

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

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I. INTRODUCTION

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

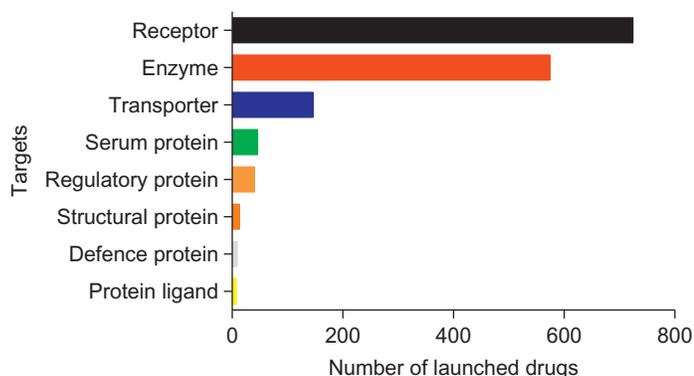


FIGURE 2.1 Number of Launched Small Molecule Drugs by Protein Target Class. As of May 2014, according to Thomson Reuters Cortellis.

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

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

II. DRUG DISCOVERY APPROACHES AND SCREENING CASCADES

A. Target Based Screening

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

B. Phenotypic Screening

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

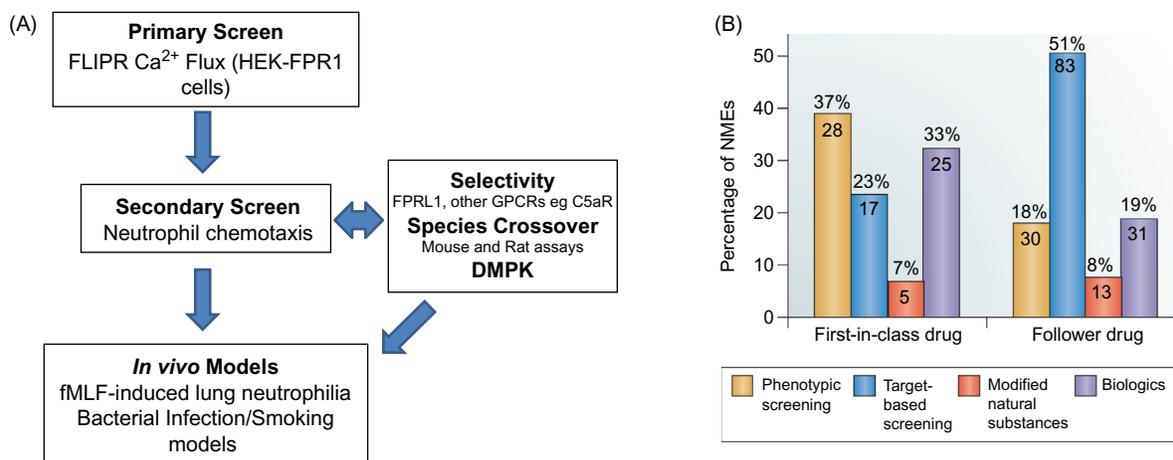


FIGURE 2.2 Screening Cascades and Drug Discovery Approaches. (A) A typical screening cascade for an early discovery program. In this instance the aim was to discover antagonists of the formyl peptide receptor, FPR1, as potential treatments for COPD. The receptor mediates a number of effects on a range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

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

III. IN VITRO ASSAYS

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

A. Primary Assays

Glossary

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

TABLE 2.1 Glossary of Key Pharmacological/Biochemical Terms

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

1. Binding assays

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

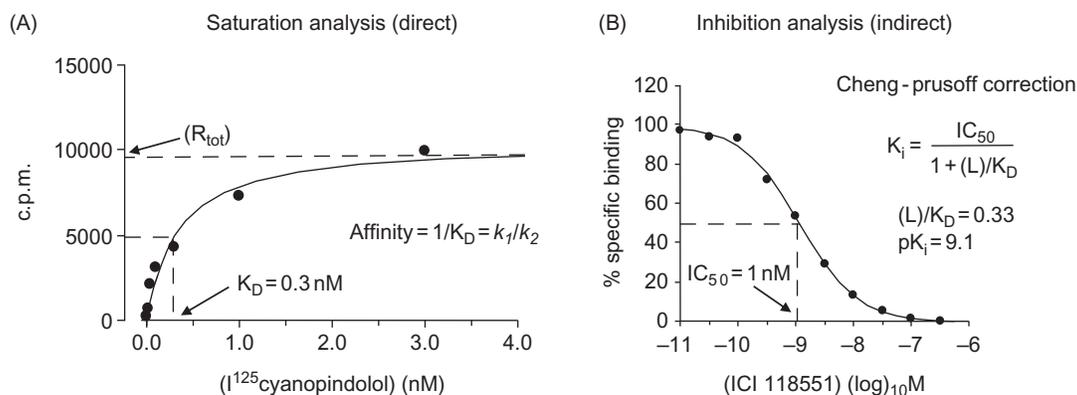


FIGURE 2.3 Binding Assays: Direct and Indirect Measurements. (A) A direct binding assay using I^{125} labeled cyanopindolol as a β_2 -adrenoceptor ligand. The curve describes a rectangular hyperbola which saturates at high ligand concentration. The ligand dissociation constant (K_D) was estimated as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

2. Binding Studies: Principles and Analysis

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

$$[LR] = \frac{[R_{tot}][L]}{[L] + K_D} \quad (2.1)$$

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

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

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

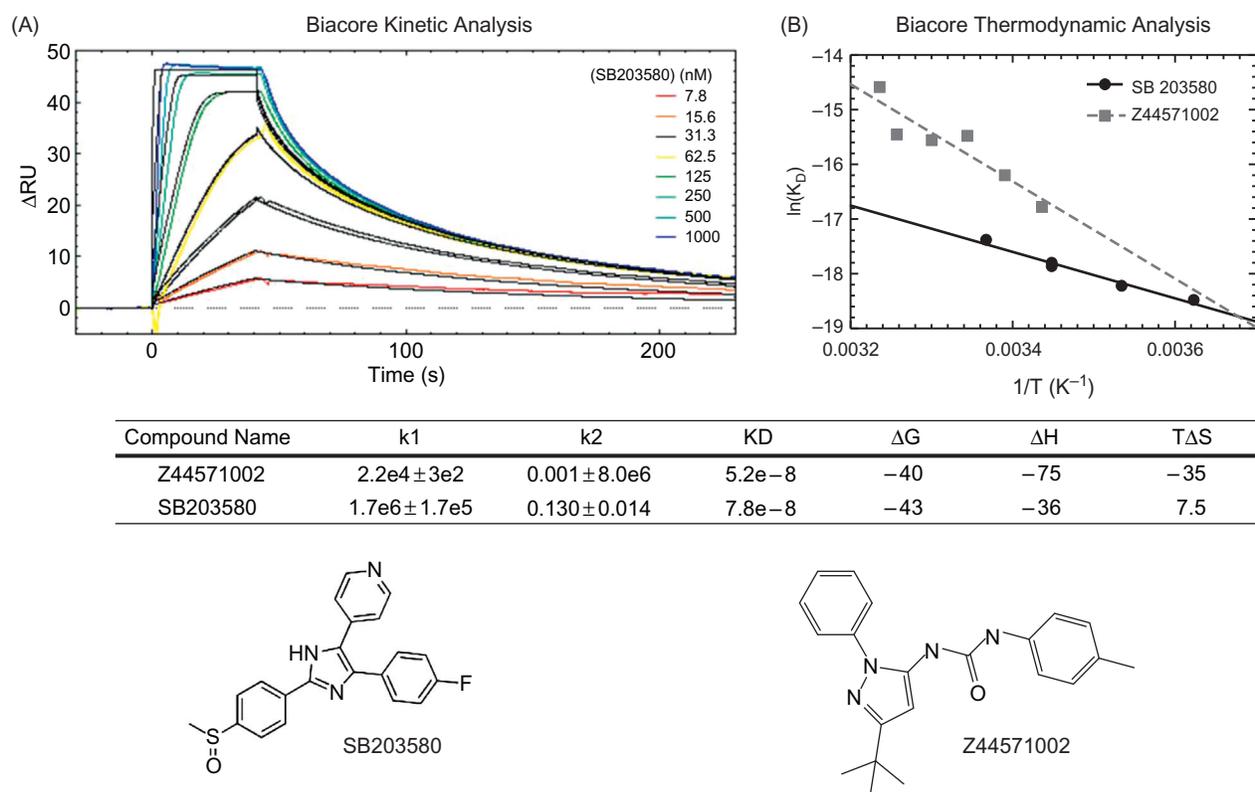


FIGURE 2.4 Binding Assays: Kinetic and Thermodynamic Measurements. Direct binding assay using the Biacore label free technique. The sensorgram (A) illustrates the time course of SB203580 binding to immobilized mitogen activated kinase p38 α . The y-axis shows the mass change resulting from compound binding to p38 α . At $t = 0$ a range of SB203580 concentrations were passed across the immobilized p38 α to measure net association, and then at $t = 50$ s compound is replaced with buffer to initiate dissociation. The table shows the association (k_1 ($M^{-1}s^{-1}$)) and dissociation (k_2 (s^{-1})) rate constants as well as the equilibrium dissociation constants (K_D (M)) for two compounds. (B) Thermodynamic analysis of two p38 α inhibitors using Biacore. Enthalpy and entropy components of binding derived from the Van't Hoff analysis are detailed in the attached table. ΔG , ΔH and $T\Delta S$ values are in kJ/mol. *Unpublished data.*

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

The kinetics of drug binding are also most easily studied in simple binding assays rather than functional assays where association and dissociation rates are more likely to be affected by diffusion barriers. Label free techniques such as Biacore are being increasingly utilized for this purpose as illustrated in Figure 2.4A for inhibitors of p38 α . Interestingly the compounds in this example have similar affinities (52 and 78 nM), but markedly different kinetics (e.g., k_1 values 2.2×10^4 and $1.7 \times 10^6 M s^{-1}$). Slow kinetics and hence longer residence times are potentially advantageous compound characteristics [7,8], therefore such measurements are becoming increasingly important in drug discovery programs. An interesting example of a clinically used drug with unusual kinetics is the muscarinic antagonist, Tiotropium. It binds M_2 and M_3 receptor subtypes nonselectively (K_i values of 0.1 to 0.2 nM), but the compound has a much slower off rate (>10-fold) at the M_3 subtype, enough to make it a physiologically selective M_3 antagonist [9].

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

$$\Delta G = \Delta H - T\Delta S \quad (2.3)$$

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

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

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

TABLE 2.2 Enzyme Assay Techniques

Enzyme class	Product	Principle	Type	Detection examples
Protein kinases	Phosphoprotein	Labeled antibody	Homogeneous	AlphaLisa
	Phosphoprotein	Labeled antibody	Multi-step	ELISA
	ADP	Coupling enzyme	Multi-step	Luciferase (luminescent product)
Metabolic enzymes	Small molecule <ul style="list-style-type: none"> no chromophore no antibody detection 	Radioactivity	Multi-step	Radiometric
		Radioactivity/Proximity	Homogeneous	Radiometric (SPA)
		Mass/charge	Homogeneous	LC-MS (RapidFire)
		Lipophilicity/charge	Multi-step	HPLC
		Coupling enzyme	Multi-step	Variety (secondary product)
Methyltransferases	Methylhistone	Labeled antibody	Homogeneous	AlphaLisa
	Methylhistone	Labeled antibody	Multi-step	ELISA
	H ₂ O ₂	Coupling enzyme	Multi-step	Peroxidase (Light-based product)
	Formate	Coupling enzyme	Multi-step	Variety (secondary product)

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

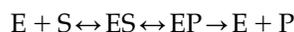
3. Enzyme Assays

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

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

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

The overall enzyme catalyzed reaction process is summarized below:



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

$$v = \frac{V_{\max}[S]}{[S] + K_m} \quad (2.5)$$

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

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

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

$$\%I = I_{\min} + \frac{(I_{\max} - I_{\min})[I]^n}{[I]^n + [\text{IC}_{50}]^n} \quad (2.6)$$

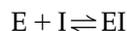
where I_{\max} is the maximal inhibition (usually ~ 100 percent), I_{\min} the minimal inhibition (~ 0 percent) and n the slope of the curve.

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

4. Types of Enzyme Inhibition and Their Analysis

A. REVERSIBLE INHIBITORS

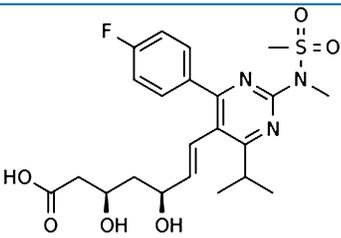
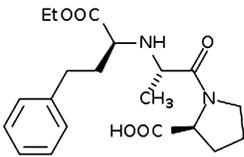
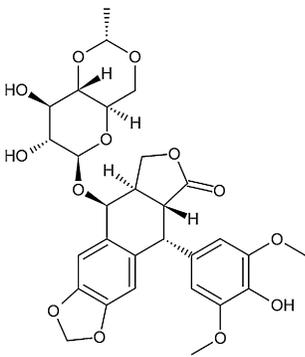
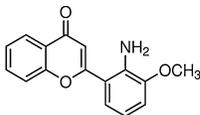
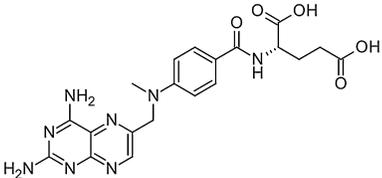
The majority of enzyme inhibitor drugs are reversible in that removal of the inhibitor (e.g., by dialysis) fully restores the enzymatic activity. Such inhibitors bind to their target enzyme through a combination of noncovalent interactions, such as hydrogen bonding or ionic, hydrophobic, and Van der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



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

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

TABLE 2.3 Examples of Reversible Enzyme Inhibitor Drugs

Example	Structure	Target
Crestor Competitive		HMG CoA Reductase
Enalapril Competitive		Angiotensin converting enzyme
Etoposide Noncompetitive		Topoisomerase II
PD 098059 Noncompetitive		MEK
Methotrexate Uncompetitive		Dihydrofolate reductase
Lithium Uncompetitive	Li ⁺	IMPase

reversible only ~9 percent inhibition would be expected. In contrast, if the inhibitor is irreversible, the jump dilution would have little effect on the enzyme-inhibitor complex and the enzyme's activity would still be inhibited by ~90 percent. Technical assay conditions may need to be fine-tuned to ensure slow dissociation is examined and that sufficient active enzyme is present post "jump" dilution. The immobilization technique depends on the ability to irreversibly immobilize the enzyme in a 96-well plate such that sufficient activity is retained for inhibitor studies. Immobilized enzyme is incubated with inhibitor and substrate to determine the pre-wash IC₅₀, followed by washing to remove substrate and inhibitor, then re-measurement of enzyme activity with substrate (post-wash). Figure 2.5 illustrates this, showing no change in IC₅₀ with washing for a mechanism-based, 2-thioxanthine, irreversible myeloperoxidase inhibitor [12], but complete loss of inhibition on washing with a reversible inhibitor [13].

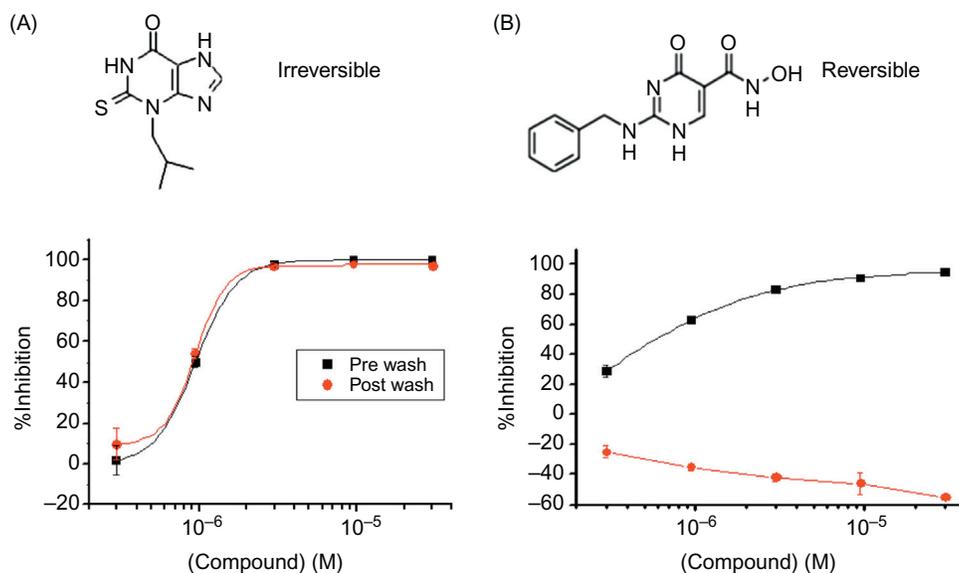


FIGURE 2.5 Reversibility of Enzyme Inhibition. MPO inhibition by an irreversible (A) and a reversible (B) inhibitor using plate-based immobilized enzyme and removal by washing. Inhibition curves are shown for both types of compound before (black circles) and after (red circles) inhibitor removal. *Unpublished data.*

B. IRREVERSIBLE INHIBITORS

In some cases, enzymes can be irreversibly inhibited through formation of a covalent bond between the enzyme and the inhibitor. Such inhibition due to the inherent chemical reactivity of an inhibitor is usually too nonspecific and promiscuous to be a useful drug mechanism. More commonly, irreversible drugs are specifically recognized by their target enzyme as “pseudo-substrates” and converted to reactive products that covalently inactivate the enzyme. This mechanism-based irreversible inhibition provides target selectivity by virtue of structural recognition of the inhibitor by the enzyme and the specific chemistry of the enzyme’s active site.



Irreversible inhibition is eventually “reversed” over days *in vivo* after inhibitor administration has stopped, by the de novo synthesis of new enzyme to replace that inactivated by the inhibitor. Examples of drugs that are irreversible enzyme inhibitors are shown in [Table 2.4](#).

The advantages of an irreversible drug are that with time it will inhibit all the enzyme such that high potencies and ligand efficiencies can easily be obtained, leading to long duration of action *in vivo*. Potential disadvantages are a higher risk of poor specificity, inability to quickly reverse *in vivo* effects if required, a need for a more complex set of *in vitro* assays to drive SAR (i.e., IC_{50} isn’t sufficient on its own), and reactive inhibitor intermediates formed during enzyme inactivation have the potential to react with other proteins to form immunogenic adducts.

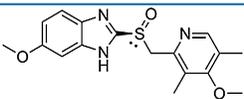
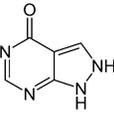
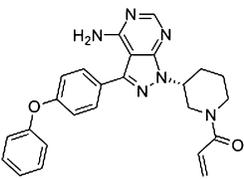
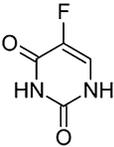
C. COMPETITIVE INHIBITORS

Inhibitors can reversibly bind to the free form of the enzyme, to substrate-bound enzyme catalytic forms, or to both, such that an inhibitor’s potency can have different relationships to $[S]$. This is summarized by the enzyme turnover reaction scheme in the presence and absence of reversible inhibitors in [Figure 2.6A](#). If inhibitor and substrate binding are mutually exclusive (i.e., inhibitor and substrate cannot bind to the enzyme at the same time), the inhibitor is competitive with respect to that substrate. Hallmark features of a competitive inhibitor are an increase in K_m , but no effect on the V_{max} as illustrated in [Figure 2.6B](#). Competitive inhibitors generally bind at the enzyme’s active site and compete with substrate for occupancy.

D. NONCOMPETITIVE INHIBITORS

A noncompetitive inhibitor exhibits affinity for both the free enzyme (E) and the enzyme-substrate complexes and thus is defined by two equilibrium constants, one for EI (K_i) and one for the ESI complex (αK_i). α describes the relative affinity of I for E and ES. For example, when $\alpha = 1$, then I has equal affinity for E and ES. Key

TABLE 2.4 Examples of Irreversible Enzyme Inhibitor Drugs

Drug	Structure	Target
Nexium		H ⁺ /K ⁺ ATPase in gastric parietal cells
Allopurinol		Xanthine oxidase
Ibrutinib		Bruton's tyrosine kinase
5-Fluorouracil		Thymidylate synthase

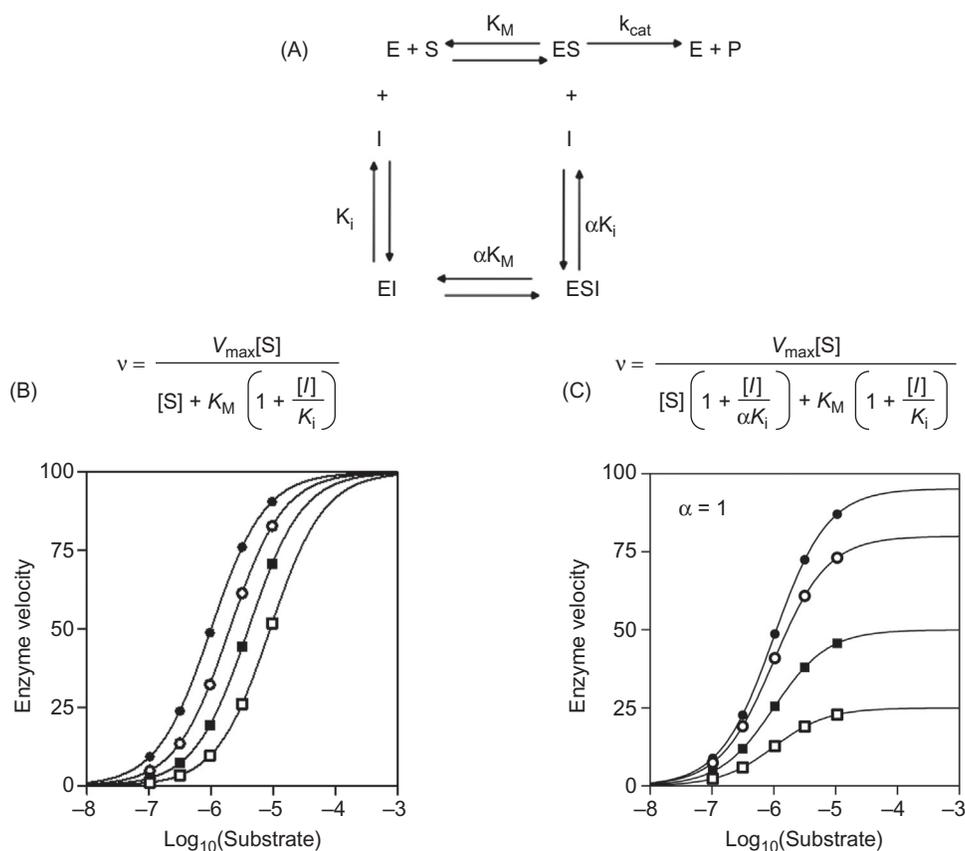


FIGURE 2.6 Competitive and Noncompetitive Enzyme Inhibition. Equations and graphs illustrating a general enzyme reaction scheme for reversible inhibitors (A), the substrate dependency of the steady state velocity for an enzyme in the presence of a range of competitive (B), and noncompetitive (C) inhibitor concentrations.

$$v = \frac{V_{\max}[S]}{[S] \left(1 + \frac{[I]}{\alpha K_i} \right) + K_M}$$

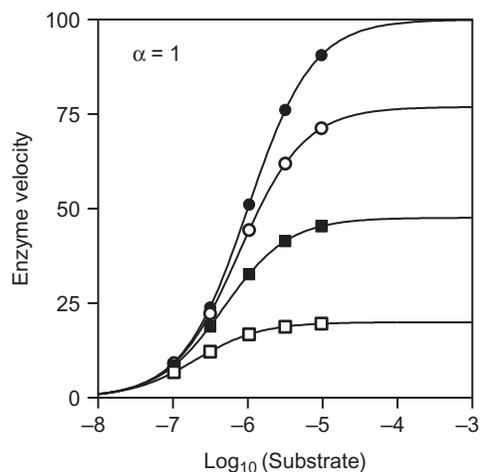


FIGURE 2.7 Uncompetitive Enzyme Inhibition. Equation and graph illustrating the substrate dependency of the steady state velocity for an enzyme in the presence of a range of uncompetitive inhibitor concentrations.

features of a noncompetitive inhibitor are no effect on substrate K_m and a decrease in V_{\max} as illustrated in [Figure 2.6C](#). Noncompetitive inhibitors tend to bind to the enzyme at sites distinct from the active site and exert their effects allosterically.

E. UNCOMPETITIVE INHIBITORS

Uncompetitive inhibitors only recognize and interact with ES and subsequent downstream catalytic species with no binding to free enzyme. Thus to exhibit enzyme binding, uncompetitive inhibitors require formation of ES and inhibition of enzyme activity is characterized by a decrease in both substrate K_m and V_{\max} (see [Figure 2.7](#)). Since uncompetitive inhibitors only block processes beyond ES formation, one might expect only V_{\max} to be suppressed with no effect on K_m , but as the inhibitor binds to and stabilizes the ES complex, it makes it more difficult for S to dissociate or be converted to product, increasing enzyme affinity for S and so reducing substrate K_m . This mode of action is attractive for drug design as the inhibitors bind to the enzyme target only when the target is active and substrate present. Uncompetitive inhibitors decrease substrate K_m and V_{\max} as well as exhibiting higher inhibition with increasing [S] as illustrated in [Figure 2.7](#). From the equations and graphs describing the three modes of enzyme inhibition ([Figures 2.6 and 2.7](#)), it can be seen that competitive (I only binds E with affinity K_i) and uncompetitive (I only binds ES with affinity αK_i) are special cases of noncompetitive inhibition (I binds both E and ES with affinities K_i and αK_i respectively).

5. Functional Assays

Binding assays as described above ([Sections IIIA\(1\) and \(2\)](#)) provide information on the affinity of compounds, but they do not generally indicate if they are agonists. That is, they do not provide information on their ability to elicit functional responses (efficacy). Such information is crucial for projects that are aiming to identify agonists as therapeutic agents, but functional assays are also important for inhibitor based projects as they allow a) compounds with unwanted agonism to be identified and b) confirmation that activity detected in binding assays translates into inhibition of functional readouts.

Traditionally pharmacologists used pieces of isolated tissue (typically smooth muscle preparations from laboratory animals) to generate functional data, but nowadays cellular systems (engineered or native cell lines) are routinely employed. The former assays have the advantage that the readout (e.g., contraction or relaxation) was often physiologically relevant, but the disadvantages of very low throughput and potential issues with species cross-over (see [Section IIID \(3\)](#)). Cellular assays have the advantage of increased throughput, allowing very large numbers of compounds to be screened. They also routinely employ the human ortholog of the protein (either endogenously expressed or engineered into a cell type that does not usually express it) ensuring that compounds chosen for progression have good activity at the clinical target. The disadvantages are that the readouts used in these assays are usually “upstream” of the physiologically relevant response and may, for various reasons, not always mirror the latter. Typical measurements include the generation of 2nd messengers such as cyclic

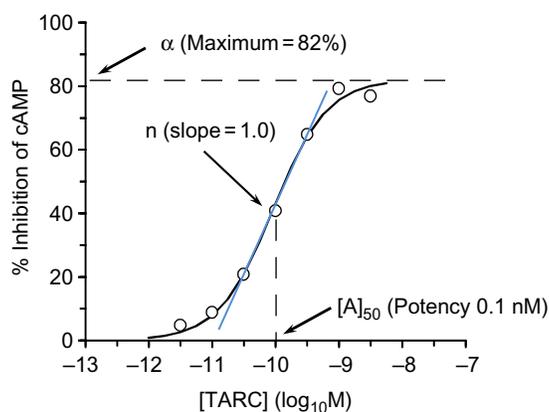


FIGURE 2.8 Agonist Concentration-Effect Curves. An agonist concentration-effect curve highlighting the parameters that define it: α (the maximum effect); $[A]_{50}$ (potency), the concentration of agonist that produces 50 percent of α ; and n , a measure of the slope. $[A]_{50}$ values are often quoted as pA_{50} ($-\log_{10}[A]_{50}$). In this example, the $[A]_{50}$ is 10^{-10} M and the pA_{50} is 10.0. Data shows TARC (CCL17) induced inhibitions of cAMP levels in CHO cells stably transfected with human CCR4. *Unpublished data.*

adenosine monophosphate (cAMP), Ca^{2+} , or inositol phosphates, and beta arrestin movement for GPCRs, ion fluxes or membrane potential changes for ion channels, and gene transcription for NHRs. The technologies for measuring such readouts are constantly evolving but include FLIPR[®], which uses a range of fluorescent dyes to measure changes in intracellular calcium concentration or membrane potential; antibody based technologies such as AlphaLISA, which can be used to detect a range of substances including cAMP; and reporter gene assays, which use fluorescent or luminescent proteins under the control of target gene promoters to assess drug-induced gene transcription. Again a major advantage of these technologies is their suitability for medium- to high-throughput screening. The nature of the readout can, however, generate difficulties in data analysis and interpretation. For example, changes in intracellular Ca^{2+} levels in response to agonists in FLIPR[®] assays are often transient in nature, which can result in failure to reach true equilibrium when potent competitive antagonists are studied. In such hemi-equilibrium cases, the antagonists appear to be insurmountable, and this has the potential to introduce errors into affinity estimations (see [14] and Section IIIA 6D).

6. Functional Studies and Their Analysis

By definition, functional studies involve analyzing agonist responses, either alone or in the presence of antagonists (or inhibitors). Central to these analyses is the generation of agonist concentration-effect curves. Their defining properties are described below. Subsequent sections describe the different classes of agonists and outline how their interaction with antagonists is analyzed to yield antagonist affinity estimates and MOA information.

A. AGONIST CONCENTRATION-EFFECT (E/[A]) CURVES

Agonist concentration-effect curves are typically sigmoidal (s-shaped) when plotted in semi-logarithmic form ($E/\log_{10}[A]$) and are described by four parameters: 1) a lower asymptote (β), which represents the basal state of the system; 2) an upper asymptote (α), which represents the maximum effect that the agonist produces in the system; 3) a location or potency ($[A]_{50}$ or EC_{50}), which represents the concentration of agonist that produces an effect equal to 50 percent of $\alpha - \beta$ and 4) a slope parameter (n), which is a measure of the gradient of the curve at the $[A]_{50}$ level. A number of computational programs are available that allow estimates of these parameters to be made by fitting experimental $E/[A]$ curve data to the following form of the Hill equation (a saturable function that adequately describes curves of varying gradients):

$$E = \beta + \frac{(\alpha - \beta)[A]^n}{[A]^n + [A]_{50}^n} \quad (2.7)$$

In practice, $\beta = 0$ in the majority of cases. That is, the basal effect level is ascribed a value of zero, and therefore most $E/[A]$ curve data can be adequately described by a 3-parameter Hill equation as illustrated in Figure 2.8. It is the analysis of how the three curve parameters (α), $[A]_{50}$, and n are affected by experimental manipulation that allows drug-receptor interactions to be quantitatively described in terms of affinity (binding) and efficacy (response-eliciting capacity).

B. FULL AGONISTS, PARTIAL AGONISTS, AND INVERSE AGONISTS

The first step in agonist action is the formation of a reversible agonist-receptor (AR) complex, a process that is generally assumed to be governed by the Law of Mass Action. Accordingly, the equilibrium concentration of

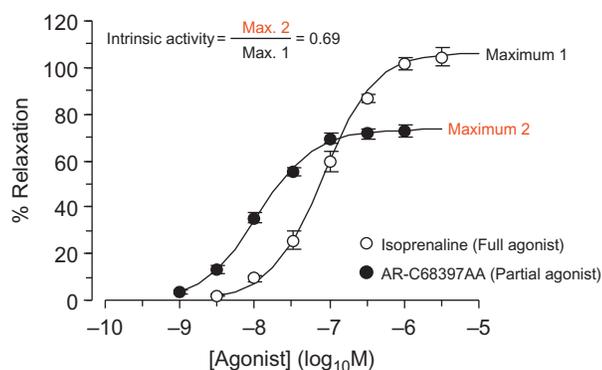


FIGURE 2.9 Full Agonists, Partial Agonists and Intrinsic Activity. Experimental data showing the β_2 -adrenoceptor mediated smooth muscle relaxing activity of the reference full agonist isoprenaline and the partial agonist AR-C68397AA in guinea pig isolated tracheal rings. The intrinsic activity of AR-C68397AA was 0.69. *Unpublished data.*

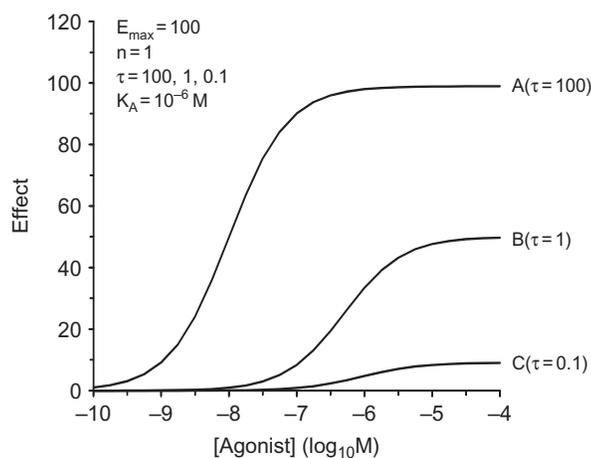


FIGURE 2.10 System Dependence of Drug Effects. Simulated curves showing how the curve parameters of an agonist change in systems with varying receptor expression. The receptor expression range is 1000-fold from curve A to curve C. In system A, the drug exhibits full agonism (IA = 1.0 and high efficacy ($\tau = 100$)); in system B, it shows partial agonism (IA = 0.5 and low efficacy ($\tau = 1$)); and in system C, it shows very weak partial agonism (IA = 0.09 and very low efficacy ($\tau = 0.1$)). In system C the drug effectively behaves as an antagonist as this level of IA is difficult to detect in most assay systems. The Operational Model of Agonism [15] was used to simulate the data. In this model, τ is a measure of the efficacy of the agonist and incorporates both drug (intrinsic efficacy) and system (receptor number ($[R_{tot}]$) and coupling efficiency) parameters.

agonist occupied receptors is a rectangular hyperbolic function (a special case of the Hill function where $n = 1$) of the agonist concentration (identical to Eq. (2.1)). This curve is defined by a maximal value of $[R_{tot}]$, the total receptor concentration, and a midpoint value of K_A , the agonist dissociation constant. K_A determines how well the agonist binds; that is, it is a measure of the affinity of the agonist for its receptors. Agonist occupancy is subsequently amplified into functional effect by the biochemical/biophysical machinery of the cell/tissue, and this is what is measured experimentally in the form of an $E/[A]$ curve. The efficiency of this transduction process can vary between agonists and across systems (i.e., it is both drug and tissue dependent). Agonist efficacy is a measure of the efficiency of the transduction process. Full agonists have high efficacies and therefore can elicit the maximum effect (E_{max}) that the test system is capable of generating. Partial agonists by contrast have low efficacy and cannot elicit a maximum response (Figure 2.9).

Measuring the efficacy (and affinity) of full agonists is not straightforward because their occupancy is efficiently converted into effect and thus the $[A_{50}]$ is much lower than the K_A . An experimental manipulation that decreases the efficacy of the agonist to a level where it behaves as a partial agonist (where $[A_{50}]$ approximates K_A) is therefore required. Irreversible antagonists have been used for this purpose as they covalently modify receptors, thereby decreasing $[R_{tot}]$ (see Section IIIA 6D). An important consequence of efficacy being both a drug- and system-dependent parameter is that an agonist can demonstrate different behaviors in different systems. Thus, a drug that exhibits partial agonism in one system may be a full agonist in another (with higher $[R_{tot}]$ or more efficient transduction machinery) or effectively an antagonist in yet another (with lower $[R_{tot}]$ or less efficient transduction machinery) (Figure 2.10).

As alluded to above, it can be difficult to measure the affinity and efficacy of agonists, and typically the information reported to the medicinal chemist is the potency ($[A_{50}]$ or, more often, pA_{50} ($-\log_{10}[A_{50}]$) and the intrinsic activity (IA) of the compound. The latter is a measure of the maximal activity of the test compound relative to a reference full agonist [16]. If the test agonist produces a maximum response less than the reference agonist, then the IA will be <1.0 . For example, in Figure 2.9 AR-C68397AA produces a maximum effect that is 69 percent of the reference full agonist isoprenaline, and thus is a partial agonist with an IA of 0.69. Such compounds are

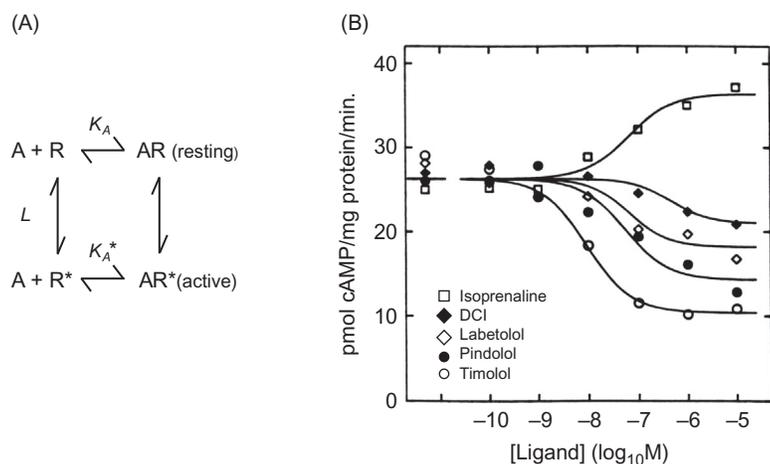


FIGURE 2.11 Inverse Agonism. (A) A simple two-state model in which the receptor exists in R (resting) or R* (active) states. In the absence of agonist (A), the distribution of the two states is governed by the equilibrium constant L . The agonist has affinities for the two states governed by the dissociation equilibrium constants, K_A and K_A^* . (B) Production of cAMP in membranes of Sf9 cells expressing the human β_2 -adrenoceptor. Isoprenaline exhibits positive agonism but all the other ligands tested show varying degrees of inverse agonism (i.e., they decrease the basal level of cAMP production). Panel (B) reproduced with permission from Chidiac et al., 1994 [20].

useful to the medicinal chemist as they help direct efforts to optimize the efficacy of compounds for therapeutic benefit. For example, identification of partial agonists were important staging posts in the development of the antagonists propranolol and cimetidine [17,18]. Finally, it is important to emphasize that the IA scale does not discriminate between full agonists (i.e., all full agonists will have an IA of 1.0 but they may have different efficacies).

Until relatively recently, agonist efficacy was considered only as a positive scalar associated with increased receptor activity. This dogma was challenged by the discovery of the phenomenon of constitutive receptor activation and compounds that showed inverse agonism [19,20]. That is, they decreased the level of constitutive activation, demonstrating negative efficacy (Figure 2.11). The most likely mechanism for inverse agonism is that such compounds have a selectively higher affinity for the inactive state of the receptor and thereby uncouple spontaneously coupled (active) receptor species [21,22]. The existence of multiple receptor states also offers an explanation for the phenomenon of "signaling bias or agonist trafficking," whereby one agonist may direct signaling to a particular cascade while another agonist may not [23]. To date, inverse agonism has largely been a property detected in genetically engineered cells systems where receptors (or modified receptors) can be expressed at supra-physiological levels. Many of the compounds that exhibit inverse agonism in such systems behave as competitive (neutral) antagonists with zero efficacy in more physiologically relevant assays. As such, the therapeutic relevance of inverse agonism remains largely unknown, but this now well-documented phenomenon has changed the way pharmacologists view drug-receptor interactions as well as resulting in the re-classification of drugs that were formerly thought to be competitive antagonists (e.g., Ranitidine and Propranolol). Importantly, designing compounds with inverse agonist properties and/or signaling bias offers the medicinal chemist further opportunities in tailoring compounds to address unmet clinical needs. The advancement of a biased μ -opioid receptor agonist (TRV130) into clinical testing as an analgesic with low side-effect potential serves as an example of how such new concepts of receptor function are being exploited [24].

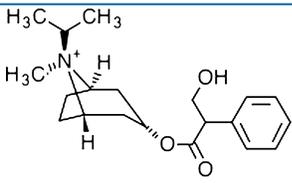
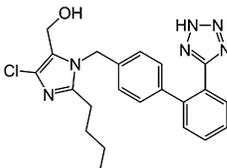
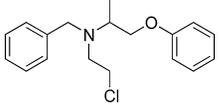
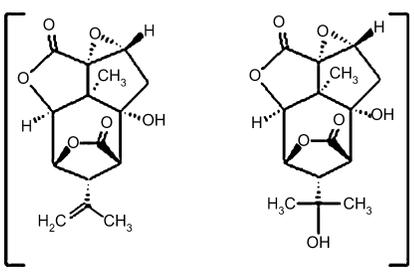
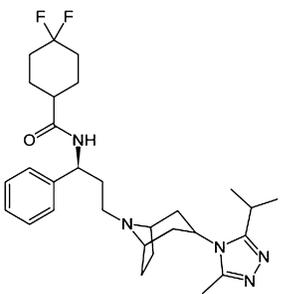
C. OPTIMIZING AGONISTS

As discussed above, agonists bind to and activate receptors. The optimization of agonist properties therefore relies on designing compounds with both good affinity and appropriate efficacy. Affinity can be measured in ligand binding assays, but functional assays are required to provide estimates of IA or efficacy. In most cases, the aim of agonist based projects is to identify high potency, high efficacy agonists so that the drug dose ultimately administered will be small and the effect large. In some instances however, partial agonists can have therapeutic advantages. Thus, if the desirable therapeutic effect is observed in a tissue with high receptor number/coupling but an undesirable side-effect is mediated in a tissue with low receptor number/coupling, a partial agonist of appropriate efficacy could produce agonism in the former but be "silent" in the latter (compare curves A and C in Figure 2.10). Finally, as described above, designing compounds that signal selectively through a particular pathway may provide further levels of therapeutic control.

D. ANALYSIS OF ANTAGONISTS

As with enzyme inhibitors, several different classes of antagonists with distinct mechanisms of action including irreversible competitive, reversible competitive, noncompetitive, and allosteric have been identified

TABLE 2.5 Examples of Various Classes of Receptor Antagonists

Example	Structure	Target
Ipratropium Competitive		M ₃ -receptor
Losartan Competitive		AT ₁ -receptor
Phenoxybenzamine Irreversible		α-adrenoceptors (nonselective)
Picrotoxin Noncompetitive		GABA _A -receptor
Maraviroc Allosteric		CCR5

(Table 2.5). Their blockade of agonist-induced effects can be surmountable (rightward displacement of the E/[A] curve with no depression of the maximum (α)) or insurmountable (depression of the maximal agonist response (α)). It is important to realize that the profile of antagonism observed can show system dependence; that is, an antagonist can exhibit surmountable activity in one assay system and insurmountable activity in another, despite having the same mechanism of action (see Figure 2.14A and B). A common example of this phenomenon is the behavior of high affinity competitive antagonists in FLIPR[®] assays in which the changes in intracellular calcium levels measured are typically transient in nature. In these circumstances, true equilibrium is not reached as the agonist does not have sufficient time to access antagonist bound receptors resulting in apparent nonsurmountable antagonism [25,26]. This contrasts with the behavior of such antagonists in systems where agonist responses are sustained (e.g., in many isolated tissue systems), true equilibrium is reached and the antagonism is surmountable.

The interaction of an antagonist with its receptors is described by a single parameter, affinity which equates to potency (unlike agonists where potency is dependent on both affinity and efficacy). By definition, antagonists have an IA = 0 in functional assays, in which their affinity is measured by studying their receptor interaction with an agonist. The affinity of antagonists can also be measured in binding assays (see Section IIIA (1)),

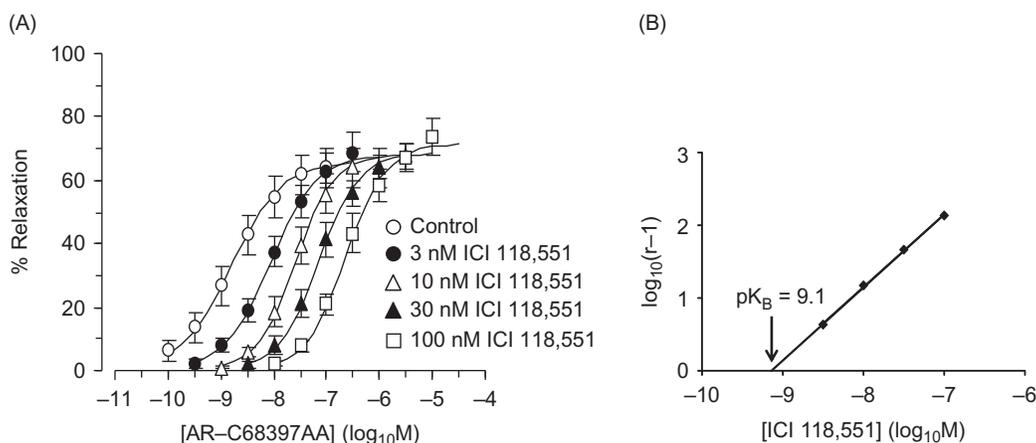


FIGURE 2.12 Competitive Antagonism in Functional Assays. Antagonism of the AR-C68397AA β_2 -adrenoceptor mediated relaxation of rabbit isolated saphenous vein by the competitive antagonist ICI 118,551 (A). Note the concentration-dependent parallel rightward displacement of the control curves. (B) Illustrates the displacements (r values) in Schild plot form derived from one of the 5 experiments that make up in the data in (A). The plot has a slope of unity and the intercept on the x-axis yields an estimate of 9.1 for the pK_B ($-\log_{10}K_B$). *Unpublished data.*

although if such systems are used it is essential to confirm lack of efficacy by subsequent testing in functional assays. The following sections discuss the properties and analysis of the various classes of antagonists.

COMPETITIVE ANTAGONISTS Reversible competitive antagonists are probably the most important class of antagonists, and a large number of clinically used drugs fall into this class. As outlined above for agonists, the first step in the action of these drugs is the formation of a reversible, relatively short-lasting, drug-receptor complex governed by the Law of Mass Action. In this mode of antagonism, the binding of the agonist and antagonist is mutually exclusive. The presence of the antagonist therefore decreases the probability that an agonist-receptor interaction will occur. To achieve the same degree of agonist occupancy—and therefore the same effect—in the presence of the antagonist as in its absence, the agonist concentration must be increased. The factor (r) by which it must be increased depends on both the concentration of antagonist ($[B]$) used and on how well it binds (K_B). This relationship, which was first described by Schild [27], is shown below:

$$r - 1 = [B]^n / K_B \quad (2.8)$$

where $r = [A_{50}] / [A_{50}]^c$ (location parameter of the $E/[A]$ curve in the presence of the antagonist/location parameter of the $E/[A]$ curve in the absence ($c = \text{control}$) of the antagonist), K_B is the antagonist equilibrium dissociation constant, and n represents the stoichiometry of the interaction between the antagonist and the receptors ($n = 1$, when one molecule of antagonist binds to one receptor molecule).

Experimentally, a K_B is estimated by studying the interaction of an agonist and antagonist over a wide range of antagonist concentrations (the wider, the better). This is necessary because drugs which are not reversible competitive antagonists may appear to be so within a narrow range of concentrations. If the antagonist is truly competitive, it should produce parallel rightward displacement (i.e., no change in midpoint slope (n) occurs) of the $E/\log[A]$ curves with no change in the maximal response (α) (see Figure 2.12A). The analysis involves fitting experimentally derived values of r at different concentrations of antagonist to the following form of Eq. (2.8) [28]. See Figure 2.12B.

$$\log_{10}(r - 1) = n \log_{10}[B] - \log_{10}K_B \quad (2.9)$$

Consistency of the data with Eq. (2.9) is judged by the finding of a linear plot with a slope (n) of 1.0. Under these conditions, the intercept on the x-axis ($\log_{10}[B]$) gives an estimate of K_B . When n is significantly different from 1, the intercept gives an estimate of pA_2 ($-\log_{10}K_B/n$). The pA_2 is an empirical estimate of antagonist affinity and equates to the negative logarithm of the concentration of antagonist that produces a two-fold rightward shift ($r = 2$) of the control $E/[A]$ curve. Nonlinearity and slopes other than unity can result from many causes. For example, a slope of greater than 1 may indicate incomplete antagonist equilibration or removal of the antagonist from the biophase (receptor compartment). A slope that is significantly less than 1 may indicate removal of the agonist by a saturable uptake process, or it may result from the interaction of the agonist with more than one receptor. In the latter case, the Schild plot may be nonlinear with a clear inflexion. All of these potential complicating factors have been described in detail previously by Kenakin [29].

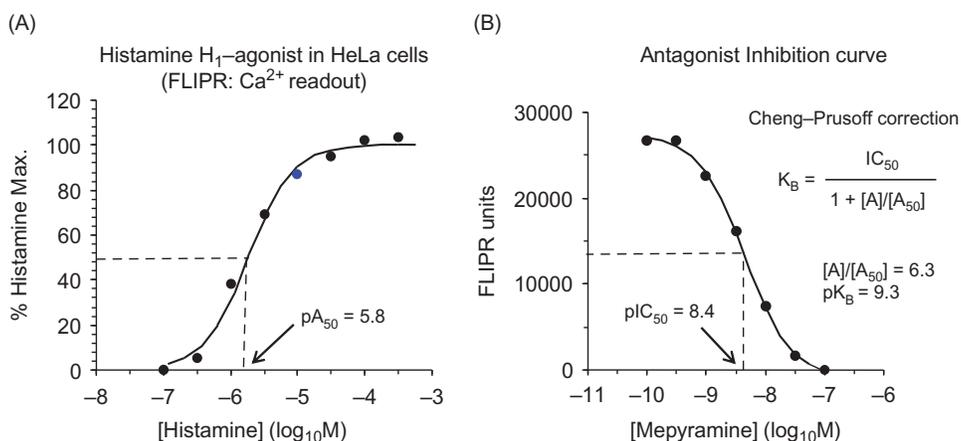


FIGURE 2.13 Cheng-Prusoff Analysis of Antagonism in Functional Assays. (A) Histamine concentration effect curve generated in HeLa cells which endogenously express the human H_1 -receptor. $10\ \mu\text{M}$ histamine (blue symbol) was chosen as the concentration of agonist to be used to assess the inhibitory effects of the H_1 -receptor antagonist mepyramine. (B) Mepyramine completely inhibited the Ca^{2+} induced histamine response and yielded a pIC_{50} value of 8.4. As $[A]/[A]_{50}$ was significantly greater than 1, the affinity (pK_B) estimate (9.3) is considerably greater than the pIC_{50} . *Unpublished data.*

Although Schild type analysis is the most robust method of assessing antagonist behavior in functional assays, the needs of modern high-throughput drug discovery programs dictate that it is used sparingly to assess the mechanism of action for priority compounds. Routine screening of antagonist properties will more likely be assessed by doing a simpler functional Cheng-Prusoff type experiment (Figure 2.13) in which the effects of several concentrations of the test compound on the response to a single concentration of agonist are studied. The experimental data can then be fitted to the following equation [30]:

$$K_B = \frac{IC_{50}}{(2 + ([A]/[A]_{50})^n)^{1/n} - 1} \quad (2.10)$$

As was outlined above for binding studies, the estimated IC_{50} is dependent on the concentration of ligand employed. In this case, the concentration of agonist ($[A]$) relative to its $[A]_{50}$ dictates the IC_{50} (and hence the estimated K_B). Practically, the experimenter usually employs a concentration of agonist that is as close to the $[A]_{50}$ as possible so that the IC_{50} is a good estimate of the K_B . The shape of the agonist $E/[A]$ curve is also important as evidenced by the inclusion of the slope parameter (n) in this form of the Cheng-Prusoff equation. When $n = 1$ the equation simplifies to a form equivalent to Eq. (2.2). Such analysis, although higher throughput, does not discriminate different modes of action of test compounds. For example, it will not differentiate competitive from noncompetitive compounds. Without additional proof that the interaction of agonist and antagonist is competitive, it is more appropriate to use the measured IC_{50} as a measurement of antagonist potency rather than calculating a K_B . As is the case with enzyme and binding assays the factor $[A]/[A]_{50}$ should be kept constant so that IC_{50} values of different compounds can be easily compared.

IRREVERSIBLE, NONCOMPETITIVE, AND ALLOSTERIC ANTAGONISTS Several other forms of antagonists have been identified and will be discussed briefly. Irreversible antagonists form covalent bonds with the receptor protein and thus prevent binding of agonists. They therefore effectively decrease the receptor pool and, by so doing, decrease the response eliciting capacity (efficacy) of the agonist, as this is dependent on $[R]_{\text{tot}}$. As equilibrium is not attained, the antagonist affinity cannot be measured, but such agents (e.g., Phenoxybenzamine) form the basis of the receptor inactivation method developed by Furchgott [31] for estimating agonist affinities and efficacies. Practically, however, the reliance of this method on the availability of suitable alkylating agents excludes its use in most receptor systems.

Noncompetitive antagonists bind to receptors and make them functionally inoperative either by preclusion of agonist binding (due to negligible dissociation of the antagonist during the response-gathering phase of the experiment) or through some other biochemical mechanism that obviates agonist effect (e.g., pore blockade of ion channels). Under these circumstances, increasing the agonist concentration cannot overcome the effect of the antagonist, and a distinctive feature of noncompetitive antagonists is the depressive effect they have on the

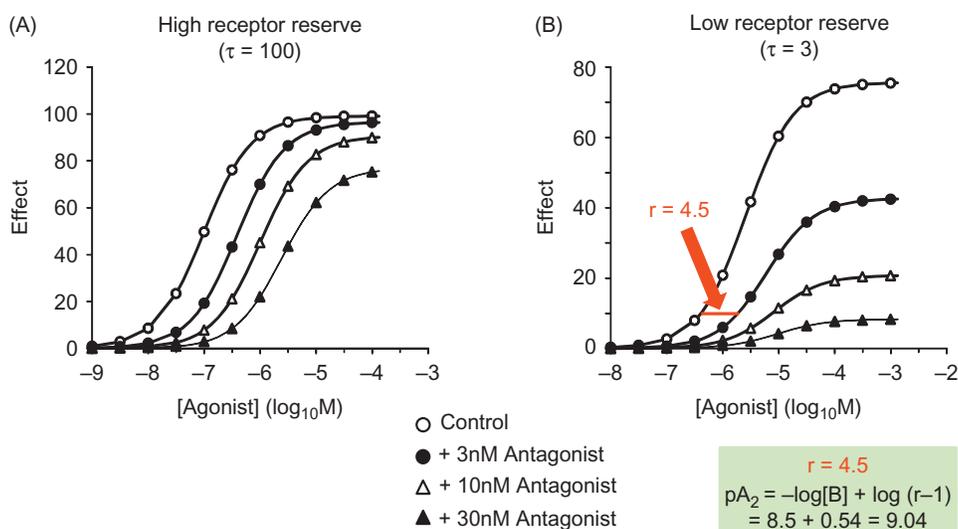


FIGURE 2.14 Noncompetitive Antagonism in Functional Assays. Simulations showing the effect of a noncompetitive antagonist on responses to the same agonist in a system with high receptor reserve (A) or low receptor reserve (B). Increasing concentrations of the antagonist (3, 10, 30 nM) cause more marked depression of the agonist maximum effect in the low reserve system. Data was simulated using a form of the Operational Model of agonism that assumes that antagonist binding precludes binding of the agonist [32]. The model parameters used were $E_m = 100$, $n = 1$, $\tau = 100$ (high reserve) or $\tau = 3$ (low reserve), $pK_A = 5.0$, $pK_B = 9.0$. Estimates of the antagonist affinity (pK_B) can be made by fitting data directly to this model or approximated as $pA_2 = -\log[B] + \log(r - 1)$ when a concentration-ratio (r) measured at low response levels is used (B).

maximal agonist response (α). The magnitude of the depression will however depend on the agonist under study and the system used. This relates to the concept of receptor reserve whereby maximum agonist effects can be achieved at low levels of receptor occupancy (binding)—for example, 10 percent occupancy may be enough to produce a maximum response and therefore there is a 90 percent receptor reserve. Receptor reserve depends on both the receptor number ($[R_{tot}]$) and the efficiency of stimulus-response coupling as well as the intrinsic efficacy of the agonist. Hence, noncompetitive antagonists will have differing capabilities to depress the maximal response to the same agonist in different systems. The same will be true for different agonists in the same system. The potency of noncompetitive antagonists can be estimated using various models but as a “rule of thumb” the pA_2 ($-\log[B] + \log(r - 1)$), as defined above for competitive antagonists, gives a reasonably accurate estimate of the antagonist affinity (pK_B) when measured at low levels of agonist response [14]. See Figure 2.14B.

All of the modes of antagonism described above are orthosteric; that is, the antagonist blocks access of the agonist to its binding site through steric hindrance. Allosteric antagonists in contrast bind to their own site on the receptor to induce a change in conformation of the receptor, which in turn alters the affinity or efficacy of the receptor for the agonist [33,34]. It is now clear that allosteric ligands can both increase and decrease the affinity and efficacy of other ligands, so allosteric modulators is a more appropriate term. Indeed, perhaps the best known therapeutically used allosteric modulators are the benzodiazepines, which increase the conductance of the GABA_A receptor. One of the key properties of allosteric modulators is their saturability of effect, which can be evidenced in functional experiments such as Schild analysis where a curvilinear plot results (Figure 2.15). Similarly, in Cheng-Prusoff type analyses, such antagonists will produce less than 100 percent inhibition of the agonist response. This behavior results from the fact that while the allosterically modified receptor may have diminished affinity (and/or efficacy) for the agonist, the agonist can still produce receptor activation in the presence of the modulator. As is evident from Figure 2.15B, use of concentration-ratios (r) at low antagonist concentrations can yield reasonably accurate estimates of compound affinity.

Allosteric modulation offers a number of potential advantages over orthosteric antagonists. First, they can modify (i.e., reduce or increase by a small amount) endogenous agonist signals without completely blocking them, thus allowing fine-tuning of responses. Second, there is the potential to increase the duration of allosteric effect by loading the receptor compartment with large concentrations of modulator. Such large concentrations will have no further effect than to prolong the saturated allosteric effect (i.e., the saturability of the allosteric ligand can be used to limit effect but increase duration). Another potential advantage of allosterism is increased selectivity. Orthosteric antagonists often have limited selectivity across receptor subtypes. For example, most

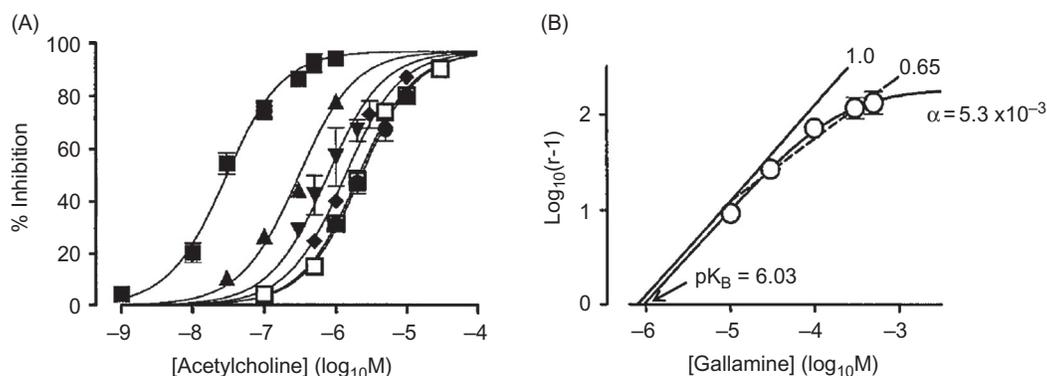


FIGURE 2.15 Allosteric Antagonism. (A) The effects of acetylcholine (ACh) on the electrically evoked contractions of the guinea pig left atrium in the absence (■) or presence of the allosteric modulator gallamine at the following concentrations: 10 μM (▲), 30 μM (▼), 100 μM (◆), 300 μM (□), and 500 μM (●). (B) The Schild plot of the data shown in (A). The solid line (slope = 1) denotes the behavior expected for a competitive antagonist, whereas the dashed line shows the best fit linear regression (and associated slope factor) through the points. The curve through the points and associated parameter estimates represent the fit to an allosteric model. The estimated pK_B was 6.03 and the α value of 5.3×10^{-3} equates to a gallamine-induced decrease in the affinity of ACh of 189-fold. Reproduced with permission from Christopoulos and Kenakin, 2002 [35].

muscarinic receptor antagonists exhibit poor selectivity between the five known subtypes (M_1 – M_5), presumably because they are competing with acetylcholine for very similar recognition sites. However, the surrounding protein structure of the receptors are sufficiently different to offer the potential for selective stabilization of receptor conformations by allosteric modulators. These potential advantages of allosteric modulators remain largely theoretical as very few such agents have to date reached the market. Nevertheless, the approval of the CCR5 antagonist Maraviroc (Selzentry[®]) for the treatment of human immunodeficiency virus (HIV) infection demonstrated the feasibility of this approach. This compound inhibits HIV entry by binding to a receptor site distinct from where the viral gp120 envelope protein binds [36,37].

Finally, although the discussion above focuses on receptors, allosteric modulation of enzyme function is a well-known phenomenon. The availability of binding sites distinct from those for the substrate again offers the potential for increased selectivity. For example, compounds designed to bind to an allosteric site in a particular protein kinase are likely to have improved selectivity over compounds targeting the ATP binding site.

B. Compound Interference in Primary Assays: Artifacts and False Positives

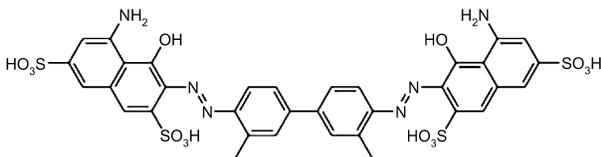
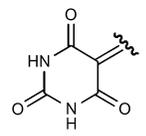
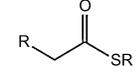
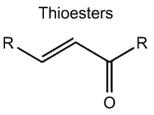
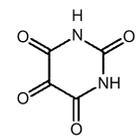
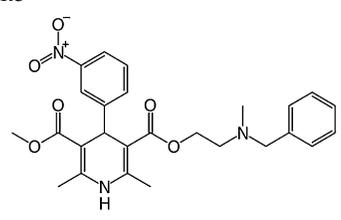
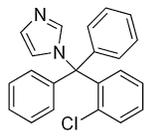
Over the last decade, high throughput screening of large compound collections has been used to successfully identify new chemical starting points for drug discovery programs, notably the CCR5 antagonist Maraviroc. However, the identification of true actives (drugs that interact specifically with the target of interest) from a HTS output has in many cases been hampered by co-detection of large numbers of “active” compounds with either undesirable and/or nonselective mechanisms (i.e., false positives). Compounds can work in an assay nonspecifically (i.e., not target related) through a variety of mechanisms, some of which are very easy to detect while others are more intractable. This is briefly summarized in Table 2.6 and reviewed elsewhere [38].

Careful design of the primary assay and selection of compound library is therefore important in minimizing the propensity to detect these undesirable promiscuous inhibitors. For example, the simple inclusion of detergent and/or protein in biochemical assays can have a profound effect on minimizing false positive detection [39].

C. Assay Biostatistics

It is not only important to understand how the biological properties of compounds are measured in various *in vitro* assay systems but also to appreciate how consistently an assay performs. This is key to defining criteria for validating compound test data, identifying SAR, and directing medicinal chemistry effort. Assay consistency is usually measured by testing a standard compound (if one is available) and various controls (usually positive and negative conditions to define signal-to-background) in each experiment. This is then used to monitor inter-assay performance on an experiment to experiment basis to ensure the assay can consistently measure the standard compound and retain an acceptable assay window. Experiments are invalidated if the assay value for the

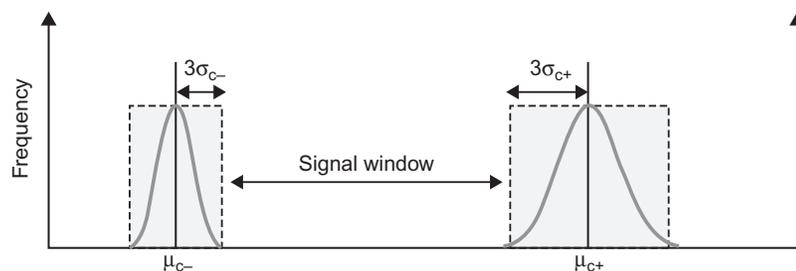
TABLE 2.6 Assay Interference Compounds

Property	Typical structures	Identification	Ease of detection
Interference with assay signal (e.g., fluorescent, singlet O ₂ quenchers)	e.g., Trypan Blue 	<ul style="list-style-type: none"> • Signal only counter-screen in absence of target • Chemical structure and properties 	Easy
Irreversible protein damage (e.g., oxidants)	e.g., Alkylidene barbiturates 	<ul style="list-style-type: none"> • Various redox assays • Chemical structure and properties 	Easy
Reactivity (e.g., acyl halides)	 Thioesters  Michael acceptor	<ul style="list-style-type: none"> • Time-dependent inhibition • Chemical structure and properties 	Medium
Activity due to impurity	Free metal ions	<ul style="list-style-type: none"> • No SAR • Re-synthesis to high purity and retest 	Medium
Chemical instability ^a	e.g., alloxan 	<ul style="list-style-type: none"> • Variable assay results that track with time after synthesis/dissolution • Activity can be linked to instability • Compound stability assay by LC/MS 	Easy
Very low solubility (usually <10 μM in aqueous)	e.g., nicardipine 	<ul style="list-style-type: none"> • Partial maximal inhibition due to lower free [compound] at high total concentrations • Tendency for assay data at high [compound] to be more variable • Test for solubility (light scattering etc.) • Increase assay [DMSO] if tolerated to improve solubility • Assay signal interference due to insolubility especially at high [compound] 	Easy
Compound aggregation ^{b,c}	e.g., Clotrimazole 	<ul style="list-style-type: none"> • Flat SAR, but not always • Nonselective within target class • Incomplete inhibition curves • No inhibition with detergent • Potency ranks with nanoparticle/aggregate detection by DLS • Insensitive to [enzyme] • Noncompetitive • Can be time dependent 	Difficult

^aDorfmueller HC, Borodkin VS, Blair DE, Pathak S, Navratilova I, van Aalten DM. Substrate and product analogues as human O-GlcNAc transferase inhibitors. *Amino Acids* 2010;40:781–92.

^bLaPlante SR, Carson R, Gillard J, Aubry N, Coulombe R, Bordeleau S, et al. Compound aggregation in drug discovery: Implementing a practical NMR assay for medicinal chemists *J Med Chem* 2013;56:5142–50.

^cSink R, Gobec S, Pečar S, Zega A. False positives in the early stages of drug discovery. *Curr Med Chem* 2010;17(34):4241.



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

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

FIGURE 2.16 Assay Biostatistics: Z' Values. A graphical representation of variation of positive (C+) and negative (C-) assay signals and how they are used to calculate Z' values. The table shows the guiding principles in using Z' values to measure assay performance. Reproduced with permission from Kenakin [41].

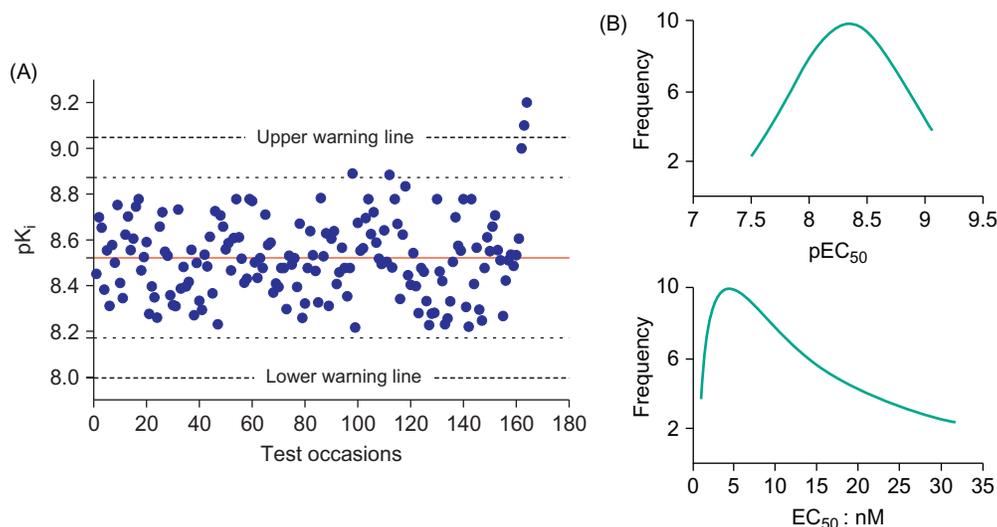


FIGURE 2.17 Assay Biostatistics: Control Charts and Averaging Biological Data. (A) Control Chart. pKi values for a compound (y-axis) run as an assay quality control over a number of occasions (x-axis). Solid line is the arithmetic mean. The dotted lines (2x SD (95 percent)) and dashed lines (3x SD (99.7 percent)) on either side of the mean are the warning lines. Data points that drift beyond these warning lines indicate concern over the quality of the assay data on these occasions. Unpublished data. (B) Log normal distribution of pEC₅₀ values (upper panel) and skewed distribution of EC₅₀ values (lower panel) converted from the same pEC₅₀ values. Reproduced with permission from Kenakin [42].

standard falls outside an acceptable range and/or the assay signal significantly changes. Typically a Z' value [40] is routinely calculated to measure both the variability of the assay window (signal–background), the signal, and the background values (Figure 2.16). For more detail see Assay Validation (<http://www.ncbi.nlm.nih.gov/books/NBK83783/>).

Control charts (Figure 2.17A) are used to monitor assay performance by tracking the standard compound pEC₅₀/pIC₅₀ values over the duration of a drug discovery project. The 95 percent confidence interval indicates the range of pEC₅₀ values within which the true population value can be expected to be found with 95 percent

certainty - this is generally a 3–4-fold range. Individual experiments in which the standard pEC_{50} falls outside this range should be investigated for any deviation in assay protocol, reagent batch, or process, and the experimental data rejected.

Overall an experiment is acceptable if the measured value (say IC_{50}) for the standard falls within an acceptable potency range (i.e., mean \pm 95 percent C.I.) and $Z' > 0.5$ for the signal and background values.

It is important to note that biological measurements are not always normally distributed and may need to be transformed prior to applying statistical tests to understand assay variability [43]. For example, IC_{50} , EC_{50} , K_i , etc. values are not normally distributed, so the mean IC_{50} of 10 μ M and 100 μ M is not the arithmetic mean, 55 μ M, but the geometric mean, 32 μ M. In contrast, pIC_{50} , pEC_{50} , pK_i , etc. are usually normally distributed, so these values should be used to interpret SAR and assay variability (e.g., SEM and SD_{n-1}) (Figure 2.17B).

A common question asked by the project chemist is, "What is the significant difference in potency between compound A and compound B as I need to know if compound A is more potent so I can design/synthesize the next compounds based on this SAR?" The best answer to this is to determine the minimal significance ratio (MSR) ideally with a set of compounds with a broad range of potencies. Essentially, the MSR is the smallest potency ratio between two compounds that is statistically significant. For a good assay, the MSR is < 3 , meaning that a 3-fold minimum in compound potency difference is significant. See <http://www.ncbi.nlm.nih.gov/books/NBK83783/> for further detail on the experimental design and statistics behind an MSR calculation. Of course, determining the MSR depends on already having inhibitors with a range of potency and is generally performed in the assay development phase or when SAR unexpectedly changes or assay performance drifts.

D. Selectivity, Cytotoxicity, and Species Cross-over.

Before compounds are progressed to more complex *in vitro* assays and to *in vivo* testing, it is important to assess their selectivity, cytotoxicity liabilities, and activity at the target protein of the model species to be used.

1. Selectivity

Selectivity is typically initially tested at closely related family members of the target of interest. Thus if the target was, for example, the purinoceptor $P2Y_{12}$, then the activity of lead compounds at the other $P2Y$ receptor subtypes would likely be assessed relatively early. As compounds are further optimized, selectivity screening widens and in this case might include other purinoceptors ($P2X$ receptors and Adenosine receptors) and ultimately a large panel of receptors, enzymes, ion channels, and transporters. Such assays can either be established "in house" or outsourced to one of the many contract research organizations that offer such screening services. Practically, there is a technical and cost limitation to the number of selectivity assays that can be run, but it is not unusual for CDs to be tested in several hundred different assays. Such testing allows compounds with good selectivity profiles to be identified, which greatly facilitates interpretation of data generated in more complex *in vitro* systems and *in vivo* models. Ultimately, the hope is that good selectivity delivers clinical candidates with excellent efficacy and minimal side-effects.

2. Cytotoxicity

A compound is cytotoxic when it damages the substructure or function of a cell, often leading to cell death, while toxicity generally refers to the damaging effects of compounds on whole organisms. Cytotoxicity is used to try to predict the *in vivo* toxicity of compounds and is often measured in parallel with drug target cell functions to gauge how specific a compound is in affecting the desired cell function; the bigger the difference in compound potency between modulation of a desirable cell function and unwanted gross cytotoxicity, the better the compound. Cytotoxicity is most often determined by simply measuring the ability of a compound to kill cells, and a variety of methods are available (Table 2.7), each with different strengths and weaknesses (see [44] for further detail). Assay selection is most often based on cost, throughput, technical capability, and target cell sensitivity.

3. Species Crossover

Usually—but not always—primary screening is carried out using the human version/ortholog of the target protein. This has the obvious advantage that compounds are screened against the ultimate target of clinical candidates. This strategy does, however, necessitate that lead compounds are subsequently tested against the other species that are typically used in drug discovery programs for both efficacy and toxicology testing. The most common species employed are mice, rats, and dogs, but other species (guinea pigs, rabbits, mini-pigs, macaques,

TABLE 2.7 Cytotoxicity Assay Guiding Principles

Cytotoxic principle	Biomarker	Detection	Pros	Cons
Cell metabolism unable to provide sufficient energy (in the form of ATP) for viable cell function	Intracellular [ATP]	CellTiter-Glo [®]	Sensitive Fast Stable signal HTS	Expensive
Oxidative metabolism (mitochondrial) unable to provide sufficient energy in the form of ATP for cell function	Dye reduction	Alamar Blue (Resazarin) MTT WST	Cheap Fast HTS	Compound interference
Loss of plasma membrane integrity such that intracellular enzymes leak out or extracellular dye leaks in	LDH/AK release Trypan Blue exclusion	ToxILght [™]	Easy Cheap	Compound interference Cell counting, so low throughput

etc.) may also be used. In many cases, compounds have good activity at the target protein in these species and therefore appropriate dosing regimens and straightforward data interpretation is possible. In instances (usually receptor targets) where poor species cross-over is observed (low or no activity at the target in typically used species), it may be necessary to investigate less commonly used species or even to initiate a parallel chemistry effort to identify compounds that do have good species cross-over.

E. Cellular and Tissue Functional Responses

The initial *in vitro* characterization of test compounds is usually carried out in assay systems designed for sensitivity, speed, reproducibility, and cost rather than physiological or pathophysiological relevance (unless phenotypic screening is being employed). The data generated is an important first stage in identifying interesting compounds, but subsequent confirmation of activity in systems with better links to the targeted disease mechanism(s) is essential for compound progression. Differences in receptor number and/or coupling strength, compound penetration, and metabolism are among the factors that may vary between the primary and more disease relevant assays and can markedly change compound activity (see above). Positive data in the latter systems significantly increases confidence that lead compounds will modulate physiological processes implicated in human disease. The systems employed will be target specific but often include a range of cellular and tissue assays. Typical examples of such assays include assessing the effects of compounds on the movement (chemotaxis) of white blood cells for anti-inflammatory targets or on the tone of airway smooth muscle for bronchodilators (Figure 2.18). Such experiments can be complemented by *ex vivo* studies as described below and also aid in the interpretation of *in vivo* experiments.

IV. EX VIVO ASSAYS

Ex vivo assays allow the effects of compounds to be studied in biological samples (e.g., tissues, blood, or cells) removed from intact animals or humans. Such experiments are very useful in that they provide evidence that the target and mechanism of action under study is operational in the species studied, as well as demonstrating drug absorption and penetration to the desired site of action. Binding studies or functional studies can be used to measure compound activity. For example, binding assays are often employed to measure receptor occupancy in brain samples. A typical experiment might involve orally administering a test compound to rats at various doses (usually a minimum of four doses plus vehicle is needed) and then terminating the animals at an appropriate time post-dose. The whole brain (or relevant area) is then removed and homogenized and the ability of the test compound in the sample to inhibit the binding of a radio-ligand to the target of interest is measured. This allows the fractional occupancy of the test compound to be plotted as a function of dose administered. In the case of functional experiments, readouts such as up-regulation of adhesion molecules or shape change of inflammatory cells in blood samples are typically used. Such readouts have the advantage that they can often also be employed in early clinical trials as proof of mechanism biomarkers. This approach has been successfully applied to several potential anti-inflammatory targets including CXCR2, stimulation of which induces neutrophil activation. Lazaar

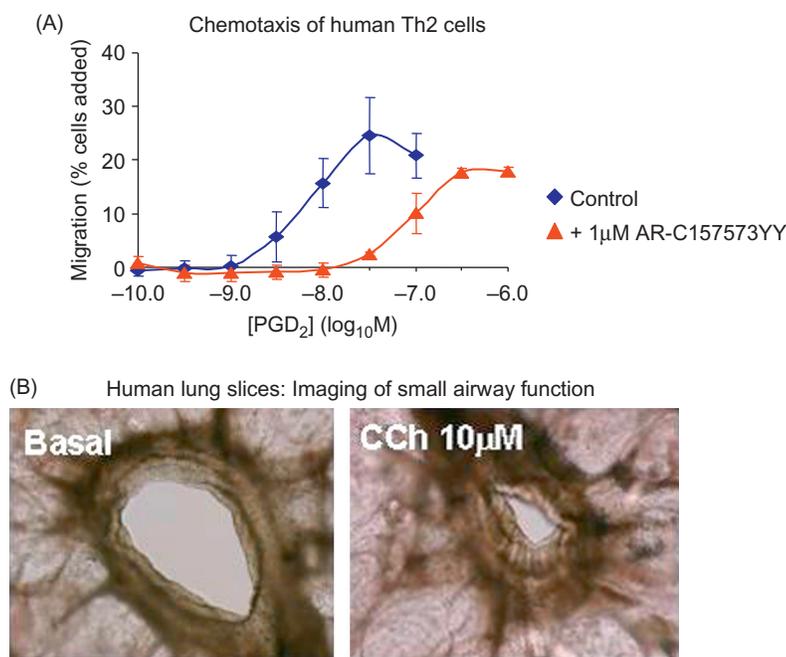


FIGURE 2.18 Disease Relevant Cellular and Tissue Functional Responses. (A) PGD_2 -induced migration of human Th2 cells. These effects are mediated by the DP_2 (CRTh2) receptor as evidenced by the blockade of the responses with the selective DP_2 receptor antagonist, AR-C157573YY ($\text{pA}_2 = 7.0$). Unpublished data. (B) Carbachol (CCh)-induced constriction of a small airway (approximately 2mm in diameter) in a slice of human lung. This response is mediated by the M_3 -muscarinic receptor and explains the utility of M_3 -antagonists such as Tiotropium in diseases such as COPD which are characterized by restricted airflow. Unpublished data.

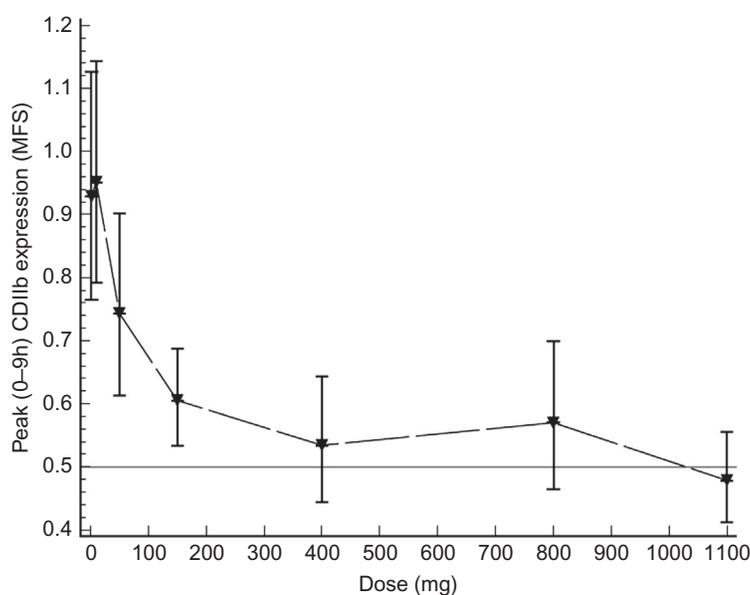


FIGURE 2.19 Ex vivo Assays. *Ex vivo* assessment of the effect of the CXCR2 antagonist, SB-656933 on CD11b expression on peripheral blood neutrophils following stimulation of whole blood samples with 30 nM CXCL1. A dose-dependent inhibition of CD11b was observed reaching maximum inhibition at concentrations of 400 mg SB-656933 and above. Reproduced with permission from Lazaar et al., 2011 [45].

and colleagues [45] used CXCL1 (GRO)-induced up-regulation of the adhesion molecule CD11b to study the *ex vivo* antineutrophilic effect of the oral CXCR2 antagonist, SB-656933, thereby allowing the effective dose of the antagonist to be estimated (Figure 2.19). By studying different time-points after dosing, *ex vivo* experiments can also provide information on the duration of action of compounds.

V. IN VIVO ASSAYS

Compounds with suitable potency, efficacy, and selectivity at the primary target and that have shown activity in disease relevant *in vitro* systems also need to have good pharmacokinetics (PK) if they are to deliver *in vivo* efficacy and duration of action. To this end, compounds are routinely tested in a range of *in vitro* assays that serve as models of drug absorption (e.g., Caco-2 cells), metabolism (e.g., rat microsomes, human hepatocytes, cytochrome P450 enzyme assays), and distribution (e.g., plasma protein and tissue binding) prior to *in vivo*

dosing. The physiochemical properties of molecules that are known to be “drug like” provide guidelines for medicinal chemists to optimize the PK properties of compounds, although this often proves more challenging than the optimization of the primary activities of potency and efficacy. The route of drug administration also dictates the properties that need to be optimized. For example, inhaled drugs ideally have low oral bioavailability, whereas oral drugs need good bioavailability. Once compounds with appropriate *in vitro* properties have been identified, they can then be assessed for *in vivo* activity as described below.

A. Pharmacokinetic Models

Pharmacokinetics is discussed in detail in Chapter 23 and mentioned here only in terms of the information such studies yield for compound optimization. The main purpose of PK models is to provide information on “dose to man” and dosing frequency. The driving force in PK is the speed with which the drug is cleared from the body. Clearance (Cl) is typically measured by following the drug concentration in plasma after intravenous administration of the drug in rats (or other species). Simplistically, the elimination of the drug from the body can be approximated by the exit of a substance from a single compartment via a first order elimination process. From this, the volume of distribution (V_d) of the drug can be estimated as the total amount of drug in the body/plasma concentration. Cl and V_d are the primary parameters required to describe PK and are related to the drug elimination rate constant (k) by:

$$k = \frac{Cl}{V_d} \quad (2.11)$$

and since drug half-life $t_{1/2} = 0.693/k$, then:

$$t_{1/2} = \frac{0.693 V_d}{Cl} \quad (2.12)$$

Thus it can be seen that a reduction in Cl leads to a slower elimination and therefore a longer $t_{1/2}$. Similarly, an increase in V_d (increased tissue binding and sequestration of drugs away from the plasma) leads to a reduction of accessibility to elimination and a subsequent increase in $t_{1/2}$. The $t_{1/2}$ determines the duration of action of the drug after a single dose but will also determine the time to reach steady state on chronic dosing since this is the mirror image of disappearance. Thus drugs with long $t_{1/2}$ may take weeks to reach steady state. Ideally, drugs are administered once or twice a day, so Cl and V_d are key parameters for the medicinal chemist to optimize. As most drugs are designed to be orally active, bioavailability (F) is another important factor in drug design. F defines the fraction of the dose that reaches the systemic circulation (i.e., is absorbed and survives first-pass metabolism in the liver). It is calculated as:

$$F = \frac{\text{Area under the curve after an oral dose}}{\text{Area under the curve after an equivalent } i.v. \text{ dose}} \quad (2.13)$$

A typical plot of plasma concentration as a function of time (i.e., PK profile) following oral dosing of a compound is illustrated in [Figure 2.20A \[46\]](#).

Ultimately, the dose and frequency of dose are aimed at keeping the drug concentration above the effective concentration in humans for as long as possible without eliciting adverse effects. Allometric scaling (which is based on body weight) can be used to predict the Cl of drugs in humans based on measurements made in preclinical species.

B. Efficacy Models

Whereas PK models measure drug concentration, efficacy models provide pharmacodynamic (PD) readouts (i.e., they measure drug effects). Such efficacy models range in complexity from simple acute readouts of mediators or cell numbers to more clinically relevant measurements such as tumor size after chronic dosing. Irrespective of the readout, they are designed to provide further confidence that the drug target being investigated is clinically relevant and its modulation will lead to a desirable clinical outcome. The species used include mice, rats, guinea pigs, rabbits, dogs, and macaques, and various routes of drug administration are employed (intraperitoneal, subcutaneous, intravenous, inhaled, oral, etc.). As described above, it is important to know that compounds under test have good activity at the target in the species under study and that the compound has a

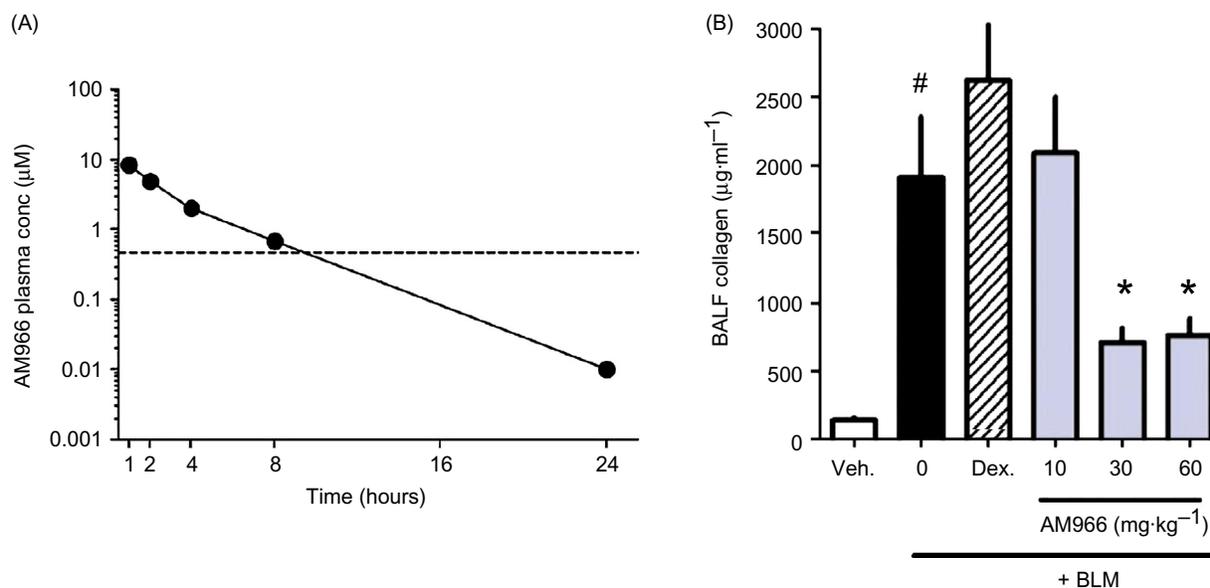


FIGURE 2.20 *In vivo* Assays. (A) PK profile of the LPA₁ receptor antagonist, AM699, after oral (10 mg/kg) administration to mice. The dashed line represents the IC₅₀ value for AM699 mediated inhibition of LPA responses in CHO cells expressing the murine LPA₁ receptor. The profile indicates that at doses of 10 mg/kg and above, the compound provides IC₅₀ coverage for ≥ 8 hours. (B) The efficacy of AM699 in a mouse 14-day bleomycin model. The compound was administered twice a day for 14 days and soluble collagen measured in the bronchoalveolar lavage fluid as a marker of fibrosis (at day 14). Dexamethasone was included as a comparative compound since steroids are used as treatments for IPF despite their dubious efficacy. Reproduced with permission from Swaney et al., 2010 [46].

PK profile that allows adequate target coverage for the duration of the PD model. In instances where the ortholog of the human target does not exist in mice or it has a different function, it may be possible to employ transgenic animals or xenografts. An example of the former approach is the transgenic expression of human ICAM-1 in mice allowing them to be infected with human rhinovirus (mouse ICAM-1 does not recognize rhinoviruses that infect humans) [47]. In the cancer field, xenografts of human tumor cells grown in mice are widely used models. Although a vast range of models are available, it should be recognized that many of the efficacy models routinely employed in drug discovery programs are somewhat poor predictors of clinical efficacy. Rather, they allow the investigators to study the effects of drugs on particular mechanisms of action that may (or may not) be relevant to the clinical disease being targeted. As an example, bleomycin-induced lung inflammation and fibrosis is widely employed as a model to study the effects of drugs aimed at treating idiopathic pulmonary fibrosis (IPF) (Figure 2.20B) [46]. Whereas the resultant lung pathology has some similarity with that observed in IPF, profound inflammation precedes the fibrosis, a sequence of events that does not appear to occur in the human disease. Nevertheless such models allow compound activity to be optimized and builds confidence that *in vitro* activity is indicative of *in vivo* activity. Ideally, compound dose-response information is generated over a wide dose range (just as it should be in *in vitro* studies), allowing estimations of ED₅₀ or ID₅₀ (effective doses of agonists or antagonists/inhibitors producing 50 percent of the maximum response) values and maximum effects to be generated and compared with *in vitro* estimates. PK measurements made during efficacy models allow PK-PD relationships to be further explored which assists in refining “dose to man” estimates made from prior PK studies.

Finally, for some diseases, useful animal models do not exist or are poorly characterized, necessitating progression of drugs straight to the clinic from *in vitro* testing. CF is a good example of this in that compounds that correct or potentiate CFTR function *in vitro* and that have good PK can readily be assessed for activity in humans using surrogates of CFTR function such as nasal potential difference or skin sweat chloride.

C. Safety Testing

At the end of drug discovery and prior to going into human clinical studies, regulatory authorities (e.g., FDA and EMA) have to be convinced that a potential new drug is safe as well as efficacious. To achieve this, data are

TABLE 2.8 Examples of Safety Testing Models

Assays	Test	Purpose
<i>In vitro</i>	Ames ^a	Bacterial assay to measure a compound's mutagenic potential—ability to change gene DNA sequence—and a predictor of carcinogenesis.
	hERG ^b Cardiac ion channel	Binding/functional assay to determine if a compound inhibits hERG activity, a predictor of cardiac arrhythmia
<i>In vivo</i>	Repeat-dose animal study	Assessing the effects of long term compound exposure on in life (e.g., weight, behavior) and terminal readings (e.g., organ histopathology)

^aMortelmans K, Zeiger E. The Ames Salmonella microsome mutagenicity assay. *Mutat Res* 2000;455(1–2):29–60.

^bSanguinetti MC, Tristani-Firouzi M. hERG potassium channels and cardiac arrhythmia. *Nature* 2006;440(7083):463–9.

submitted from nonclinical pharmacological, pharmacokinetic and toxicological animal studies. At this stage, an understanding of the nonclinical safety profile is essential and should aim to cover three areas:

- establish a safe initial dose level of the first human exposure
- identify parameters for clinical monitoring of potential adverse effects
- special toxicity (e.g., genotoxicity, carcinogenicity, reproduction toxicity)

Generally, a tailored combination of *in vitro* and *in vivo* animal tests is performed (see Table 2.8 for examples), which will include studies of the drug's toxicity on organs targeted by that compound, as well as determining if there are any long term carcinogenic effects or toxic effects on mammalian reproduction.

Importantly, it is assumed that animals and humans respond to administered chemicals in essentially the same way and that exposing animals to the maximal level of compound possible is a valid approach to predicting low incidence human toxic responses. These data are then used to determine a “No Observable Adverse Effect Level” (NOAEL) and then a safe starting dose is estimated for human clinical trials by allometry (scaled by animal/human, size/shape differences).

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