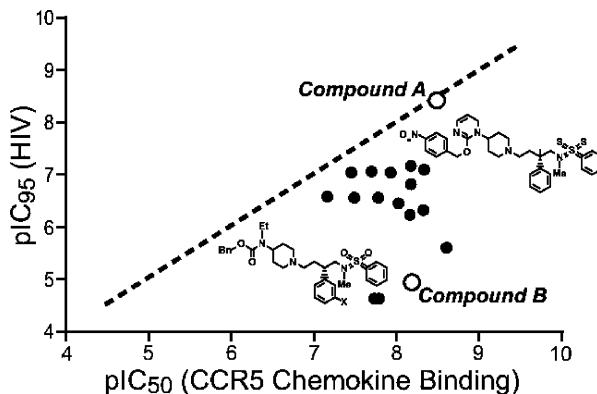


FIGURE 8.9 Correlation between blockade of chemokine binding to CCR5 (abscissae as pK_i values) and 95% inhibition of HIV infection as pIC_{95} (ordinates) for a series of CCR5 antagonists. It can be seen that compound A is nearly equiactive as a blocker of chemokine binding ($pK_i = 8.5$) and HIV infection ($pIC_{95} = 8.4$; ratio of affinities = 1.3), whereas structural analogs (filled circles) clearly differentiate these activities. For the structure B shown, the chemokine-blocking activity has been somewhat retained ($pK_i = 8.2$), whereas the HIV-blocking activity largely has been lost ($pIC_{95} = 4.9$; ratio of affinities = 3020). Data drawn from [6].

The major requirements for a screen are high sensitivity and a large signal-to-noise ratio for detection of effect. This latter factor concerns the inherent error in the basal signal and the size of the window for production of biological effect. A large detection window for response (i.e., difference between basal response and maximal agonist-stimulated response) is useful but not necessary if the random error intrinsic to the measurement of biological effect is low. A smaller maximal detection window, but with a concomitant lower random error in measurement, may be preferable. Since the vast majority of compounds will be exposed to HTS only once, it is critical that the assay used for screening has a very high degree of sensitivity and accuracy. These factors are quantified in a statistic called the Z' factor [7].

The Z' factor calculates a number that is sensitive to the separation between the mean control values for HTS (background) and mean of the positive sample as well as the relative standard deviations of both of those means. In validating a screen, a number of negative controls (background signal) and positive controls (wells containing a ligand that gives a positive signal) are run; this process yields a mean value. A positive control mean signal (μ_{c+}) (for example, the maximal response to an agonist for the target receptor), with accompanying standard deviation (denoted σ_{c+}) and negative control signal (background noise, no agonist) denoted μ_{c-} (with σ_{c-}), are generated with a standard positive control drug (i.e., full agonist for the receptor). The bandwidth of values 3σ units either side of the mean is designated the *data variability band*, and the width of the spread between the two means ($+3\sigma$ units) is denoted the *separation band*.

(or dynamic range) of the screen. It is assumed that 3σ units represent a 99.73% confidence that a value outside this limit is different from the mean (see Chapter 12 for further discussion). An optimum screen will have a maximum dynamic range and minimum data variability band (see Figure 8.10A). It can be seen that problems can occur with either a large intrinsic standard error of measurement (Figure 8.10B) or small separation band (Figure 8.10C). Interestingly, an efficient and accurate HTS can be achieved with a low separation band (contrary to intuition) if the data variability band is very small (see Figure 8.10D). The Z' factor (for a control drug of known high activity for the assay target, this is referred to as a Z' factor) calculates these effects by subtracting the difference between the means from the sum of the difference of the standard deviations of the means divided by the difference between the means:

$$Z' = \frac{|\mu_{c+} - \mu_{c-}| - (3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|} = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|} \quad (8.1)$$

Table 8.2 shows the range of possible Z' values with comments on their meaning in terms of high-throughput screening assays.

The calculation of Z' values for experimental compounds can yield valuable data. Values of Z' for test compounds are calculated in the same way as Z' values except the μ_{c+} and σ_{c+} values are the signals from the test compounds (denoted μ_s and σ_s for test sample) and μ_{c-} and σ_{c-} from the assay with no test compounds run (i.e., controls for noise, denoted μ_c and σ_c for controls). While the Z' indicates the robustness and detection capability of the screen (calculated with known active compounds), a value of Z' for a set of unknown compounds also can test other

FIGURE 8.10 Representation of Z' values. (A) Shaded areas represent distribution of values for control readings (no drug) and the distribution for readings from the system obtained in the presence of a maximal concentration of standard active drug. The signal window for this assay is the separation between the distributions at values $3 \times$ the standard deviation of the mean away from the mean. (B) A representation of an assay with a low Z' value. Though there is a separation, the scatter about the mean values is large and there is no clear window between the lower and upper values. (C) An assay with a low signal window. This assay has a low Z' value. (D) An assay with a low signal window but correspondingly low error leading to a better Z' value.

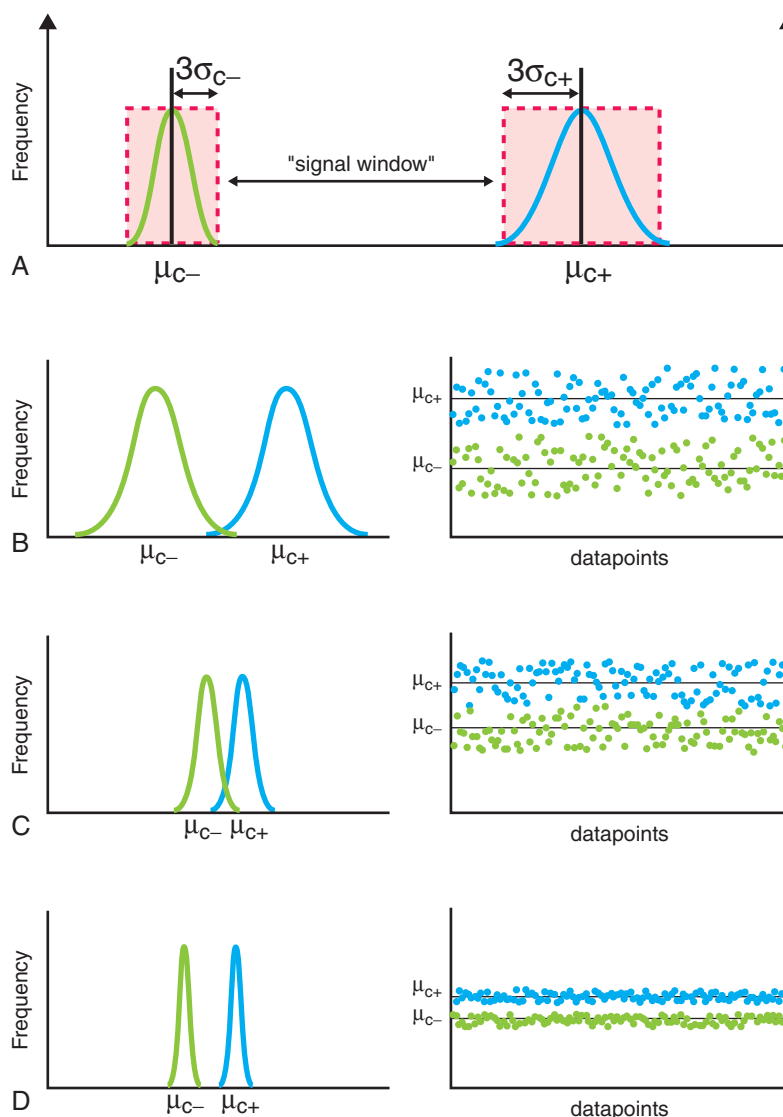


TABLE 8.2 Z' Values and High-Throughput Screening Assays

Z' Value	Description of Assay	Comments
$Z' = 1$	No variation ($\sigma = 0$) or infinite band of separation	Ideal assay
$1 > Z' \geq 0.5$	Large dynamic range	Excellent assay
$0.5 > Z' > 0$	Small dynamic range	Adequate assay
0	No band of separation, σ_{c+} and σ_{c-} touch	Dubious quality
< 0	No band of separation, σ_{c+} and σ_{c-} overlap	Impossible for screening

From [7].

factors related to the screen such as the concentration at which the compounds are tested and/or the chemical makeup of the compound set. For example, Figure 8.11A shows a screen with an excellent Z' value ($Z' = 0.7$), and Z' values for a set of test compounds run at two concentrations; it can be seen that the higher concentration yields a higher signal and variation (possibly due to toxic effects of the high concentration). This, in turn, will lead

to a lower Z' factor. Similarly, Figure 8.11B shows distributions for two chemical libraries; it can be seen that there is a clear difference in the quality of the assay with these two sets of compounds, indicating a possible inherent property of one of the chemical scaffolds leading to variability in the screen. In effect, the quality of the compound set can be quantified for this assay with a value of Z' [7].

Of major importance for HTS is sensitivity to weak ligands. As discussed in Chapter 2, functional systems generally amplify responses as the signal is measured distal to the agonist-receptor interaction. For this reason, agonist screens utilizing end organ response are preferred (i.e., melanophore function, reporter assays). In contrast, the sensitivity of antagonist screening can be controlled by adjustment of the magnitude of the agonism used to detect the blockade. At least for competitive ligands, the lower the amount of stimulation to the receptor the system, the more sensitive it will be to antagonism. This effect is inversely proportional to the window of detection for the system. On one hand, as large a window of agonist response as possible is preferred to maximize signal-to-noise ratios. On the other hand, too large a window may require a strong agonist stimulation that, in turn, would create insensitivity to antagonism. This can be offset by screening at a higher concentration of antagonist, but this can introduce obfuscating factors such as toxic effects of high concentrations of weakly active compounds. Thus,

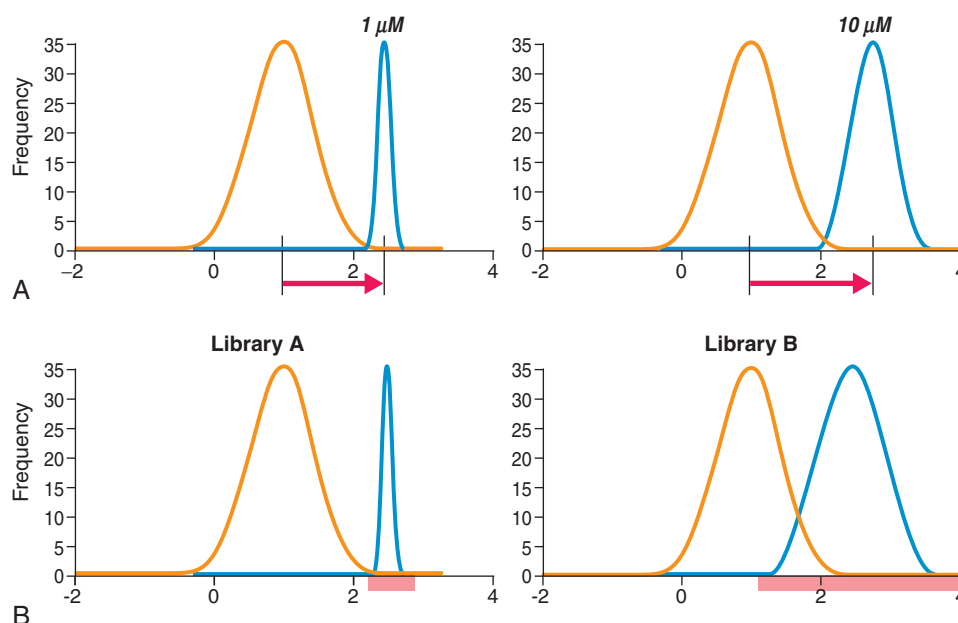


FIGURE 8.11 Distributions for various screens. (A) The larger distribution represents inactive compounds, while the smaller one shows a small sample with values greater than the mean of the total compound library. Distributions are shown for two concentrations tested from this library. It can be seen that, while the mean of the higher concentration is slightly farther away from the control distribution, the error is also much greater, leading to a lower Z' value. (B) The results of single concentration of two compound libraries are shown. It can be seen that library A has a smaller standard error about the mean and therefore is a higher-quality library for potentially active molecules.

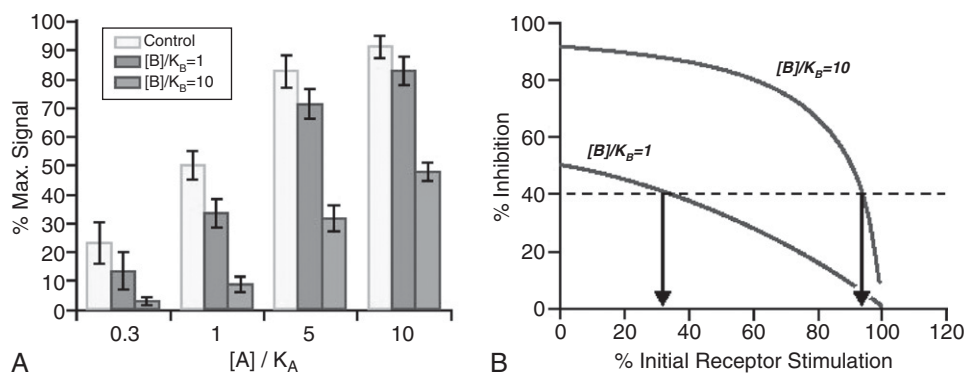


FIGURE 8.12 Antagonism of single concentration stimulation (either functional or radioligand binding) by two concentrations $[B]/K_B = 1$ and 10 of a simple competitive antagonist in screening experiments. (A) Various levels of receptor stimulation in the absence of antagonist (open bars), in the presence of a concentration equal to the K_B and $10 \times K_B$ antagonist (shaded bars) — see box in figure. (B) Percent inhibition (ordinates) of initial receptor stimulation (abscissae) produced by two concentrations of antagonist. If it is assumed that a minimum of 40% inhibition of initial signal is required for adequate detection of antagonism, then the receptor stimulation levels must not be greater than those that produce 33% and 90% receptor–receptor activation (or initial radioligand binding B_0 value) in the HTS for antagonist concentrations of $[B]/K_B = 1$ and 10 , respectively.

for antagonist screening, it becomes a trade-off of strength of agonist stimulation against concentration of antagonist. An optimal screening assay must adjust for maximal sensitivity and minimal variability. Figure 8.12 shows some potential scenarios for single concentration inhibition of different levels of agonist stimulation by different concentrations of an antagonist. It can be seen that the maximal sensitivity to antagonism is observed with low levels of receptor stimulation (Figure 8.12A, see $[A]/K_A = 0.3$). However, the standard deviation of the signal is large enough to interfere with the determination of antagonism. As the magnitude of the receptor stimulation increases ($[A]/K_A = 1.5$, and 10), the standard deviation of the signal ceases to be a problem, but there is less inhibition of the signal. This can be overcome by increasing the concentration of antagonist (Figure 8.12A, filled bars); Figure 8.12B shows the relationship between the initial level of receptor stimulation and the percent inhibition of that signal by an antagonist. If it is assumed that a 40% or greater inhibition of the signal is unequivocal for detection of antagonism, then it can be seen from this figure that the initial level of receptor stimulation cannot exceed 33% maximum for screening antagonist concentrations at the equilibrium dissociation constant (K_B) and <90% maximum stimulation for antagonist concentration = $10 \times K_B$.

From the standpoint of sensitivity to antagonist, a receptor stimulation level of 50% is optimal for functional studies. However, in view of signal-to-noise factors and the need for a clear window of inhibition, an 80% level of stimulation often is employed. In this regard, binding may hold some advantages since the window of detection for a binding assay with a low level of nsb may be

greater than that for a functional assay. Figure 8.13 shows the antagonism by a concentration of antagonist of $[B] = K_B$, of a dose-response curve for receptor stimulation of 80% (function; see Figure 8.13A) and receptor binding level of 10%. It is assumed that both of these initial levels of receptor stimulation yield adequate windows of detection for the respective assay formats. It can be seen that the concentration of antagonist produces 50% inhibition of the binding and only 23% inhibition of the functional signal, that is, the binding assay format is more sensitive to the antagonism. A re-expression of this effect in terms of the minimal potency of antagonist that each screen could detect (assuming that a 40% inhibition is required for detection) indicates that the binding assay would be capable of detecting antagonists with a $K_B \geq 8 \mu\text{M}$, while the functional assay would detect only antagonists of $K_B \geq 3 \mu\text{M}$ (a 2.7-fold loss of sensitivity). It should be stressed that binding and function have been somewhat arbitrarily assigned these two levels of receptor stimulation.

The association of an assay format need not be associated with the sensitivity. In practice, if the functional signal-to-noise level were high, there would be no need to turn to radioligand binding to increase sensitivity of the screen. Similarly, if the nsb levels of the binding screen were high, the level of initial B_0 values for screening would need to be increased to levels comparable to functional assays (i.e., 50% stimulation), and the advantage of binding over function would be lost. In general, sensitivity is not the major factor in the choice of screening format.

The process of tracking screening hits and determining which chemical series is likely to produce a fruitful lead

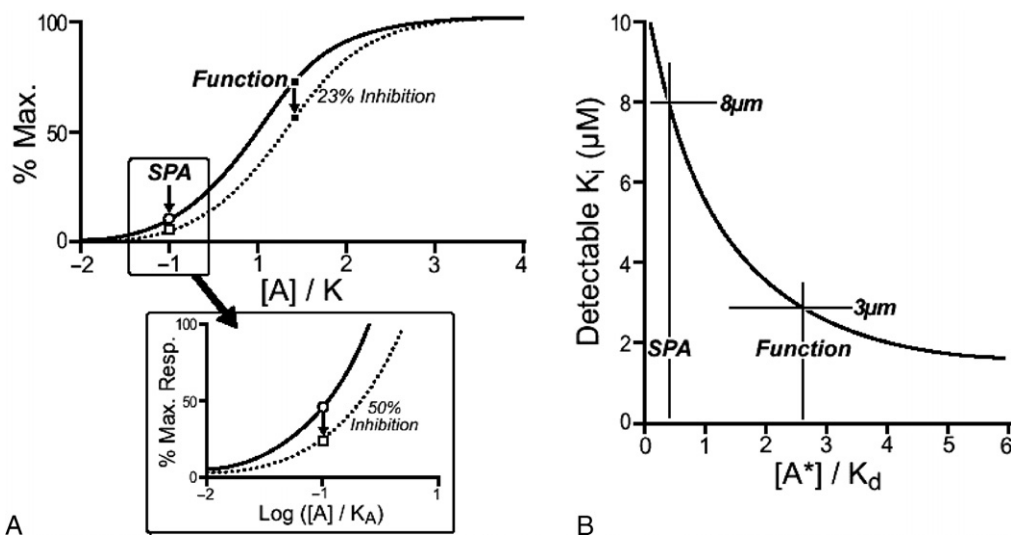


FIGURE 8.13 Windows of detection for antagonism. A twofold shift in a dose-response curve (either to an agonist in a functional study or a radioligand in a saturation binding study) will be perceived differently in different regions of the dose-response curve. Thus, a concentration that produces 80% response will be blocked 23% while a concentration that produces only 10% will be blocked by a factor of 50%. Therefore, the lower the initial signal input to an antagonist assay, the more sensitive it will be to antagonists. In general, functional assays require stronger input signals to achieve acceptable windows (usually an EC₈₀ agonist concentration) than do binding studies (such as scintillation proximity assays, or SPAs). Inset shows where a 10% maximal initial radioligand binding signal can still yield a useful window for observation of antagonism. (B) Ordinate axis shows the lowest potency of hypothetical antagonists that are detectable in an assay (assume 50% blockade of initial signal) as a function of the signal strength used for the assay. If it is assumed that a minimal signal strength for functional assays is $[A]/K_d = 2.5$ while that for an SPA can be lower ($[A]/K_d = 0.5$), it can be seen that the binding assay will detect weaker antagonists ($IC_{50} < 8 \mu M$) than will the functional assay (must be $IC_{50} < 3 \mu M$).

involves the verification of activity within a series of related structures. While the absolute potency of the hit is clearly important, it is recognized that factors such as selectivity, favorable physicochemical properties, absence of toxophores (pharmacophores leading to toxicity: *vide infra*), and the capability for the rapid production of chemical analogs are also very important features of lead molecules. For this reason, the concept of “ligand efficiency” has been used to evaluate the worth of screening hits. This idea converts ligand affinity to the experimental binding energy per atom (so-called *Andrews binding energy* [8]) to normalize the activity of ligand to its molecular weight [9]. It has been estimated that a maximum affinity per atom for organic compounds is $-1.5 \text{ kcal mol}^{-1}$ per non-hydrogen atom ($\Delta G [\text{free energy of binding}] = -RT \ln K_d / \text{number of nonhydrogen atoms}$) [10].

Before discussion of the drug discovery process following lead identification, it is relevant to discuss variations on the theme of hit identification. Screening traditionally has been based on finding a defined primary biological activity, that is, receptor-based agonism or antagonism of physiological effect. Such an approach presupposes that all potentially useful receptor activity will be made manifest through these effects. However, some receptor activities may not be mediated through G-protein activation. For example, the CCK antagonist D-Tyr-Gly-[(Nle^{28,31},D-Trp³⁰)

cholecystokinin-26-32]-phenethyl ester actively induces receptor internalization without producing receptor activation [11]. This suggests that screening assays other than simple agonism and/or antagonism may be useful for the detection of ligand activity.

A similar idea involves the modification of screening assays for the detection of special ligands. For example, certain inhibitors of enzyme function trap the enzyme in dead-end complexes that cannot function; this is referred to as *interfacial inhibition* [12]. Thus, inhibitors such as brefeldin A and camptothecin target a transient kinetic intermediate that is not normally present in a non-activated protein. Screening assays designed to detect these types of inhibitor have a small concentration of substrate in the medium to produce the enzyme transition state (the target of the interfacial inhibitor). Similarly, topoisomerase assays have been designed to identify transient trapping of catalytic-cleavage complexes. Interestingly, such inhibitors may offer an added measure of selectivity since they are active only when both partners of a physiological interaction are present and target only this interaction.

This has particular relevance to allosteric modification of receptors. As described in Chapter 7, the fraction of receptor bound to an agonist [A], expressed in terms of the presence of an allosteric modulator [B], is given as

$$\frac{[AR]}{[R_{\text{tot}}]} = \frac{[A]/K_A(1 + \alpha[B]/K_B)}{[B]/K_B(\alpha[A]/K_A + 1) + [A]/K_A + 1}. \quad (8.2)$$

This leads to the expression for the observed affinity (expressed as equilibrium dissociation constant of the ligand-receptor complex) of the modulator as

$$K_{\text{obs}} = \frac{K_B([A]/K_A + 1)}{\alpha[A]/K_A + 1}. \quad (8.3)$$

It can be seen from Equation 8.3 that the concentration of the probe molecule ($[A]/K_A$) affects the observed affinity of the modulator. This can have practical consequences, especially when allosteric potentiators are the desired chemical target. Just as an allosteric potentiator will increase the affinity of the probe molecule (agonist, radioligand), the reciprocal also is true; namely, that the agonist will increase the affinity of the receptor for the modulator. This can be used in the screening process to make an assay more sensitive to potentiators. For example, for a potentiator that increases the affinity of the agonist 30-fold ($\alpha = 30$), the observed affinity of the modulator will increase by a factor of 15.5 when a small concentration of agonist ($[A]/K_A = 1$) is present in the medium. Such modification of screening assays can be used to tailor detection for specific types of molecules.

Finally, as a corollary to the screening process, there are thermodynamic reasons for supposing that any ligand that has affinity for a biological target may also change that target in some way (i.e., have efficacy). This is because the energetics of binding involve the same forces responsible for protein conformation, that is, as discussed in Section 1.10 in Chapter 1, a ligand will bias the natural conformational ensemble of the receptor. This can be simulated with a probabilistic model of receptor function [13, 14] described in Chapter 3. One of the main predictions of this model is that the same molecular forces that control ligand affinity also control efficacy, and thus they are linked. Under these circumstances, the binding of a ligand may well have thermodynamic consequences that result in a receptor species with different reactive properties towards the cell, that is, the ligand may also have efficacy. As discussed in Chapter 2, this efficacy may not be a conventional stimulation of cellular pathway but rather may involve a changing behavior of the receptor toward the cell, such as a change in the ability to be phosphorylated, internalized, or otherwise altered. The important point is that the theory predicts an efficacy that may not be observed experimentally until the correct pharmacological assay is used, that is, all possible “efficacies” of ligands should be looked for in ligands that bind to the receptor. This can be demonstrated by simulation using the probabilistic model.

Figure 8.14 shows calculated values (see Equations 3.32 and 3.33 in Chapter 3) for affinity (ordinates) and efficacy (abscissae) for 5000 simulated ligands; the probabilities are random, but it can be seen that there is a correlation between affinity and efficacy. The calculations

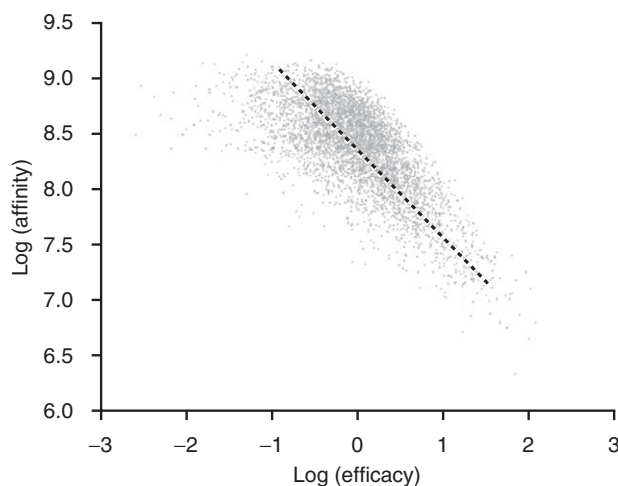


FIGURE 8.14 Simulation for 5000 theoretical ligands with calculated efficacy (Equation 3.3) and affinity (Equation 3.2). It can be seen that efficacy and affinity are correlated, suggesting that all ligands that have been shown to bind to a receptor should be extensively tested for possible efficacy effects on the receptor directly, through agonist effects on the receptor, or through changes in constitutive behavior of the receptor itself. Redrawn from [15].

show that the energy vectors that cause a ligand to associate with the protein also will cause a shift in the bias of protein conformations, that is, the act of binding will cause a change in the nature of the protein ensemble. This suggests that if a ligand binds to a receptor protein, it will in some way change its characteristics toward the system. This has implication in screening since it suggests that all compounds with measured affinity should be tested for all aspects of possible biological activity, not just interference with the binding of an endogenous agonist [15]. This, in turn, argues that a screen that detects fundamental changes in the receptor protein might be an effective method of detecting molecules that bind to the receptor. For example, resonance techniques such as FRET (fluorescent resonance energy transfer) and BRET (bioluminescence resonance energy transfer) take advantage of the fact that energy-sensitive probes alter their wavelength of emission when their relative proximity changes; if two such probes are engineered into a receptor protein, then a change in the conformation of the protein alters the relative positions of the probes and the conformation change can be detected (see Figure 8.15). For example, cyan (CFP) and yellow (YFP) variants of green fluorescent protein allow the transfer of energy from light-excited CFP to YFP (for FRET). In a variant technique, CFP is replaced by light-emitting luciferase (BRET); this approach reduces the background signal but also causes a loss of sensitivity [16]. Replacement of YFP with small fluorescein-derivative FIAsh binds to short cysteine-containing sequences to allow the use of a label much smaller than GFPs [17]. A screen that can detect generic binding of any molecule to the receptor through BRET or FRET then allows the reduction of potential molecules from the order of millions to perhaps a few thousand. This is a much more manageable number to pursue specific

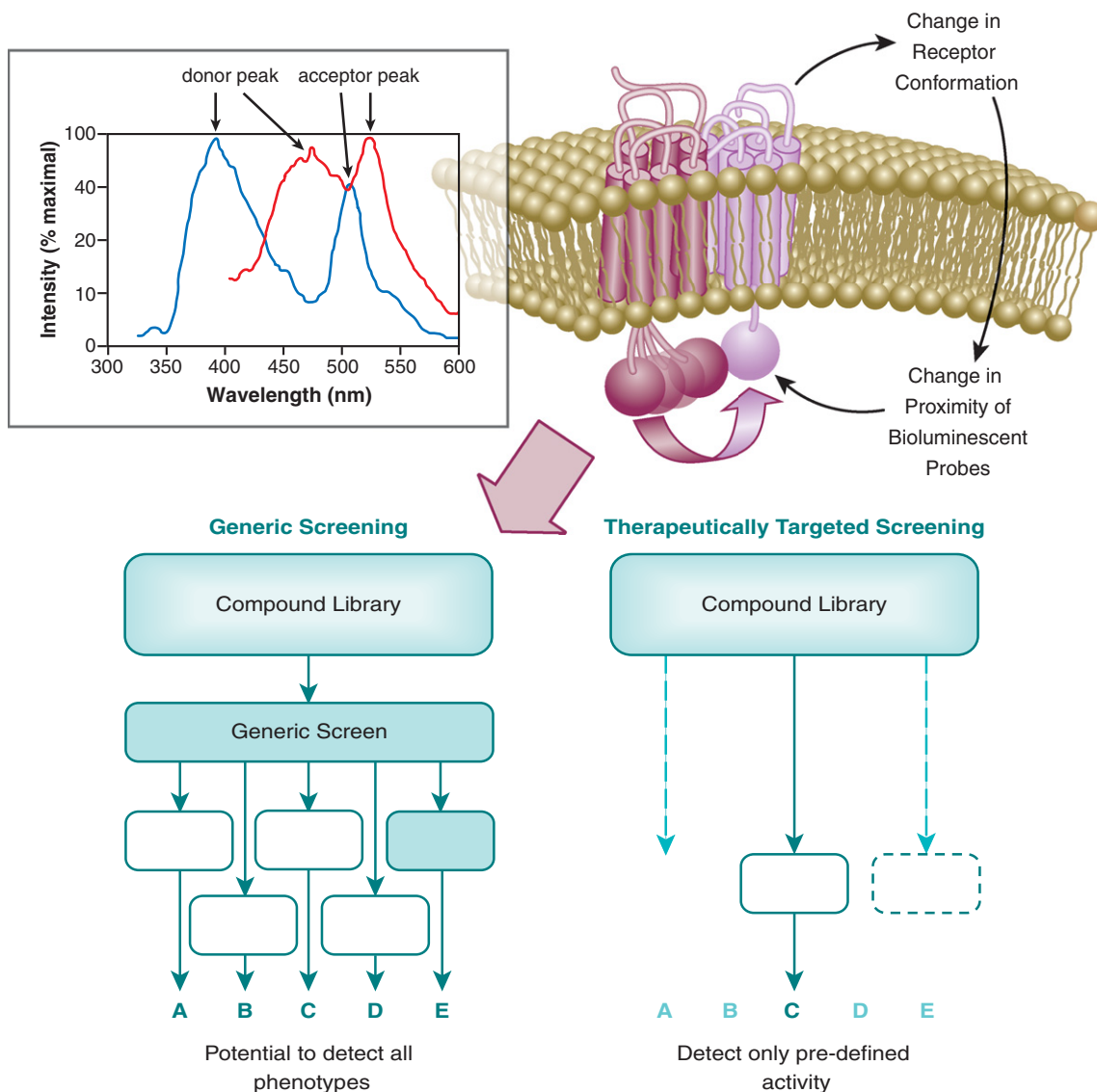


FIGURE 8.15 “Generic” screening using bioluminescence resonance energy transfer (BRET), which detects changes in receptor conformation through ligand binding. Two probes are placed on the receptor protein, which have a characteristic bioluminescence signal that changes when the distance between them is altered. Changes in receptor conformation cause a change in the relative position of the probes, which then causes a change in the luminescence signal. This type of assay detects all compounds that bind to the receptor and cause a conformational change; as discussed in the context of the probabilistic model of receptor function, this could essentially entail all compounds that bind to the receptor (see Chapter 3). This detection is based on the principle that the ligand-bound receptor is thermodynamically different from the unliganded receptor. Secondary testing of the subset of binding molecules (a much smaller set than the original library) can then sort compounds with respect to function. A contrasting approach uses a therapeutically relevant screen, where a specific receptor-coupling pathway is chosen for detection, depends on the assumption that the pathway is all that is required for therapeutic activity. With this approach, ligands with unknown potential may not be detected, and the strategy may not be successful if the chosen pathway is the incorrect one.

activities that may be therapeutically relevant (Figure 8.15). This is an alternative to presupposing the therapeutically relevant receptor coupling (i.e., cyclic AMP) and screening on that basis. For example, the β -blocker propranolol does not produce elevation of cyclic AMP and thus would not be detected as an agonist in a cyclic AMP assay. However, in assays designed to detect ERK (extracellular signal-related kinase) activation, propranolol is an active ERK agonist [18]. These data underscore the importance of the assay in drug detection.

8.4 DRUG DISCOVERY AND DEVELOPMENT

Once hits have been identified, they must be confirmed. The test data obtained from a screen form a normal distribution. One criterion for determining possible active molecules is to retest all initial values $>3\sigma$ units away from the mean; this will capture values for which there is $>99.3\%$ probability of being significantly greater than the mean of the population (see Figure 8.16). The distribution of the apparently active

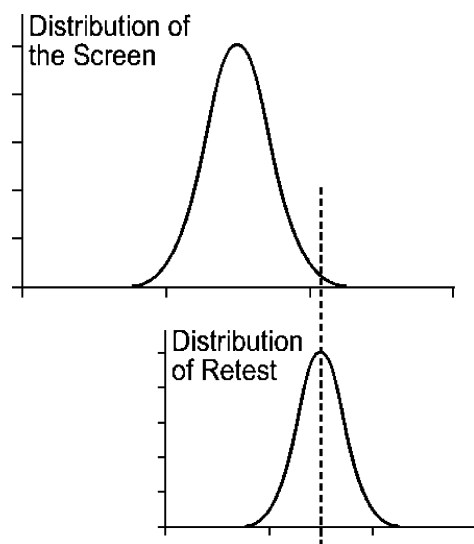


FIGURE 8.16 Confirmation of initial hits in the HTS. Top panel shows the distribution of values from a single test concentration of a high-throughput screen. The criteria for activity and subsequent retest are all values >3 standard error units away from the mean (dotted line). The process of retesting will generate another distribution of values, half of which will be below the original criteria for activity.

compounds, when retested, will have a mean centered on the 3σ value for the distribution of the total compound set. It can be seen that 50% of these will retest as active (be greater than 3σ units away from the initial total compound set mean). Therefore the compounds that retest will have a 99.85% probability of having values greater than the mean of the original data set. The criteria for retest may be governed by practical terms. If the hit rate is inordinately high, then it may be impractical to test all hits that give values $>3\sigma$ units from the mean; a lower (having a greater probability of retest) number of “hits” ($>4\sigma$ or 5σ units away from the mean) may need to be tested to reduce the retest load.

Another important concept in the process of early confirmation of lead activity is ligand-target validation. The first, and most obvious, criterion for selective target interaction is that the ligand effect is observed in the host cell only when the target is present. Thus, in a cell-based assay using cells transfected with receptor, the response to a putative agonist should be observed only in the transfected cell line and not in the host cell line (or at least a clearly different effect should be seen in the host cell line; see Figure 8.17).

There are two general types of observable biological response: agonism and antagonism. The lead optimization process is the topic of Chapter 10, where specifics of the methods and theory of determining molecular activity are outlined. However, there are common issues for all drug discovery programs where pharmacology plays a central role; it is worth considering these.

Table 8.3 shows some of the major issues that drug discovery teams deal with throughout a discovery–development program. Two of the first tasks for these teams

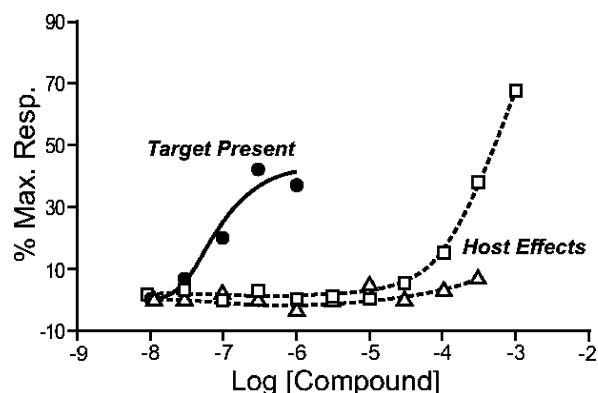


FIGURE 8.17 Ligand-target validation. Dose-response curves to a putative agonist for a therapeutic target on cell lines transfected with the target receptor (filled circles) and on cell lines not transfected with the target receptor (dotted lines, open circles, and open triangles). The open symbol curves reflect nonspecific and nontarget-related effects of the compound on the host cell line. The clear differentiation between the target curves and the host curves indicate a specific effect on the therapeutically relevant target.

TABLE 8.3 Issues at Various Stages of Drug Discovery and Development

A. Early Discovery Phase

- Accomplish target validation (is this worth the effort?)
- Identify biological reagents and assay design for
 - Screening
 - Lead optimization
 - Animal orthologues of target
- Develop animal models for efficacy.
- Design critical path and lead criteria.
- Create information technology system for data analysis and data visualization tools.
- Run the screen; identify hits and assess chemical tractability.

B. Lead Optimization Phase

- Identify tractable scaffold candidate for chemistry.
- Synthesize numerous analogues for enhancement of activity and selectivity.
- Identify SAR for primary activity and selectivity.
- Explore all facets of scaffold for intellectual property protection and follow-up.
- Explore possible spin-offs for other indications.
- Attain activity with druglike properties to achieve candidate selection (first time in humans).

C. Clinical Development Phase

- Define NOAEL (no observed adverse effect level) and MRSD (maximum recommended starting dose) for clinical trial.
- Synthesize numerous analogues for enhancement of activity and selectivity for follow-up candidate(s).
- Explore other clinical indications.

are to define the lead criterion for success and the critical path designed to get there. Table 8.4 shows some example lead criteria in terms of chemistry, pharmacology, and pharmacokinetics. A critical path can evolve throughout a program being more concerned with discovery, quantification, and optimization of primary target activity in the early stages and more on required druglike properties of molecules (pharmacokinetics) and issues of safety pharmacology in later stages. One important aspect of a critical path is the type of assay that controls progress; a clear simple readout is required. In contrast, assays that do not necessarily control compound progress (so-called “texture” assays that more fully describe a compound but do not furnish critical data for progression) should not be on the critical path since these type of data tend to obscure development. Also, the proper placement of assays is important because progression assays placed early on in the path may preclude exploration of chemical scaffolds that will later define flexible structure-activity relationships that can be used to optimize pharmacokinetics and/or eliminate safety issues. However, if these assays are placed too near the end of the critical path (i.e., near the point where the structure-activity relationships are defined in detail), then a “dead end” may be reached whereby a progression-stopping activity may be encountered without sufficient options for alternate structures.

In the lead optimization phase of discovery and development is the iterative process of testing molecules, assessing their activity, and synthesizing new molecules based on that data (determining a structure-activity relationship, SAR). If there is a single index of activity, then the attainment of an improved potency (as determined by statistics) is a useful approach. One way to do this is to test the molecules repeatedly, determine a mean value with a measure of variation (standard deviation), and use those measurements to determine a confidence limit for that estimate. One proposed confidence limit that rapidly leads to comparison of multiple estimates is the 84% confidence limit of a mean [19]. For example, if four measurements yield a mean estimate pIC_{50} of 7.1 with a standard deviation (s_x) of 0.13, then the 84% confidence limits can be calculated as

$$\text{Confidence limit} = s_x \bullet t_{0.16} \bullet (n)^{-1/2}, \quad (8.4)$$

where the $t_{0.16}$ is the value for 84% confidence limits and the standard deviation based on a sample (s_x) is

$$s_x = \sqrt{\frac{n \sum x^2 - (\sum x)^2}{n(n-1)}}. \quad (8.5)$$

For this example, $t = 1.72$, therefore the 84% confidence limits for this estimate are $7.1 \pm (1.72 \times 0.13) = 7.1 \pm 0.22 = 6.9$ to 7.32 . This means that 84% of the

TABLE 8.4 Lead Criteria

Chemical

- Novel active structures (activity not due to impurity).
- Search prior art and correct analysis of hit composition.
- Demonstrable SAR (activity can be quantified and associated with specific changes in chemical structure).
- Druglike physicochemical properties, stable, fulfilment of “Lipinski rules,” and good solubility.
- Chemically tractable scaffolds, not complex (amenable to analogue synthesis).

Biological

- Confirmed pharmacology for determination of affinity, efficacy, target geography, and kinetics of interaction with target in system-independent manner.
- Demonstrable interaction with target (pharmacological validation with no effect in absence of target).
- Selective for target with acceptable liability.
- Defined genetic polymorphisms (<2% population).

Preferred Features

- There is a number of tractable hit series.
- There is good permeation potential ($\log P_{APP}$ value > -5.0 desirable).
- Blood-Brain Barrier Entry potential, usually desirable — see Chapter 9.
- No evidence of induction or binding to CYP450s — see Chapter 9.
- *In vitro* metabolic stability (i.e., S9 metabolism $<50\%$ at 1 hr) — see Chapter 9.
- There are sites available to modify pharmacokinetics that do not affect primary activity *in vivo*, a generally good pharmacokinetic profile.
- There is low protein binding.
- No genotoxicity evident.
- There is 100-fold separation between potency at primary target and cytotoxicity.

Strategic

- Existence of acceptable intellectual property (determined IP position and competitive landscape).
- Target is therapeutically relevant (strong association between target and disease in literature), not associated with toxicology.

TABLE 8.5 Primary Activity Data for a Series of Compounds

#	Compound	pIC ₅₀	STD	84% conf. limit
1	ACS55542	7.1	0.13	6.81 to 7.38
2	ACS55549	7.25	0.13	6.67 to 7.23
3	ACS55546	6.9	0.15	6.57 to 7.3
4	ACS55601	7.36	0.17	7 to 7.73
5	ACS55671	7.2	0.16	6.85 to 7.55
6	ACS55689	7.75	0.16	7.4 to 8.5
7	ACS55704	7.5	0.07	7.35 to 7.65
8	ACS55752	7.8	0.14	7.49 to 8.1
9	ACS55799	7.65	0.1	7.43 to 7.87
10	ACS55814	7.86	0.12	7.6 to 8.1

time, the true value of the pIC₅₀ will lie between those values based on this estimate. The significance of the 84% confidence limits lies in the statistical evidence that it may be concluded that two samples from different populations (i.e., two pIC₅₀s are different) if their 84% confidence limits do not overlap [19]. This provides a simple method of sorting through a series of compounds to determine which changes in chemical structure produce statistically significant improvements in activity. For example, Table 8.5 shows a series of pIC₅₀ values for a range of related compounds; these data are shown graphically in Figure 8.18. It can be seen from these data that significant improvements in potency, from the base compound 1, are achieved with compounds 6, 8, 9, and 10.

It is imperative to have a simple unambiguous scale of activity to guide SAR, but there can be more than one such guide required (multivariate SAR). For

example, if two related targets or activities are involved and selectivity between the two is required, then the scale of absolute activity and the ratio between two activities (selectivity) are relevant [20]. Table 8.6 shows the activity of 10 compounds with activities on two receptors; the aim of the program is to optimize the activity on receptor A and minimize the concomitant activity on receptor B (optimize the potency ratio of A to B). The standard deviation for the ratio of activities on A and B is given by

$$s_{A/B} = \sqrt{\frac{(n_A - 1)s_{xA}^2 + (n_B - 1)s_{xB}^2}{n_A + n_B - 2}}. \quad (8.6)$$

The corresponding confidence limit on the selectivity ratio is given as

$$\text{Confidence limit} = t \bullet s_{A/B} \sqrt{\frac{1}{n_A} + \frac{1}{n_B}}. \quad (8.7)$$

With the assessment of the error on the ratio comes the possibility to statistically assess differences in selectivity between compounds. For example, for given compounds 1 and 2, the standard deviation of the selectivity is given as

$$s_{\text{diff}} = \sqrt{\frac{df_1 s_{(A/B)1}^2 + df_2 s_{(A/B)2}^2}{df_1 + df_2}}, \quad (8.8)$$

where $df_1 = N_1 - 2$ where N_1 is the sum of the values used to calculate selectivity 1 and $df_2 = N_2 - 1$ where N_2 is the sum of the values used to calculate selectivity 2. This, in turn, allows the calculation of the confidence limits for the selectivity of compounds as

$$\text{Confidence limit} = t \bullet s_{\text{diff}} \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}. \quad (8.9)$$

Just as the effects of changes in chemical structure on the primary activity could be rapidly tracked through overlap of 84% confidence limits of the primary pIC₅₀s, the effects of structural changes on selectivity can be tracked

FIGURE 8.18 Graphical display of data shown in Table 8.5. The first compound in the series had a pIC₅₀ of 7.1 (shown in red); bars represent 84% confidence limits. Compounds 2 to 5 had estimates of 84% confidence limits that cross the 84% limits of the original compound, therefore no improvement in activity was produced by these changes in structure. However, compounds 6, 8, 9, and 10 (in blue) had means and 84% confidence limits that were different from that of the original compound, therefore these represent improvements in activity.

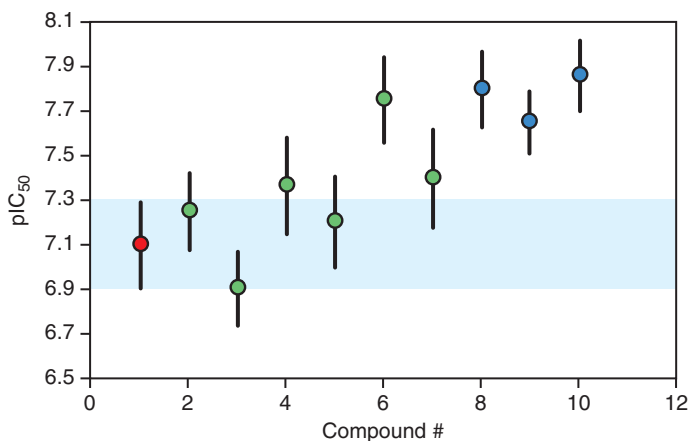


TABLE 8.6 Primary Activity Data + Selectivity Data for a Series of Compounds

	Compound	pIC ₅₀ Recept. A	STD _A	n _A	pIC ₅₀ Recept. B	STD _B	n _B	ΔpIC _{50A-B}	STD _{A/B}	84% c.l. of selectivity
1	ACS66002	6.95	0.310	10	6.32	0.360	19	0.625	0.434	0.38 to 0.87
2	ACS68013	7.49	0.201	4	5.86	0.250	14	1.630	0.279	1.4 to 1.86
3	ACS62071	8.18	0.269	14	8.63	0.360	18	-0.451	0.443	-0.68 to -0.22
4	ACS64003	8.67	0.168	9	6.12	0.320	21	2.553	0.346	2.35 to 2.75
5	ACS60052	9.12	0.260	17	9.04	0.290	14	0.084	0.426	-0.14 to 0.30
6	ACS58895	9.38	0.200	10	8.32	0.330	9	1.064	0.419	0.78 to 1.35
7	ACS61004	8.00	0.140	8	7.90	0.320	7	0.100	0.388	-0.2 to 0.4
8	ACS64021	7.80	0.160	6	8.30	0.210	5	-0.500	0.319	-0.8 to -0.2
9	ACS67091	8.40	0.110	7	7.90	0.340	7	0.500	0.391	0.19 to 0.8
10	ACS68223	8.90	0.130	8	7.85	0.250	6	1.050	0.328	0.78 to 1.3

ΔpIC_{50A-B} = logarithm of the ratio of potencies for Receptor A vs. Receptor B.

STD_{A/B} = standard deviation of the selectivity of activity of Receptor A vs. Receptor B according to Equation 8.8.

84% c.l. of selectivity = the 84% confidence limits of the selectivity according to Equation 8.9.

through overlap of 84% confidence limits on selectivity. The data shown in Table 8.6 and Figure 8.19 illustrate a complication of multivariate SAR. Specifically, there might be separate SAR for primary activity and selectivity, making integration of both activities into one

molecule difficult. As seen in Figure 8.19A, the most potent compound is not the most selective.

The type of critical path and whether primarily single variate or multivariate SAR is operative sometimes depends on the type of drug the program is aimed to deliver.

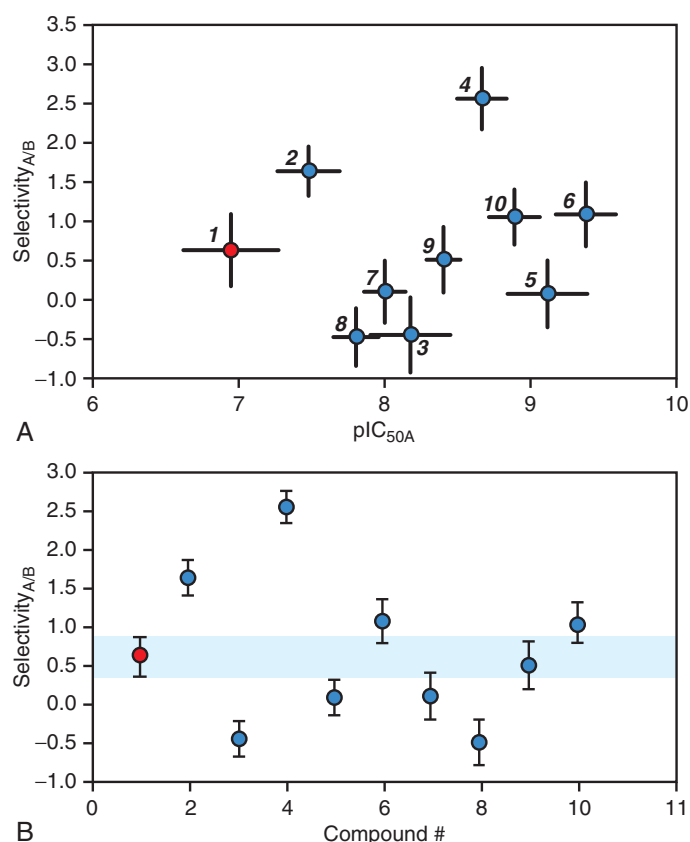


FIGURE 8.19 Multivariate structure activity relationships. (A) Compound data summarized in Table 8.6 expressed as the pIC₅₀ for the therapeutically relevant activity (activity A) as abscissae and the logarithm of the selectivity of the same compound for activity A versus B (high number is favorable) as ordinates. Bars represent standard deviations. Compound 1 (red) represents the original molecule in the active series. Note also how the most selective compound (compound 4) is not the most potent compound (compound 6). (B) Graph representing the logarithms of the selectivity of the compounds shown in panel A with bars showing 84% confidence limits. Compounds with 84% confidence limits outside of the limits of the original compound (compound 1 in red) represent compounds either less selective (compounds 3, 5, 8), of equal selectivity (compounds 6, 7, 9, 10) or greater selectivity (compounds 2, 4).

A therapeutically useful drug may simply be an improvement over existing therapy in the class. The primary questions to be answered are the following:

- Is the molecule active at primary target? (Potency and efficacy.)
- Is the molecule promiscuous? (Selectivity.)
- Is the molecule toxic? (Safety pharmacology.)
- Is the molecule absorbed, distributed, and does it have sufficient $t_{1/2}$? (Adequate druglike qualities and pharmacokinetics.)

A slightly more rigorous or novel approach may be required for the delivery of a drug that will be novel in the class or a completely new therapeutic entity. When the program is focused on such a chemical target, the preceding questions are still relevant, as well as a few additional questions:

- Is the molecule different from previous molecules and all other available therapy?
- Does this molecule incorporate the newest knowledge of disease and pharmacology?

Another feature of this latter type of program is the need for more critical path assays to define and differentiate unique activity.

8.4.1 Safety Pharmacology

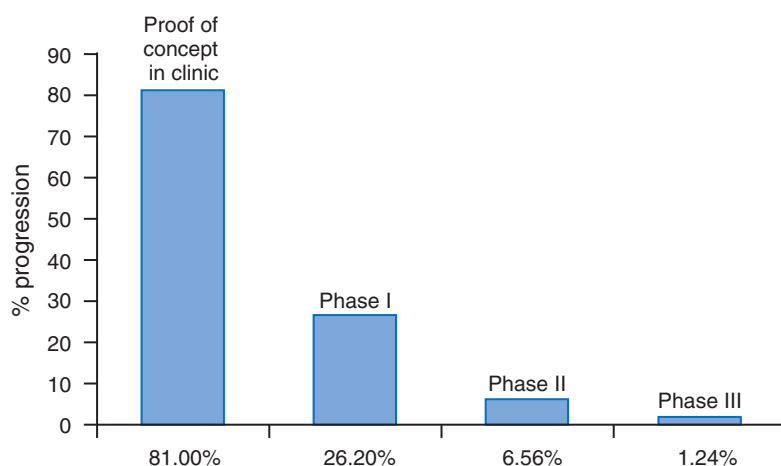
For the remainder of this chapter, it is assumed that the hit from screening has been through the lead optimization process to the point where it can be considered a drug candidate. As shown in Figure 8.1, the next stages involve the developability of the molecule(s) in terms of pharmacokinetics, pharmacodynamics, and propensity for adverse drug reactions.

The preceding discussion involves the elucidation of the primary hit and lead activity, obviously a crucial step in the drug discovery process. However, there are numerous other reasons why a molecule with good primary

activity may still fail as a drug, and it is becoming increasingly clear that the factors that lead to this failure need to be addressed as early as possible in the lead optimization process. Figure 8.20 shows the outcome of a risk analysis for the probability of a new compound emerging as a drug; it can be seen that attrition is extremely high. An active molecule must be absorbed into the body, reach the biological target, be present for a time period sufficient for therapeutic activity, and not produce untoward side effects. It will be seen that an important part of the lead optimization process is to incorporate these properties into the primary lead molecule early on in the process [21]. One reason this is important is that the concepts involved are, in some cases, diametrically opposed. For example, while low molecular weight is a known positive property of drugs, the lead optimization process generally results in increased molecular weight as pharmacophores are added to increase potency. For this reason, the concept of “lead likeness” [22] can be used to determine the suitability of lead molecules for beginning the lead optimization process (*vide infra*). The problems involved in introducing lead likeness into screening hits is exacerbated by the fact that, as analogs become more potent, there is less tolerance for chemical analoging to improve physicochemical properties. In fact, it is a general observation that there often are relatively minor differences between leads and launched drug candidates (see Figure 8.21) [29]. On the other hand, there is abundant evidence to show that apparently very minor changes in chemical structure can impose large effects on biological activity (see Figure 8.22).

New drugs must be efficacious, reach the site of action, and do no harm; this latter condition is the subject of drug liability studies. For the decade 1991–2000, new drug registration was a mere 11% of compounds submitted for first in human studies with toxicity and safety issues accounting for approximately 30% of the failures. There are clear “zero tolerance” toxicities and those that are tolerable with tolerance depending on the indication, patient population (i.e., age and gender), length of

FIGURE 8.20 Attrition of molecules as they are taken through the clinical testing procedure. It can be seen that very few become drugs (1.34%). Redrawn from [21].



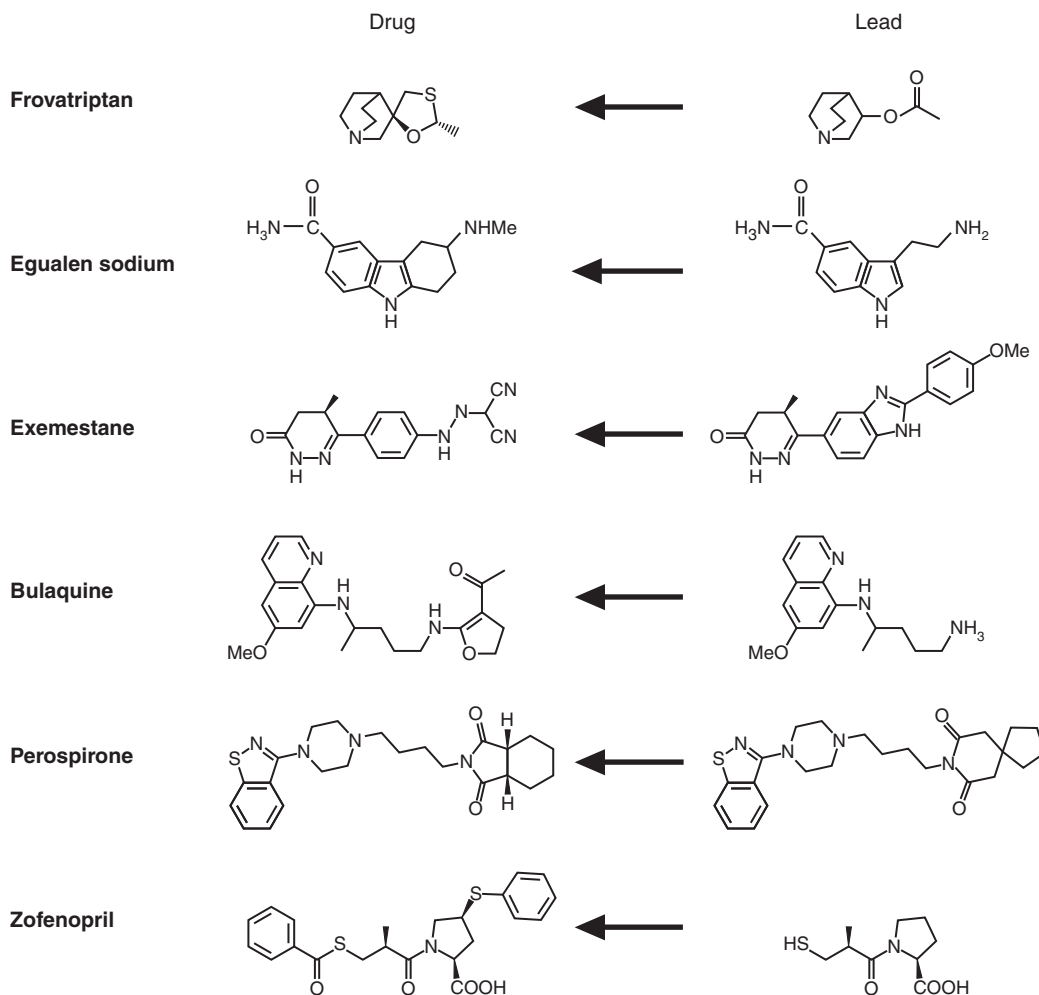


FIGURE 8.21 Structural relationships between the initial lead for a molecule and the eventual drug. It can be seen that changes in structure are, in some cases, not extensive. Data shown for frovatriptan [23], egualen sodium [24], exemestane [25], bulaquine [26], perospirone [27], and zofenopril [28]. Drawn from [29].

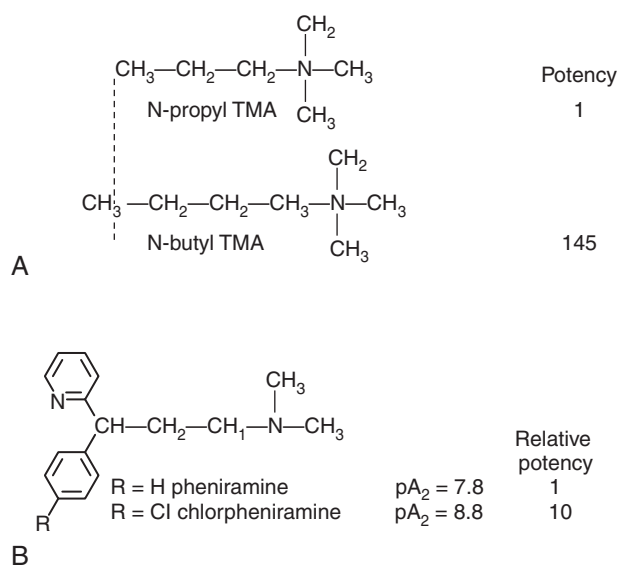


FIGURE 8.22 Small changes in the chemical structure of N-propyl tetramethylammonium and pheniramine produce 145- and 10-fold increases in potency respectively.

treatment, and seriousness of illness. Table 8.7 shows a number of common side effects of drugs when tested in clinical trial. Toxicity is assessed in a number of ways; a commonly used index is the therapeutic index, which is the ratio of the concentration of drug required to produce 50% maximal therapeutic effect (or therapeutically active in 50% of the population) and the concentration producing 50% toxic effect (toxic in 50% of the population); see Figure 8.23A. Another, and more stringent, scale is the “margin of safety,” which is the ratio of drug that is 99% effective over the concentration that produces 1% incidence of toxic effect (Figure 8.23A). The margin of safety of some commonly used drugs can be strikingly low; for example, Figure 8.23B shows the incidence of side effects with theophylline with a less than 2-fold margin between effect and incidence of mild side effects to a 3.5-fold margin between effect and serious side effects [30]. Side effects commonly arise from exaggerated effects at the primary target (mechanism-based toxicity), or problems with dosing, prolonged use, or cytotoxicity

TABLE 8.7 Major Adverse Side Effects Associated with Clinical Use of Drugs

Cardiovascular	Hematology	Renal
arrhythmias	agranulocytosis	nephritis
hypotension	hemolytic anemia	nephrosis
hypertension	pancytopenia	tubular necrosis
congestive heart failure	thrombocytopenia	renal dysfunction
angina, chest pain	megaloblastic anemia	bladder dysfunction
pericarditis	clotting, bleeding	nephrolithiasis
cardiomyopathy	eosinophilia	
Dermatology	Musculoskeletal	Respiratory
erythemas	myalgia, myopathy	airway obstruction
hyperpigmentation	rhabdomyolysis	pulmonary infiltrates
photodermatitis	osteoporosis	pulmonary edema
eczema		respiratory depression
urticaria		nasal congestion
acne		
alopecia		
Endocrine	Metabolic	Ophthalmic
thyroid dysfunction	hyperglycemia	disturbed color vision
sexual dysfunction	hypoglycemia	cataract
gynecomastia	hyperkalemia	optic neuritis
Addison syndrome	hypokalemia	retinopathy
galactorrhea	metabolic acidosis	glaucoma
	hyperuricemia	corneal opacity
	hyponatremia	
Gastrointestinal	Neurological	Otological
hepatitis, hepatocellular damage	seizures	deafness
constipation	tremor	vestibular disorders
diarrhea	sleep disorders	
nausea, vomiting	peripheral neuropathy	
ulceration	headache	
pancreatitis	extrapyramidal effects	
dry mouth		
		Psychiatric
		delirium, confusion
		depression
		hallucination
		drowsiness
		schizophrenia, paranoia
		sleep disturbances

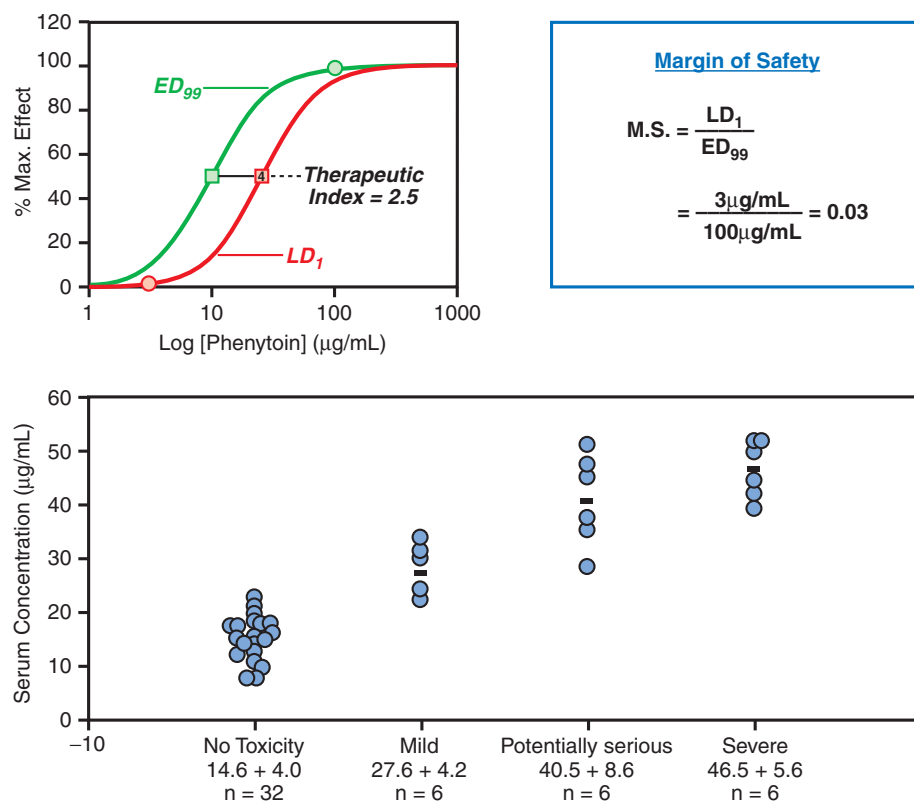


FIGURE 8.23 Expressions of relative safety of drugs. (A) Dose-response curves for phenytoin therapeutic activity (green) and toxicity (red). Shown also are the ED₅₀ values used to calculate therapeutic index and ED₉₉ values used to calculate margin of safety. (B) Toxic effects of theophylline illustrating the narrow margin between the no toxic effect dose (14.6 μg/mL), mild toxic effects (1.9-fold>), potentially serious side effects (2.8-fold>), and severe side effects (3.2-fold>). Data redrawn from [30].

Margin of Safety

$$\text{M.S.} = \frac{\text{LD}_{50}}{\text{ED}_{99}}$$

$$= \frac{3 \mu\text{g/mL}}{100 \mu\text{g/mL}} = 0.03$$

(i.e., hepatotoxicity and bone marrow toxicity). Table 8.8 shows some classifications of toxicity. Effects on receptors, ion channels, and liver metabolic enzymes also account for major drug liabilities. In most cases, such as effects on receptors, the untoward effects are a direct

result of the receptor activation (or blockade). Table 8.9 shows some cardiovascular side effects commonly associated with some 7TM receptors [31]. In some cases, the receptor activity belies effects that are not obvious. For example, muscarinic m3 receptor activity has been associated with type 2 diabetes [32].

Clearly it would be advantageous to detect possible safety issues with candidate molecules as early in the selection process as possible so as to not waste time and resource on the development of drugs that will fail in the clinic. As with pharmacokinetic *in vitro* testing (*vide infra*), there are a number of simple *in vitro* tests that can be done to detect future safety issues. For example, promiscuous receptor activity is a potential problem with drugs, therefore rapid *in vitro* tests on panels of receptors known to be associated with toxic effects can be done on candidate chemical scaffolds. Table 8.10 shows a short list of “repeat offenders” in the receptor world that have been associated with a range of toxic effects in humans. Similarly, hydrophobic drugs have been shown to have affinity for calcium channels and, notably, potassium channels. This latter activity is a clear liability since blockade of the hERG potassium channel can lead to cardiac QTc prolongation and a condition called *torsades de pointes*, a potentially fatal cardiac arrhythmia

TABLE 8.8 Classifications of Toxic Effects

Type of Toxicity	Example
Undesired expected effects	Digital tremor with β-agonist bronchodilators due to β ₂ -adrenoceptor stimulation
Desired excessive effects	Insulin-induced hypoglycemic reaction
Undesired unexpected	Hypertensive crisis for treatment of depression with MAO inhibitor: consumption of cheddar cheese and beer (tyramine)
Poorly predictable	Drug allergies, idiosyncratic, mutagenesis, carcinogenesis, drug dependency

TABLE 8.9 Some Cardiovascular Targets Associated with Adverse Drug Effects

Target		Possible Adverse Drug Effects	
adenosine A1	bradycardia	AV-block	renal vasoconstriction
adenosine A2a	hypotension	coronary vasodilation	platelet aggregation
adenosine A3	mediator release		
α_{1a} -adrenoceptor	hypertension	orthostatic hypotension	inotropy
α_{1b} -adrenoceptor	orthostatic hypotension		
α_{2a} -adrenoceptor	hypertension	possible hyperglycemia	
α_{2b} -adrenoceptor	hypertension	cardiac ischemia	vasoconstriction
	central ↓ blood pressure		
α_{2c} -adrenoceptor	hypertension	cardiac ischemia	skel. muscle blood flow
β_1 -adrenoceptor	cardiac inotropy	heart rate	ventricular fibrillation
	bronchospasm		
β_2 -adrenoceptor	fascil. cardiac arrest	impairs cardiac perform.	
angiotensin AT ₁	hypertension	cell proliferation, migration	tubular Na ⁺ resorption
bradykinin B ₁	nociception	inflammation	cough
bradykinin B ₂	nociception	inflammation	cough
CGRP	hypocalcemia	hypophosphatemia	
Ca ²⁺ channel	hypotension		
dopamine D ₁	induces dyskinesia	vasodilatation, schizophrenia	↓ coordination
endothelin ET _a	vasoconstriction	cell proliferation	aldosterone secretion
endothelin ET _b	vasoconstriction	cell proliferation	bronchoconstriction
histamine H ₃	↓ memory, sedation	vasodilatation	↓ GI motility
muscarinic m1	Δ blood pressure	↓ GI secretion	
muscarinic m2	vagal effects	Δ blood pressure	tachycardia
muscarinic m3	vagal effects, salivation	Δ blood pressure, dry mouth	↓ ocular accommodation
muscarinic m4	vagal effects, salivation	Δ blood pressure	facilitates D1 stim.
NE transporter	adrenergic hyperreactivity	facilitates α-activation	
nicotinic Ach	autonomic functions	palpitations, nausea, sweating	tremor, ganglionic function
NPY ₁	venous vasoconstriction	↓ gut motility, gastric emptying	anxiogenic
K ⁺ channel (hERG)	cardiac QTc prolongation		
K ⁺ channel [ATP]	hypotension, hypoglycemia		
5-HT _{2b}	cardiac valvulopathy		
5-HT ₄	facilitates GI transit	mechanical intestinal allodynia	
Na ⁺ channel (site 2)	cardiac arrhythmia		
thromboxane a ₂	vascular constriction	bronchial constriction	allergic inflamm, platelet ag.
vasopressin V _{1a}	vasopressor		
vasopressin V _{1b}	vasopressor, anxiogenic		

Taken from [31].

TABLE 8.10 Some General Seven Transmembrane Receptors Noted for Producing Toxic Effects

General Tox	GI Tox	CV Tox	CV Tox
5-HT _{2A}	5-HT _{1A}	5-HT ₄	Muscarinic m3
5-HT _{2B}	5-HT _{1p}	α_{1A} -adrenoceptor	Muscarinic m4
α_{1A} -adrenoceptor	5-HT _{2A}	α_{1B} -adrenoceptor	Nicotinic Ach
α_{1B} -adrenoceptor	5-HT _{2B}	α_{2A} -adrenoceptor	NPY ₁
α_{2A} -adrenoceptor	5-HT ₃	α_{2B} -adrenoceptor	Thromboxane A2
Adenosine 2A	5-HT ₄	α_{2C} -adrenoceptor	Vasopressin V _{1a}
Adenosine A1	α_{2A} -adrenoceptor	Adenosine 2A	Vasopressin V _{1b}
β_1 -adrenoceptor	α_{2B} -adrenoceptor	Adenosine A1	
β_2 -adrenoceptor	α_{2C} -adrenoceptor	Adenosine A3	
Bradykinin B2	CCK2	Angiotensin AT1	
Cannabinoid CB1	Dopamine D2	β_1 -adrenoceptor	
Dopamine D2	δ -opioid	β_2 -adrenoceptor	
Histamine H1	EP2	Bradykinin B1	
μ opioid	EP3	Bradykinin B2	
Muscarinic m1	Gastrin	Cannabinoid CB1	
Purinergic P2Y1	Histamine H2	CGRP	
	μ opioid	Dopamine D2	
	Motilin	Endothelin A	
	Muscarinic m2	Endothelin B	
	Muscarinic m3	Histamine H3	
	SST1	Muscarinic m1	
	VIP	Muscarinic m2	

GI = gastrointestinal.
CV = cardiovascular.

(see Figure 8.24). Other promiscuous targets are the pregnane X-receptor, a nuclear receptor associated with regulation of cytochrome P450 enzymes. Induction of PXR can have large effects on metabolism, drug–drug interactions, multidrug resistance, and transport mechanisms. Cytochrome P450 enzymes are particularly susceptible to drug activity due to their broad substrate specificity. Four of these enzymes, CYP3A4, CYP2C9, CYP2C19, and CYP2D6 account for 80% of known oxidative drug metabolism [33]. Blockade of these enzymes can lead to detrimental interactions with other drugs. For example, the antihistamine terfenadine was high affinity for the hERG channel (leading to serious liability). This drug is

rapidly metabolized and the metabolite fexofenadine is weakly active at the hERG channel. However, in the presence of other drugs that interfere with terfenadine metabolism (cytochrome enzymes), this antihistamine poses a serious risk of life-threatening arrhythmia.

Drug-induced mutagenicity, whereby a drug induces mutation of DNA transcription products, can be a devastating liability since such effects can lead to cancer. Also, the effects may not be detected until very late in the drug development process. In fact, their detection may require use of the drug in very large populations, larger than those practical for any Phase III clinical trial. Therefore, early *in vitro* prediction of such effects can be extremely

Blockade of the HERG Channel

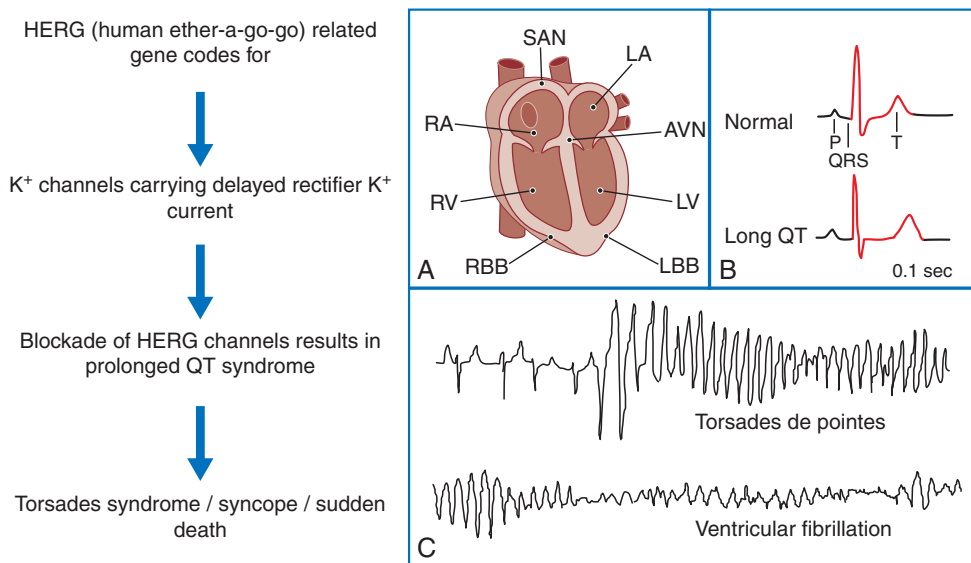


FIGURE 8.24 Schematic diagram showing sequence of events beginning with the blockade of the hERG potassium channel in the heart that causes QT-interval prolongation that eventually can lead to a potentially fatal arrhythmia called *torsades de pointes*.

important. One general test that has been used is an *in vitro* genetic toxicology test to determine mutagenic properties of a compound called the *Ames test*. Devised by a group led by Bruce Ames in the 1970s at Berkeley, California, it utilizes defective (mutant) salmonella that is unable to use external histidine for growth. When these bacteria are cultured in absence of histidine, they do not grow unless a mutation causes revertant (back mutation) that enables it to grow in the absence of histidine. In addition, a mixture of revertants is used that contains liver enzymes to produce possibly mutant metabolites. Mutations are also facilitated by introduction of genes responsible for lipopolysaccharide synthesis to make the cell more permeable to chemicals. This test is simple, rapid, and quite predictive, although not perfect. For example, dioxin causes cancer but is not positive in the Ames test. Table 8.11 shows some common *in vitro* tests available to detect toxicity in compounds at an early stage of development.

Another rapid potential method of detecting safety issues is pharmacophore modeling of “antitargets” [34]; these can be used to “virtually screen” for potential problematic drug activity. Figure 8.25 shows some known “toxicophores” associated with mutagenicity (and hence, a risk for the production of cancer). Such data can assist medicinal chemists as they produce analogues for candidate selection. As seen in Figure 8.25B, while such modeling can potentially predict mutagenicity [35], these predictions are not absolute (i.e., compounds A and C are mutagenic but compound B, although predicted to also be mutagenic, is not).

TABLE 8.11 Some *In Vitro* Assays for Estimating Toxicity

Toxicity	Assay	Potential Benefits
Cytotoxicity	MTT assay	~Measures tetrazolium salt reduction to gauge cell viability
K ⁺ channel inhibition	hERG Assay, dofetilide binding	~Measures propensity to cause life-threatening Torsades de Pointes
Mutagenicity/carcinogenicity	Ames Test	~Potential for compound to cause mutations that could lead to cancer
Receptor profiles	Binding/function <i>in vitro</i> assays	~Gauge interaction with receptors commonly regarded to mediate harmful cardiovascular, GI, and CNS side effects

Toxicophore name	Substructure representation	Example compound	
aromatic nitro			A cas: 51630-58-1
aromatic amine			
three-membered heterocycle			
nitroso			B cas: 146795-38-2
Unsubstituted heteroatom-bonded heteroatom			
azo-type			C cas: 1028-11-1
aliphatic halide			
polycyclic aromatic system			

FIGURE 8.25 Chemical functional groups that have been associated with toxicity in drugs. These pharmacophores can be used to predict potential mutagenic activity that can, in turn, lead to cancer. These data predict mutagenicity for compounds A, B, and C; mutagenic activity was verified only for compounds A and C, not B, illustrating how there can be exceptions to these predictions. Data drawn from [35].

8.5 CLINICAL TESTING

The final, but most expensive and labor-intensive step in the drug discovery process, is the testing of candidates in humans in a clinical trial setting. This is done in phases of increasing intensity and rigor. Phase I clinical trials explore the first-time exposure to humans to measure tolerance and safety in human volunteers. These trials consist of rising dose studies to determine maximum tolerated dose via expected route of administration. In addition, pharmacokinetic studies may include multiple dosing in preparation for the next step in the process; namely, Phase II trials. At this stage there may be patient involvement to more accurately reflect targeted population (i.e., geriatric, healthy patients to toxic cancer drugs) to detect special effects such differences in tolerance (i.e., schizophrenics are 200 times more tolerant of the side effects of haloperidol than are healthy volunteers).

Should a candidate demonstrate positive effects in Phase I trials, then Phase II trials (initial clinical study for treatment efficacy and continued study of safety) are

initiated. These trials are divided into two separate stages: Phase IIa trials are limited to determine some degree of efficacy, while Phase IIb trials are more extensive and expensive including a larger number of patients (100–200). At this stage, biochemical and physiological indices of efficacy are sought in a double-blind (neither patient nor clinicians know which group receives drug and which receives a placebo) setting. In addition, to a placebo arm, the FDA often requires a positive control arm (known drug, if available). If the positive control arm fails to show efficacy, the trial is a failure.

Phase III clinical trials are critical and require full-scale treatment in several medical centers. The design of these trials compares the test candidate to known treatment and placebo in a double-blind manner. The dosage used in these trials is critical as these determine regulatory decisions and marketing. The number of patients can be several hundred to thousands, and assessments of drug interactions are made at this stage.

While new drugs are approved after completion of successful Phase III trials, there is yet another stage beyond

drug approval. Thus, Phase IV clinical trials consist of postmarketing surveillance. At this point, there is monitoring of adverse effects and additional long-term large-scale studies of efficacy. There is monitoring of additional indications at this stage as well. Pharmacoeconomic data also are obtained to convince health-care payers that the new drug offers significant benefit over existing therapy (time to recovery, quality of life).

8.6 CHAPTER SUMMARY AND CONCLUSIONS

- The drug discovery process can be divided into four subsets: acquisition of chemical drug candidates, pharmacodynamic testing of large numbers of compounds (screening), optimization of pharmacokinetic properties, and optimization of pharmaceutical properties.
- Potential chemical structures for drug testing can originate from natural products, design from modeling the active site of the biological target, modification of natural substances, hybridization of known drugs, or random screening of chemical diversity.
- There is evidence to suggest that druglike structures exist in clusters in chemical space (privileged structures); identification of these can greatly enhance success in screening.
- Large-scale sampling of chemical space can be achieved with high-throughput screening. This process involves the design of robust but sensitive biological test systems and the statistical sifting of biological signals from noise. The Z' statistic can be useful in this latter process.
- Surrogate screening (utilizing similar but not exact therapeutically relevant targets) can lead to dissimulation in screening data, especially for allosteric molecules. For this reason, frequent reality testing with a therapeutically relevant assay is essential.
- The importance of the definition of lead criteria and critical paths is discussed as well as the differences involved in following single- and multiple-variate structure activity relationships.
- Active molecules also must not have toxic side effects and must have favorable pharmaceutical properties for qualification as useful drugs. There are a number of *in vitro* assays that can furnish early data to detect overt toxicity, especially for *torsades de pointes* and mutagenicity.

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