Examining Dose-Response Relationships

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Forward

This book is intended to be provide general information and assistance in understanding dose-response relationships as they pertain to altered gene expression or reporter activity elicited by nuclear receptors. However, the concepts are generally applicable to other areas as well, in-so-much as any graded dose-response relationship follows the same basic shape and is governed by the same principles.

Receptor Theory

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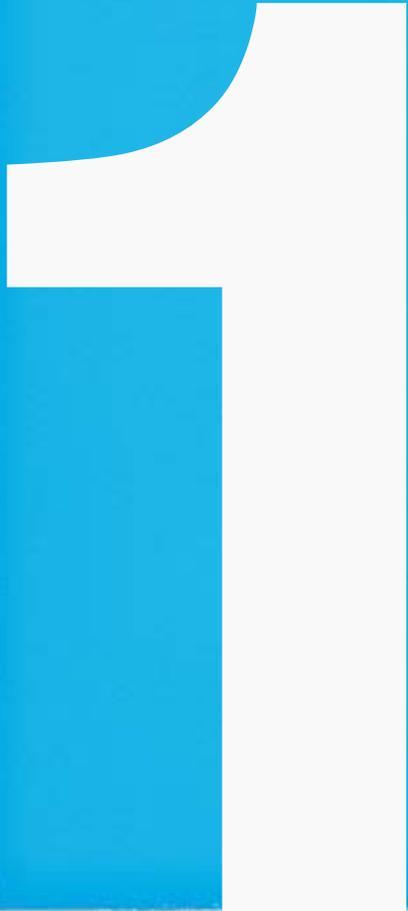
The shape of dose-response curves is governed to a large extent by equations that describe bi-molecular interactions. These interactions were initially described for ligand-receptor interactions and hence are termed "Receptor Theory".

Introduction

A key concept in biological sciences including pharmacology and toxicology is that bioactive small molecules such as drugs, hormones, toxins/toxicants and nutrients must achieve adequate concentration at a target site in order to elicit a biological response. For many chemicals, the ultimate site of action is a cognate protein or "**receptor**". The main criteria for the operational term "receptor" are the functions of recognition and transduction. By this definition, a receptor must recognize a distinct chemical entity and translate information from that entity into a form that the cell can interpret, and alter its state accordingly. This altered state may be a change in permeability, activation of a guanine nucleotide regulatory protein or an alteration in the transcription of DNA. To differentiate a receptor from an enzyme, the recognition unit should not chemically alter the small molecule and, to differentiate from a binding protein, a receptor must produce a biochemical change and transmit the signal. Often the receptor is a protein and a single component of a large complex of macromolecules that may include other proteins, RNA and DNA.

The quantification of the interaction between a macromolecule and a **xenobiotic** is often called receptor theory. As will be discussed later in the chapter, the development of receptor theory predates many of the modern techniques of molecular biology and coincides with developments in analytical biochemistry, basic enzymology and pharmacology. The study of structure-activity relationships and modifications of chemicals to fit the active site of the macromolecule have become standard in the pharmaceutical industry and are an important part of modern pharmacology and toxicology. Also, the basic tools of quantification and characterization of a bimolecular interaction is applicable to many disciplines including enzymology (Michaelis complex between enzyme and substrate), immunology (formation of antibody-antigen complex), pharmacology (drug-receptor complex) and toxicology (toxicant-receptor complex).

Much of the conceptual framework regarding receptor theory evolved from pharmacology and the investigation of drug action. Consequently, the historical account of the development of receptor theory contains many references to drugs as opposed to hormones, neurotransmitters and xenobiotics. The term **ligand** (L) is used interchangeably with drug in this case, and simply denotes a chemical that binds with affinity and specificity to a receptor. Drugs and certain xenobiotics presumably bind to

receptors designed for interaction with endogenous hormones and neurotransmitters. By way of definition, **agonists** are analogous to endogenous hormones and neurotransmitters, in the sense that they elicit a biological effect, although the effect elicited may be stimulatory or inhibitory. In contrast, **antagonists** are defined as agents that block receptor-mediated effects elicited by hormones, neurotransmitters, or agonist drugs by competing for receptor occupancy or by interfering with agonist binding in other ways. Antagonists may not have an endogenous, physiologically-relevant counterpart in the strict sense of a competitive inhibitor of receptor occupancy.

 It is also worth noting that ligands, whether naturally-occurring, pharmaceutical, dietary or environmental, have the same basic mechanism of interaction with receptors. The ligands as discussed herein are small chemicals that associate with the protein receptor, irrespective of the source of the molecule. We will discuss how this bi-molecular interaction is studied as well as the pertinent endpoints and approaches used to define the ligand-receptor complex. The interpretation of this data will vary based on whether the small molecule of interest is a drug versus a pollutant, for example, but the steps taken to derive meaningful measurements of affinity are identical.

Law of Mass Action

The concept of receptor-mediated events dates back to the late 1800s and helped explain, qualitatively, the selectivity and saturability of drug action. However, it was not until A.J. **Clark** (1885-1941), performed his research that the quantitative nature of receptor theory emerged. Clark postulated that drugs combine with their receptors at a rate dependent on the concentration of drug and receptor. Similarly, the resulting drug-receptor complex breaks down at a rate proportional to the number of complexes. Most analyses of ligand binding experiments are based on this simple model:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} LR$$
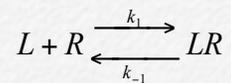
where L is the ligand, R is the receptor and LR is the ligand-receptor complex.

The model is based on several simple ideas:

1. Binding occurs when ligand and receptor collide due to diffusion, and when the collision has the correct orientation and sufficient energy. The rate of association (number of binding events per unit of time) equals [L][R]k_1 , where k_1 is the association rate constant in units of $M^{-1}min^{-1}$.

2. Once binding has occurred, the ligand and receptor remain bound together for a certain amount of time that is influenced by the affinity of the receptor and ligand for one another. The rate of dissociation (number of dissociation events per unit time) equals [L]k_{-1}, where k_{-1} is the dissociation rate constant expressed in units of min^{-1}.

3. After dissociation, the ligand is the same as before binding. As noted earlier, this is to differentiate a receptor from an enzyme.

4. Equilibrium is reached when the rate at which new LR complexes are formed equals the rate at which these complexes dissociate. Equilibrium may be more appropriately termed apparent equilibrium or steady state, since the achievement of true equilibrium is often not possible in pharmacologic systems.

At equilibrium, LR complexes form at the same rate that they dissociate:

$$[L][R]k_1 = [LR]k_{-1}$$

which may be rearranged to define the equilibrium dissociation constant **Kd**.

$$Y = \frac{[LR]}{[R]_{TOT}} = \frac{[LR]}{[R]+[LR]} = \frac{[L]}{[L]+K_d}$$ (Equation 2)

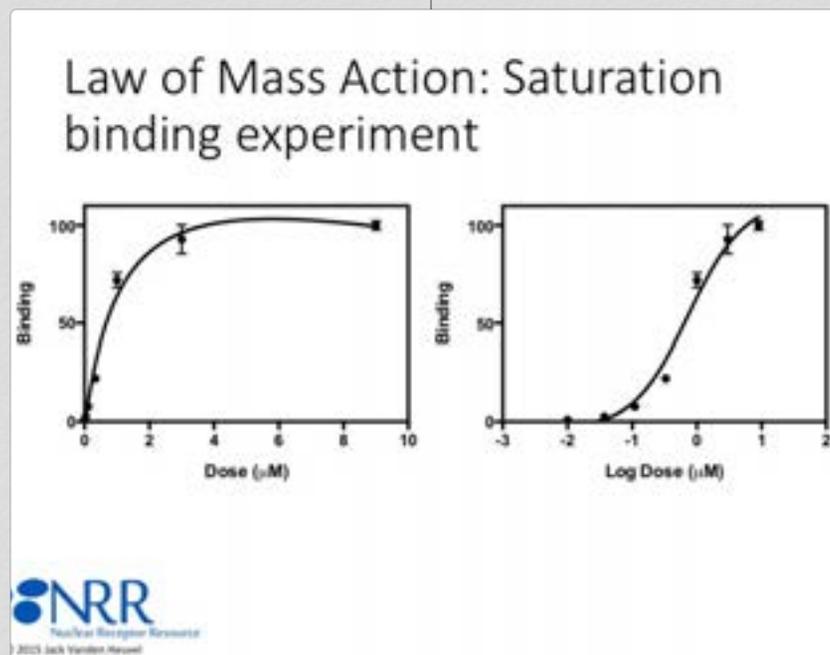$$\frac{[L][R]}{[LR]} = \frac{k_{-1}}{k_1} = K_d$$ (Equation 1)

The K_d, expressed in units of moles/liter or molar, is the concentration of ligand that occupies half of the receptors at equilibrium (see below). A small K_d means that the receptor has a high affinity for the ligand whereas a large K_d means that the receptor has a low affinity for the ligand. Often K_d, the equilibrium dissociation constant, is confused with k_{-1}, the dissociation rate constant. However, they are obviously not the same, as denoted by the fact that their units are different.

The law of mass action predicts the fractional receptor occupancy at equilibrium as a function of ligand concentration. **Fractional occupancy (Y)** is defined as the fraction of all receptors that are bound to ligand:

FIGURE 1.1 Saturation binding curves

Law of mass action predicts a rectangular hyperbola as shape of curve. Note that the data shown above is identical, but is plotted with the X-axis normal (left) or log (right) scaling.

This equation predicts the following. When no ligand is available, the occupancy equals zero. When the concentration of ligand is very high (many times K_d), the fractional occupancy approaches (but never reaches) 100%. When [L]=K_d, the fractional occupancy is 50%. Equation 2 predicts that the approach to saturation as ligand concentration increases is quite slow. When the ligand concentration equals four times its K_d, it will only occupy 80% of the receptors at equilibrium. The occupancy rises to 90% when the ligand concentration equals 9 times the K_d. It takes a concentration equal to 99 times the Kd to occupy 99% of the receptors at equilibrium. (See)

Assumptions of the law of mass action

The law of mass action is a convenient and simple model to describe a bimolecular interaction. However, in order to coerce a complex biological interaction into a simple model, several assumptions or criteria must be met. Although termed a "law", the law of mass action is simply a model based on the following assumptions:

1.   All receptor sites are equally accessible to ligand. In the simplest model, all receptor sites are considered to have equal affinity for ligand and to be independent. That is, occupancy of some receptor sites does not alter the binding to other, unoccupied sites (cooperative binding is not present).

2.   The two reactants (receptor and ligand) are either free or bound to ligand. The model ignores any states of partial binding. Also, the measured products do not include degraded, metabolized or other unavailable forms of drug or receptor. Importantly, non-specific binding must be accounted for such that the concentration of the free or bound ligand is measured accurately. Non-specific binding includes any non-receptor site for ligand binding that would diminish the free concentration of the chemical being examined.

3.   Binding is reversible. The association between a ligand and receptor depends only on the interaction of ligand and receptor and dissociation only on the breakdown of the ligand receptor complex.

Receptor binding experiments

Brief outline of saturation binding experiments

It is beyond the scope of the present review to describe in details the methods used to assess receptor binding. Suffice it to say, that the following are key parts of the experiment:

Choice of Label

In order to examine the binding of ligand and receptor, one of these constituents must be easily quantified. The most common means to quantify a ligand is to add an isotope to form a **radioligand** or to add a fluorescent tag to the ligand. Often, to differentiate from the unlabeled ligand, the radio- or fluorescence-labeled molecule is shown as L*.

The incubation

Critical components of the incubation include the labeled ligand, a source of receptor (crude membrane preparation, recombi-

nant protein etc.) and a suitable buffer. Also, the incubation must proceed for a sufficient amount of time, at an appropriate temperature, until enough drug-receptor complex is formed to be detectable.

Separation of bound from free ligand

To determine the quantity of LR accumulated, one must have a method for determining the incubation that permits the resolution of L from LR. For most experiments that use radiolabeled ligands, the free and bound forms of drug may be easily separated by filtration where the free L flows through the filter and the LR complex is captured. There are some methods deemed "homogeneous" such as TR-FRET and Fluorescence Polarization (FP) that rely on distinct properties of the free fluorescent molecule compared to the bound complex and do not require physical separation techniques.

Nonspecific binding

In addition to ligand binding to receptors of physiological interest, ligands often bind to nonreceptor sites. Any interaction with a non-receptor site would be considered **nonspecific binding** and is found at excess over true, specific interactions with the receptor. When performing radioligand binding experiments, a measure of both total and nonspecific binding is performed, and

specific (receptor) binding is calculated as the difference (See Interactive 1.1)

As shown in Figure 1.1, the relationship between receptor occupancy ([LR]) and drug concentration is hyperbolic when a saturable receptor population binds to ligand in a freely reversible, bi-molecular interaction. Often the results is examined by linear transformation of the experimental data. The advantage of such transformations is that K_d, and receptor density (B_{max} or R_{tot}) can be easily identified. However, if the drug concentrations examined are insufficient, as define empirically, linear transformations may give a distorted view of the binding phenomena. In this section, both non-linear and linear transformations will be discussed. All plotting methods must meet the following criteria: (1) Assumptions of the law of mass action, as

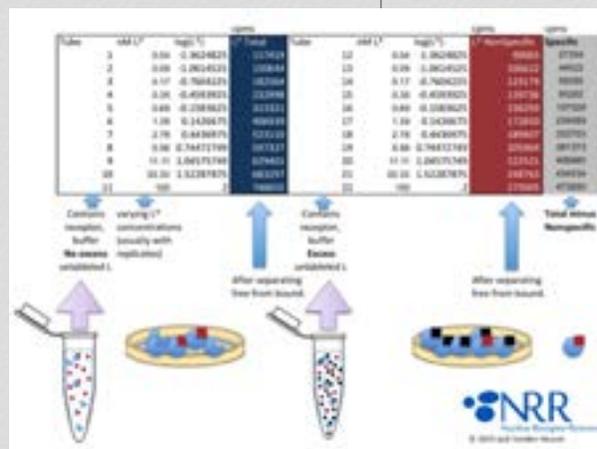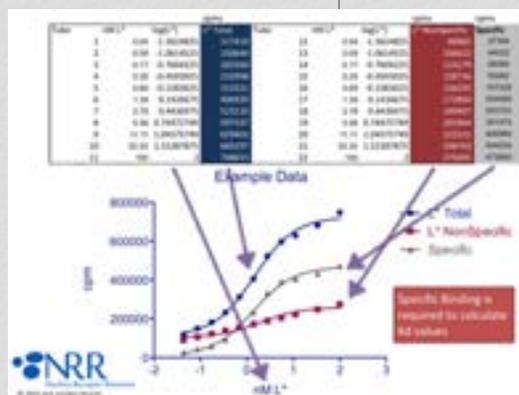**FIGURE 1.2** Determining specific binding: Experiment Set-up

FIGURE 1.3 Lorem Ipsum dolor amet, consectetur

Calculating specific binding, top panel

described above, are met; (2) The incubation and separation techniques are appropriate; and (3) Nonspecific binding has been adequately measured.

Plotting saturation binding data

Saturation binding experiments measure specific binding at equilibrium at various concentrations (often 6-12) of the radioligand to determine receptor number and affinity (Figure 1.2.). Use of drug concentrations that allow binding to approach saturation is crucial for accurate examination of the interaction between drug and receptor. The next consideration is that of nonspecific binding. As mentioned above, a good rule-of-thumb is to use a concentration of unlabeled ligand at 100 times the K_d. When examining binding in the presence of $100K_d$, the amount of [LR] detected is considered nonspecific, and as shown in Figure 1.3 is linear with respect to free ligand concentration. Total binding is the amount of [LR] detected in parallel samples without

this competitor included in the incubation. The difference, (total – nonspecific) is the **specific binding**, and is the data that should conform to the law of mass action. It is this binding that we will examine in more detail.

One of the criteria stated previously for a physiologically relevant receptor is that the binding sites are saturable. To assess saturability, the characteristics of binding as a function of increasing concentrations of radioligand are determined. The saturation binding curve can described by equation 2

$$Y = \frac{[LR]}{[R]_{TOT}} = \frac{[LR]}{[R]+[LR]} = \frac{[L]}{[L]+K_d}$$ (See Equation 2)

which can be rearranged to the following,

$$[LR] = \frac{[L][R]_{tot}}{K_d +[L]}$$ (Equation 3)

Equation 3 is the description of a rectangular hyperbola $y = \frac{ax}{b+x}$. An important consequence of this equation representing a rectangular hyperbola is that the horizontal asymptote is R_{tot} or sometimes referred to as B_{max}. Thus B_{max} can only be obtained at infinite concentrations of L. This will be of importance when linear transformations are discussed. Other important pieces of information can be obtained from equation 3. The term K_d is a measure of the affinity of the receptor for ligand and is the con-
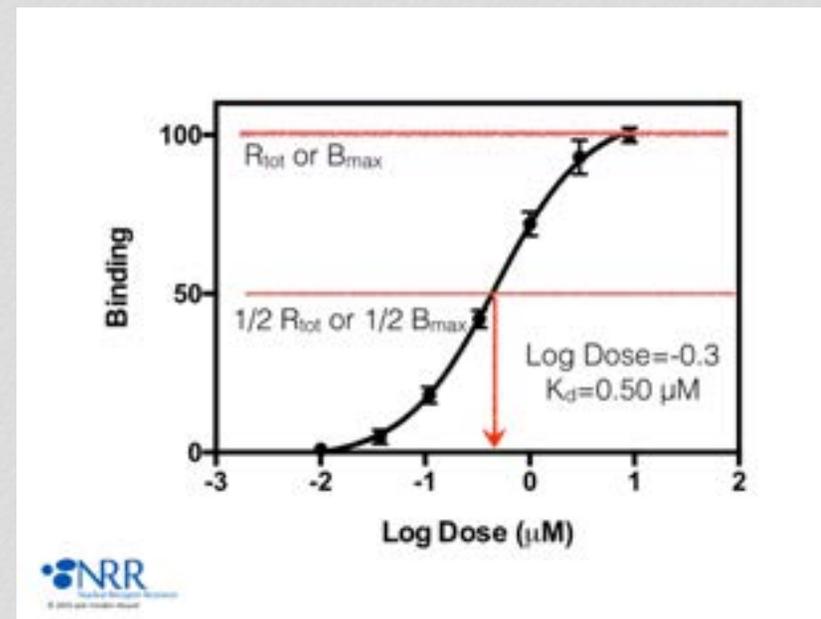
Estimating Bmax and Kd from specific binding data

centration of ligand that occupies one-half of the maximal binding sites. This can be easily derived from equation 3 if [LR] is replaced by and solving for K_d (the result being K_d=[L]). The estimation of B_{max} and K_d can be solved by either linear transformation or by non-linear regression, which is described below. (see Figure 1.4).

Linear Transformation: Scatchard plots

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The most common linear transformation of binding data is the **Scatchard plot**. In this plot, the X axis is specific binding (usually labeled "bound") and the Y axis is the ratio of specific binding to concentration of free radioligand (usually labeled "bound/free"). Some of the terms used in Scatchard plots are:

B=Bound (LR): Concentration of ligand in the incubation that is *specifically bound* to the receptor at equilibrium

F=Free (L): Concentration of free ligand present in the incubation at equilibrium, often estimated by the concentration of the drug added to the incubation

K_d : Equilibrium dissociation constant. In the Scatchard plot, the slope of the line is equal to K_d^{-1}. K_d is expressed in the same units as the drug concentration (i.e. molarity).

Bmax (Rtot): Maximum number of binding sites in the incubation at equilibrium, or total receptor concentration. B_{max} is expressed in the same units as the specific binding, usually as a concentration relative to amount of protein in the incubation (i.e. fmoles/mg protein).

$$(B/F) = -\frac{1}{K_d}(B) + \frac{B_{max}}{K_d}$$ Equation 4 (Scatchard Equation)

When making a Scatchard plot, you have to choose units for the Y axis. One choice is to express both free ligand and spe-

FIGURE 1.5 Scatchard Plot
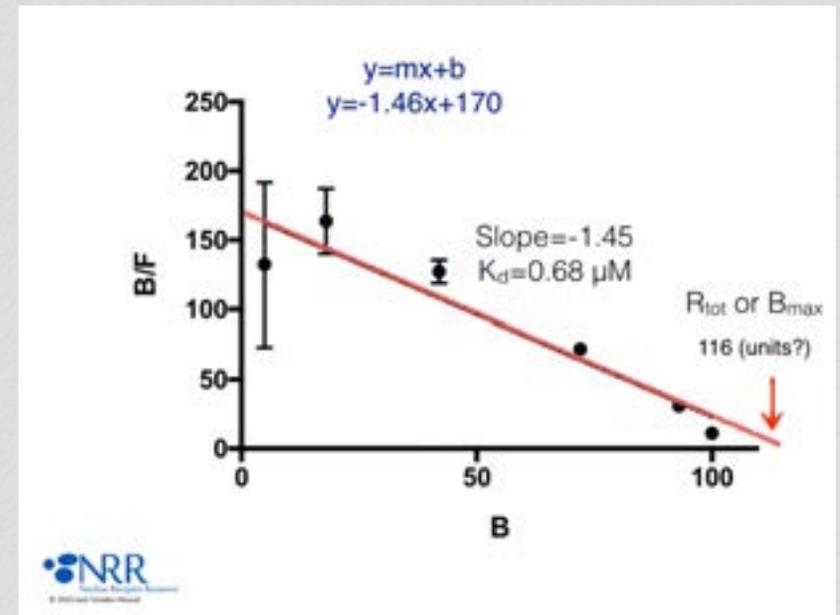
Using linear transformation to estimate K_d and B_{max}

cific binding in counts per minute (cpm) so the ratio bound/free is a unitless fraction. The advantage of this choice is that you can interpret Y values as the fraction of radioligand bound to receptors. If the highest Y value is large (greater than 0.10), then the free concentration will be substantially less than the added concentration of radioligand, and the standard analyses are not appropriate. The disadvantage is that the experimenter cannot interpret the slope of the line without performing unit conversions. An alternative is to express the Y axis as fmol ligand bound per mg protein per concentration (nM). While these val-

ues are hard to interpret, they simplify calculation of the Kd which equals the reciprocal of the slope. The specific binding units cancel when the slope is calculated. The negative reciprocal of the slope is expressed in units of concentration (nM) which equals the K_d. (see Figure 1.5).

Non-linear regression

Equilibrium specific binding at a particular radioligand concentration equals fractional occupancy times the total receptor number (Bmax or Rtot):

$$Specific\ Binding = [LR] = \frac{[L] \bullet B_{max}}{K_d + [L]}$$ Equation 5

This equation describes a rectangular hyperbola or a binding isotherm. As before, [L] is the concentration of free radioligand, and is plotted on the X axis. B_{max} is the total number of receptors expressed in the same units as the Y values (i.e., cpm, sites/cell or fmol/mg protein) and K_d is the equilibrium dissociation constant (expressed in the same units as [L] usually nM). Typical values might be a B_{max} of 10-1000 fmol binding sites per milligram of protein and a K_d between 10 pM and 100 nM.

To determine the B_{max} and K_d, the most accurate method is to fit the specific binding data to equation 5 using nonlinear regression. There are currently several resources available for performing non-linear regression and several statistics and plotting programs will perform the analysis. An excellent source for a detailed description of non-linear regression may be found at the GraphPad website www.graphpad.com.. For the more adventerous, you can use Excel with the Solver add-in to do basically the same thing. The equation being fit is Y=Bottom + (Top--Bottom)/(1+10^((LogEC50-X))).

See the table below with a hypothetical example to compare the results from the each of the methods described.

Method	K_d	B_{max}
Estimation from plot	0.5	100
Scatchard	0.68	116
Nonlinear regression (Prism)	0.51	107
Non-linear regression (Excel)	0.47	107

Competitive binding experiments

Why use a competitive binding curve?

Competitive binding experiments measure the binding of a single concentration of labeled ligand in the presence of various concentrations of unlabeled ligand. To quantify the potency of drugs in competing for the receptor, the **IC50** (concentration that inhibits 50% of the specific radioligand binding) value is determined for each competitor. Competitive binding experiments have several advantages to direct binding assays and have been used for the following types of experiments:

•Validate a direct binding assay. Competition binding experiments are an excellent way to examine the physiological significance of a direct binding assay. In this type of analysis the radioligand is competed with drugs whose potencies are known from functional experiments. Demonstrating that these drugs bind with the expected potencies, or at least the expected order of potency, helps prove that the radioligand has identified the correct receptor.
•Determine whether a drug binds to the receptor. Thousands of compounds can be screened to identify drugs that bind to the receptor simply by determining if they cam effectively compete with a known ligand. This can be faster and easier than other screening methods. In fact, this is the most common way the

pharmaceutical companies are identifying novel ligands for important receptor systems.
•Investigate the interaction of low affinity drugs with receptors. Binding assays are only useful when the radioligand has a high affinity ($K_d < 100$ nM). A radioligand with low affinity generally has a fast dissociation rate constant, and will not stay bound to the receptor while you wash the filters or pellet the complex.

Performing the experiment

The experiment is done with a single concentration of radioligand, usually equal to the K_d concentration of that ligand for the receptor as determined in previous studies. A higher concentration will increase the sensitivity of the assay and decrease counting error but will also increase non-specific binding and time to equilibrium.

As with direct binding experiments, the incubation should reach equilibrium. However, the reaction is more complicated in the presence of an inhibitor, often at high concentrations. To ensure that a steady-state has been reached, the incubation proceeds for 4-5 times the half-life of the radioligand for receptor dissociation as determined in an off-rate experiment. In order to have a complete profile of the competition, typically 12-24 concentrations of unlabeled compound spanning about six orders of magnitude are examined.

Analyzing competitive binding data

Visual inspection of competition of radioligand binding reveals a hyperbolic curve, with the most potent ligands inhibiting specific binding at lower concentration than less potent chemicals Figure 1.6. The plateau at the top of the curve (i.e. the radioligand binding in the absence of the competing unlabeled drug), represents total binding. Note that total binding is not the same as B_{max} since the receptor is not fully saturated (if using a K_d concentration of radioligand). The bottom of the curve is a plateau equal to nonspecific binding (NS) with the difference between the top and bottom plateaus being specific binding. The IC_{50} is the concentration of unlabeled drug that blocks half the specific binding.

Competitive binding curves are described by this equation:

$$[LR]_I = NS + \frac{Total - NS}{1 + 10^{\log[I] - \log IC_{50}}}$$ Equation 6

FIGURE 1.6 Competitive binding curves
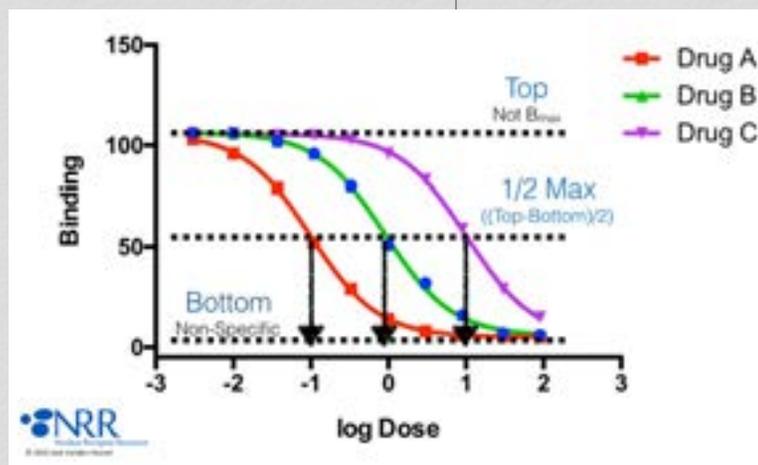
Estimating IC50 values from inhibition binding curves. Note, the "Top" is not equivalent to Bmax

where [LR] (Y-axis) is the amount of binding measured at each [I] (X-axis). Nonlinear regression is used to to fit your competitive binding curve to determine the $\log(IC_{50})$. In order to determine the best-fit value of IC_{50}, the nonlinear regression problem must determine the 100% (total) and 0% (nonspecific) plateaus.

Calculating the K_I from the IC_{50}

The IC_{50} value is not equivalent to K_d for the competitor (or K_i) and is dependent on the amount of radioligand in the incubation. Therefore, the IC_{50} value can vary between studies whereas K_i is an apparent constant. The K_i can be calculated from IC_{50} using the **Cheng and Prusoff** method

$$K_i = \frac{IC_{50}}{1 + \frac{[L*]}{K_d}}$$ Equation 7

where Ki is the equilibrium dissociation constant for the inhibitor and Kd is the equilibrium dissociation constant for the radioligand L*. Several assumptions were made in the derivation of the Cheng and Prusoff equation that must me met.

1.   The radioligand and the inhibitor must interact with receptor according to the law of mass action. That is, the binding of both chemicals should be reversible and directed at a single population of R. Whether the law of mass action has been met can be determined from an indirect Hill plot where the slope equals –1.

2.   The concentration of L added should equal the amount free. There should not be ligand depletion ([LR]<10%) and the concentration of receptor is much less than Kd

3.   The incubation has reached equilibrium for radioligand and all concentrations of the competitor.
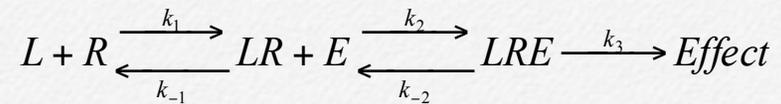
Receptor-mediated effects

2

The dose-response of a cell to a ligand is more complicated than predicted by direct binding assays. While it is assumed the ultimate response is proportional to the amount of ligand-receptor complex formed, direct proportionality may not be observed. Therefore, it is often difficult to determine K_d values for a ligand-receptor interaction based solely on the response of that cell.

Quantitation of receptor responses

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR + E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} LRE \xrightarrow{k_3} Effect$$

Why examine receptors based on responses?

The interaction between the receptor and ligand is the first step in the elicitation of a biological response. In fact, there are three components of a receptor system: the ligand, the receptor and the effector (E) (see pathway to the right). The effector may be an enzyme, an ion or a transcription factor and it transmits the biophysical interaction of ligand and receptor into a biochemical or molecular signal. Physiological receptors are linked to the signal transduction apparatus of the cell. Ultimately, the dose-response of a cell to a ligand is more complicated than predicted by direct binding assays. While it is assumed the ultimate response is proportional to the amount of ligand-receptor complex formed, direct proportionality may not be observed. Therefore, it is often difficult to determine K_d values for a ligand-receptor interaction based solely on the response of that cell.

Beyond adding complexity, the effector system amplifies the response. Therefore, detection of a receptor-mediated event is often easier examined than direct binding *per se*. This is the major reason why receptors are often characterized by their responses prior to the implementation of direct binding assays. Sensitive assays for detecting influx or efflux of Na^+, K^+, Ca^{2+} or Cl^-, activation of adenylate cyclase, phosphorylation of a receptor or effectors, alterations in mRNA expression, have been developed. In addition, binding of a chemical to a receptor says nothing about the response elicited by the ligand. That is, antagonists and agonists may bind with the same affinity to a receptor (in fact antagonists are often more avidly associated) but are coupled to the effector molecular in a different manner. Therefore, receptor responses are of more physiological relevance than direct binding assays in isolation.

Graded dose-response curves: Definitions

Graded dose-response curves can be constructed for responses that are measured on a continuous scale, eg, heart rate, blood pressure, LDH release, altered gene expression.

Definitions of Pharmacologic Effects

Agonist Drug which binds to the same receptor as the endogenous compound and produces the same type of signal as the endogenous hormone/neurotransmitter.

Antagonist Drug which binds to the same receptor as the endogenous compound and inhibits the signal produced by the activating hormone/neurotransmitter.

Partial Agonist Drug which when maximally bound to the receptor causes a sub-maximal response.

Inverse Agonist (Negative Antagonist) Drugs which cause a decrease in basal receptor activity i.e. in the absence of agonist.

Affinity A measure of the strength of interaction between a receptor and its cognate ligand; K_D is defined as the concentration at which half of the receptor is occupied.

Efficacy A measure of the efficiency in which a bound ligand activates its target receptor's signal transduction/biological response.

Potency An overall measure of the ability of a ligand to activate its target receptor. Related to the EC_{50} ;the concentration at which a half maximal effect is achieved.

Graded dose-response curves relate the intensity of response to the size of the dose, and hence are useful for characterizing the actions of drugs. In fact, for every graded dose-response relationship there are a variety of different measures of activity that can be used to describe a compound (see Figure 2.1 and the text box above). The two key features are that of efficacy and potency, and how they are experimentally defined will be

FIGURE 2.1 Graded dose-response relationship
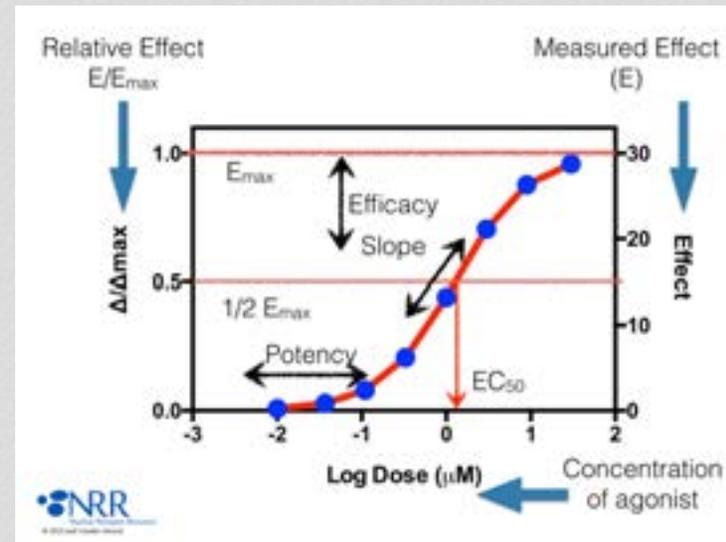
Key information that can be achieved through a dose-response study is efficacy and potency

described subsequently. Although the shape of the dose-response curve is identical to that of the receptor occupancy, there are some very important distinctions. First and foremost, both efficacy and potency are variables that are dependent on experimental conditions. Although the response observed may be related to the fractional occupancy, the relationship may be complicated. In other words, binding of a drug to a cognate receptor is an essential component of the biological response, but the coupling or transduction of this event will determine the type and extent of the effect observed. For example, ligands can in-

teract with a receptor and act as agonists, partial agonists, antagonists or inverse agonists (see Figure 2.2) depending in some cases on cellular context. For each of these types of responses, we will need to describe the drug action in terms of potency, efficacy and in some cases slope.

FIGURE 2.2 Types of ligands
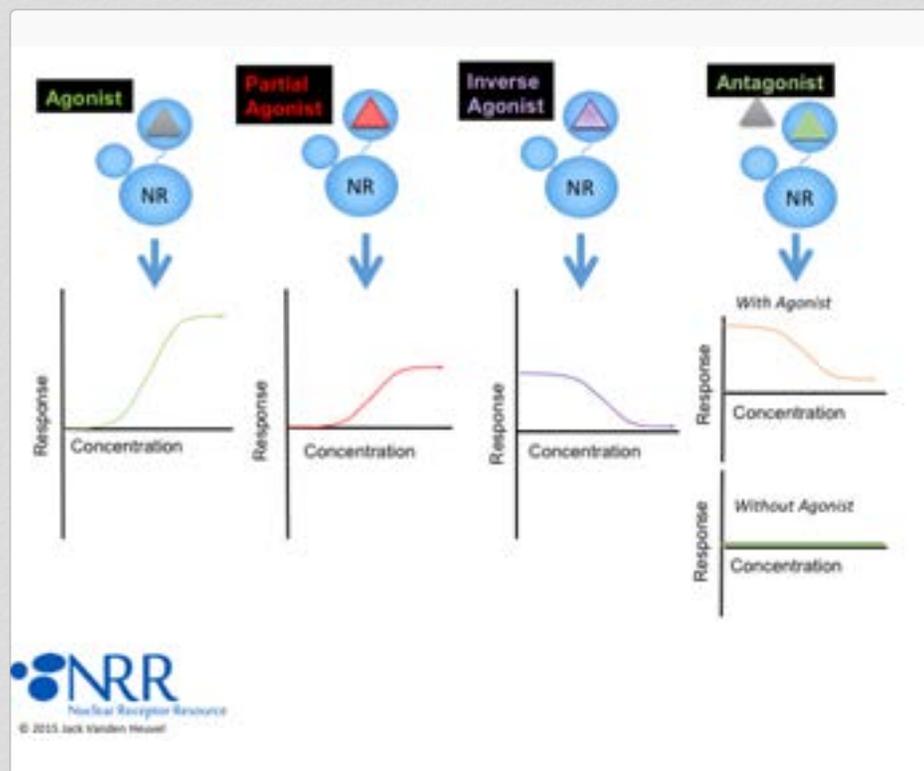
Key concepts

Initially, it was proposed that Clark's equations would hold true when measuring a biological response, and not just for examining simple biomolecular interactions. However, this simple relationship between occupancy and effect does not adequately predict many biological responses. In particular, dose-response relationships to drugs and other chemicals tend to have a steeper slope than predicted. Also, the existence of **spare-receptors** shows that maximal responses do not require full occupancy. Finally, some ligands do not result in the same maximal effects seen with other compounds, despite reaching 100% occupancy. Thus, different liganded receptors have different abilities to generate a signal than others (i.e. full and partial agonists exist). To explore further, examine the following pathway.

$$L + R \xrightleftharpoons[k_{-1}]{k_1} LR \xrightarrow{k_e} Effect$$
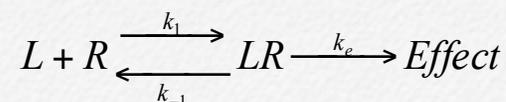
Thus, if k_e (the rate constant for coupling, or of the effector system) is known, for any given [LR] the extent of the response can be predicted. But the actual quantitation of this term is very difficult.

$$Response\ (\Delta) = f([LR]) = f(\frac{[L][LR]_{max}}{K_d + [L]})$$

If examining the most simple case with a full agonist and no spare receptors, the maximal response Δ_{max} will occur at $[LR]_{max}$

$$\frac{\Delta}{\Delta_{max}} = \frac{LR}{LR_{max}} = \frac{[L]}{K_d + [L]}$$

Equation 8

However, a non-linear relationship between receptor occupancy and biological response must be appreciated.

$$Response = f(S) = fe\frac{LR}{LR_{max}}$$

$$Intrinsic\ Efficacy\ (\varepsilon): e = \varepsilon \bullet LR_{max}$$

$$Response = f(\varepsilon \bullet LR_{max} x \frac{LR}{LR_{max}}) = f(\varepsilon[LR])$$

Equation 9

where e is efficacy, ε is intrinsic efficacy and S is the stimulus applied to the tissue or cell. The introduction of the term ε effectively divided efficacy into two components, a ligand-dependent (e) and a tissue-dependent (LR_{max}) term.

Again, for our ability to compare dose-response relationships, the two parameters we are most interested in are potency and efficacy. Although determining a K_d and a B_{max} for each compound has advantages of being constants only dependent on physical properties of the constituents, they are difficult to obtain experimentally. For this reason, we will focus on the *Sigmoi-*

dal E_{max} model, as described below. For more information on estimated K_d and B_{max} from responses, the reader is directed to an excellent textbook: Limbird, LE Cell surface receptors: A short course on theory and methods, 2nd edition edition. Boston, MA: Kluwer Academic Publishers; 1996. p.238.

Analyzing graded dose-response relationships.

Sigmoid E_{max} Model

The difficulty in determining K_d values from biological responses had lead many to find alternative approaches or to simply the models in such a way so that comparisons can be made between compounds, at least when examined in the same tissue at the same time. Instead of determining constants (K_d and B_{max}) that are transferable regardless of condition, the aim is to find terms of potency (EC_{50}) and efficacy (peak effect or "span") that have less universal applicability but can be used for evaluation and comparison within a system.

As shown in Equation 8, $\dfrac{\Delta}{\Delta_{max}} = \dfrac{LR}{LR_{max}} = \dfrac{[L]}{K_d + [L]}$

We can replace the contact K_d with a more experimentally achievable parameter EC_{50}.

$$\frac{\Delta}{\Delta_{Max}} = \frac{[L]}{EC_{50} + [L]}$$ (Equation 10)

For some responses where the response in the absence of ligand is non-zero (i.e. blood pressure, basal gene expression etc.), the equation can be changed to the following:

$$\text{Effect } (\Delta) = \Delta_0 + \frac{\Delta_{Max}[L]}{EC_{50} + [L]}$$ (Equation 11)

The E_{max} model assumes that there is a single binding site for the ligand and that the maximal response will be achieved when the ligand concentration is high (many times the EC_{50}) and fractionally occupancy approaches 100%. Values for EC_{50} and peak effects can be estimated from the log(dose)-response curve, as outlined in Interactive 2.1. However, non-linear regression is prefered to solve for EC_{50}, Δ_0 and Δ_{max}. (See Interactive 1.1 or Interactive 1.1 for examples; the equation being fit is derived from Equation 11 and is Y=Bottom+(Top-Bottom)-/(1+10^((LogEC50-X)).) Note, EC_{50} is the concentration of agonist that gives a response half way between Δ_0 and Δ_{max} . A meaningful parameter is the amount of change seen, a measure associated with efficacy, is **span** which is simply Δ_{max}-Δ_0.

Most dose-response curves have a standard slope of 1.0, which is indicative of a single binding site per receptor. A model that does not assume a standard slope but rather fits the Hill Slope from the data, is called a Variable slope model.

$$\text{Effect } (\Delta) = \Delta_0 + \frac{\Delta_{Max}[L]^n}{EC_{50}{}^n + [L]^n}$$ (Equation 12)

Where n=**Hill Slope**, which describes the steepness of the family of curves. A Hill Slope of 1.0 is standard, and you should consider constraining the Hill Slope to a constant value of 1.0. A Hill slope greater than 1.0 is steeper, and a Hill slope less than 1.0 is shallower. Non-linear regression can be used to fit the four parameters to the data (requires more data points for fitting Equation 12 versus Equation 11; Y=Bottom + (Top-Bottom)-/(1+10^((LogEC50-X)*HillSlope)).

Operational model of drug action

The model described above are based on the assumption that there is a direct proportionality (not necessarily linear) between pharmacologic effect and number of occupied receptors. However, as mentioned above, the existence of spare receptors and partial agonism confound this simple model. The Operational Model described by Black and Leff (Proc. R. Soc. Lond. B, 220: 141-162, 1983) addresses these problems. In this case, the hyperbolic function to relate receptor occupancy and pharmacologic effect is shown as:

$$\text{Effect } (\Delta) = \frac{\Delta_{MaxSystem}[LR]}{K_A + [LR]}$$ Equation 13

The term $\Delta_{Maxsystem}$ refers to the maximum effect achievable in the system (i.e. with full agonist) and K_A is the concentration of occupied receptors required to produce half of $\Delta_{Maxsystem}$. The new term $\Delta_{Maxsystem}$ is used to differentiate from the maximal effect achieved by each ligand, which of dependent on the efficacy of different agonists. By substituting from Equation 5, the full operation model is achieved:

$$\text{Effect } (\Delta) = \frac{\Delta_{MaxSystem}[L][R_{tot}]}{K_d K_A + ([R_{Tot}] + K_A)[L]}$$ Equation 14

A new parameter, the intrinsic efficacy (τ) is defined as:

$$\tau = \frac{[R_{Tot}]}{K_A}$$ Equation15

This τ is a measure of the efficiency of the transduction of occupied receptors into a pharmacologic effect. It can be seen from the equation above that the effects approaching $\Delta_{Maxsystem}$ can be achieved at relatively low concentrations of occupied receptors if the K_A is low (i.e. τ is high). Rearranging these equations results in

$$\text{Effect } (\Delta) = \frac{\Delta_{MaxSystem}[L]\tau}{K_A + (1 + [L])\tau}$$ Equation 16

The operational model helps to differentiate between properties of the ligand (affinity, intrinsic efficacy) and the properties of the

system (receptor density, $\Delta_{Maxsystem}$) and how they work in concert to produce the effect. For example, when L is much greater than K_d, the Δ_{Max} is dependent on both system and drug properties as follows:

$$\Delta_{Max} = \Delta_{Maxsystem}\left(\frac{\tau}{\tau+1}\right)$$ Equation 17

and

$$K_A = \frac{\Delta_{Maxsystem}\tau}{(1+\tau)}$$ Equation 18

When τ is large, the Δ_{Max} is approximately $\Delta_{Maxsystem}$ and EC_{50} is much less than K_A, meaning full agonism. When τ is small, Δ is less than Δ_{max} and EC_{50} is approximately equal to K_A, meaning partial agonism or, for very small τ, competitive antagonism.

For non-rectangular hyperbolic dose-response curves (i.e. with different Hill slopes), if receptor occupancy is still assumed to be non-cooperative, an alternative transducer function to equation Equation 16 is required.

$$Effect\ (\Delta) = \frac{\Delta_{MaxSystem}[LR]^n}{K_A{}^n + [LR]^n}$$ Equation 19

$$\Delta_{Max} = \Delta_{Maxsystem}\left(\frac{\tau^n}{\tau^n+1}\right)$$ Equation 20

To determine the affinity of a partial agonist, use the operational model to globally fit the dose-response curves of both a full ago-

nist and the partial agonist. The data from the full-agonist determines the maximum possible effect. Knowing that, the fitting can determine the affinity of the partial agonist. Non-linear regression can be used to fit the following equations:

Model:

operate (OP)= $(((10^{\wedge}logK_A)+(10^{\wedge}X))/(10^{\wedge}(log\tau+X)))^{\wedge}n$

Full Agonist: Δ= Basal + $(\Delta_{maxsystem}$-Basal$)/(1+10^{\wedge}((LogEC_{50}$--X)*n))

Unknown Agonist Δ = Basal + $(\Delta_{max}$-Basal$)/(1+OP)$

Receptor Antagonism

Quantitation of Pharmacologic/ Toxicologic Antagonism

The use of antagonists to block receptor responses represents a classical approach in pharmacology and a major mechanism by which chemicals cause toxicity. There are 5 basic types of antagonism: 1). **Functional antagonism**. This type of inhibition often results from stimulation of two pathways with opposite function (i.e. sympathetic versus parasympathetic nervous systems). Functional antagonism results from action via two distinct receptors and cannot be examined by the methods described herein; 2). **Competitive antagonism**. A competitive antagonist is binding to the receptor in roughly the same physical space as the agonist, precluding the latter from interacting with the receptor to elicit a biological response. A competitive antagonist is reversible, with a rate of dissociation that is relevant to the time-frame of the experiments; 3). **Irreversible antago-**

nism. As with the competitive antagonist, the irreversible antagonist binds to the same region of the receptor as the agonist. However, in this instance, the rate of dissociation is extremely slow or practically non-existent. 4). **Noncompetitive antagonism**. Receptors are complicated macromolecules that may interact with small molecules in a variety of ways. A noncompetitive antagonist is defined as a chemical that binds to the receptor at a site other than the agonist-binding pocket, yet binding precludes the binding of the agonist. This is often caused by the antagonist producing a conformational change in the binding pocket, and; 5). **Mixed Antagonism**. A combination of any of the type of antagonism listed above.

Competitive Antagonism

Competitive antagonists are ligands that compete with agonists, usually for a common binding site in a receptor. The interaction of agonist (L), competitive antagonist (I) with receptor (R), is described using the following scheme.

$$L + \begin{array}{c} I \\ + \\ R \\ {\scriptstyle Ki} \uparrow\downarrow \\ IR \end{array} \xrightarrow{\;Kd\;} LR \xrightarrow{\;Ke\;} Effect$$
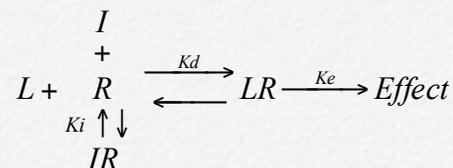
Note that in this instance, the inhibitor-receptor complex is ineffectual, and cannot couple occupancy with a biological response. The formation of the ligand-receptor and the inhibitor-receptor complex may be described by the Clark equations and have equilibrium dissociation constants of K_d and K_i respectively. As a result of IR formation, there are fewer receptors available for LR formation.

$$\frac{\Delta}{\Delta_{max}} = \frac{LR}{LR_{max}} = \frac{[LR]}{[R]+[LR]+[IR]} = \frac{\dfrac{[L]}{K_d}}{1+\dfrac{[L]}{K_d}+\dfrac{[I]}{K_I}}$$

Equation 21

This can be rearranged (divide by [L]/K$_d$):

$$\frac{\Delta}{\Delta_{max}} = \frac{1}{1+\dfrac{K_d}{L}(1+\dfrac{[I]}{K_i})}$$

Equation 22

As you can see, the extent of antagonism depends on the agonist and antagonist concentration, as well as their dissociation constants, K_d and Ki. In the presence of competitive inhibitor I, fractional occupation [LR]/[LR]$_{max}$ decreases as a consequence of an increase in the apparent equilibrium dissociation constant of the agonist L. This decrease is from a value of K_d in the absence of inhibitor to a value of K_D (1+[I]/Ki) in the presence of I.

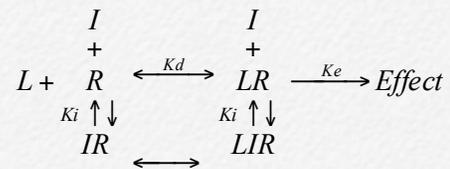**FIGURE 2.3** Agonist response in presence of a competitive inhibitor
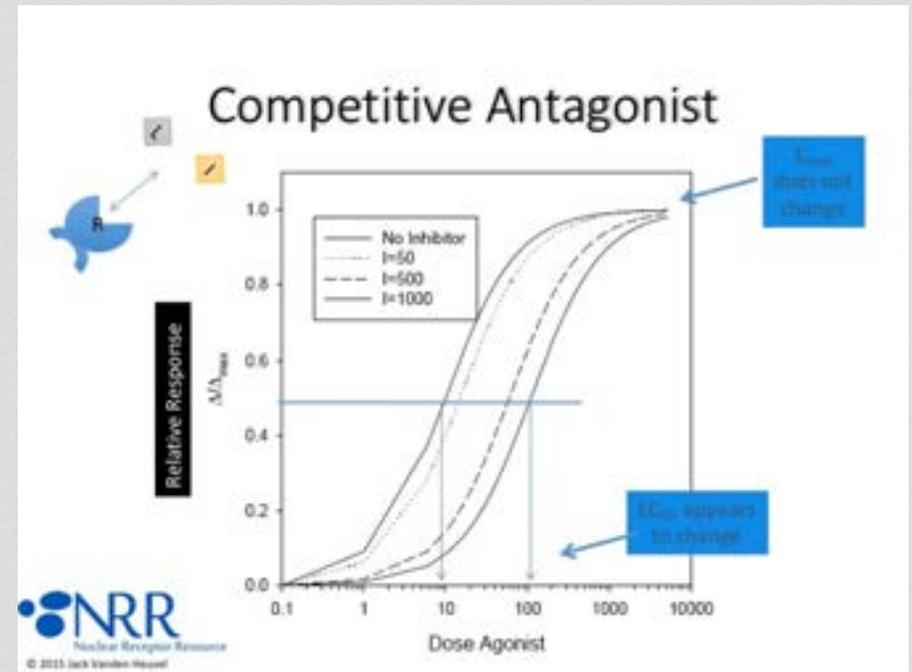
Dose response curve of an agonist with increasing concentrations of a competitive antagonist (0, 50, 500 or 1000)

The increase in apparent dissociation constant yields an equation with the same shape dose-response curve, only shifted to the right with no effect on maximal response (see).
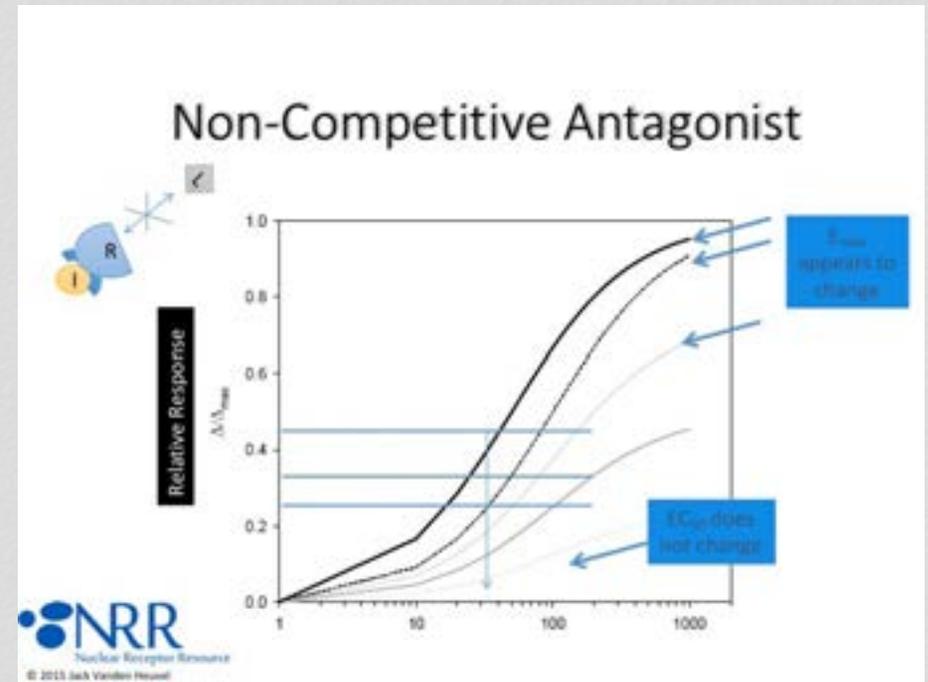
Noncompetitive Antagonism

A noncompetitive antagonist interacts with the receptor, but this interaction takes place at a site different than that of the agonist. Upon binding to the noncompetitive antagonist, the affinity of the receptor for the agonist is altered, possible the result of a conformational change in the protein structure. The interaction of agonist (L), noncompetitive antagonist (I) with receptor (R), is described using the following scheme.

$$
\begin{array}{ccccc}
I & & I & & \\
+ & & + & & \\
L + \quad R & \xrightarrow{\ Kd\ } & LR & \xrightarrow{\ Ke\ } & Effect \\
\scriptstyle Ki\ \uparrow\downarrow & & \scriptstyle Ki\ \uparrow\downarrow & & \\
IR & \longleftrightarrow & LIR & &
\end{array}
$$

An antagonist of this sort produces quantitative changes in the agonist dose-response curves: The maximal response is decreased while the EC_{50} of the agonist does not change (Figure 2.4). The antagonist does not alter the concentration of bound agonist (LR+LRI) but the LRI complex is nonfunctional. The response of the agonist may be derived as follows:

FIGURE 2.4 Agonist response in the presence of a non-competitive inhibitor

Dose response curve of an agonist with increasing concentrations of a non-competitive antagonist (0, 50, 500 or 1000)

$$\frac{\Delta}{\Delta_{max}} = \frac{k_e[LR]}{[R]_{Total}} = \frac{k_e[LR]}{[R]+[LR]+[IR]+[ILR]}$$

Which can be rearranged to :

$$\frac{\Delta}{\Delta_{max}} = \frac{k_e[L]}{([L]+K_d)(1+\frac{[I]}{K_I})}$$

Equation 23

The response is decreased by a factor whose magnitude is increased by [I] and low values of K_i (i.e. high affinity). For non-competitive antagonists $K_i = IC_{50}$, the concentration of antagonist that inhibits the response by 50%.

The dose-response relationship of the agonist at one concentration in the presence of increasing doses of i. nhibitor were discussed in Chapter 1 <u>Interactive 1.1</u>

Comparing dose-response curves

Comparison of Dose-Response Curves using Sigmoid E_{max} and Operational Models

Let's take the hypothetical example of the testing of three unknown drugs (labeled Drug A, B and C) against a reference compound (see Figure 2.5). From this graph, there are some generalities that can be made. First, Drugs A-C are all partial agonists (they do not achieve the same Δ_{max} as the reference compound. The rank order of efficacy is Reference>Drug C>Drug A>Drug B. The potency, in terms of EC_{50}, of these compounds appears to be Drug B>Drug A>Reference>Drug C. To quantify these differences, we will use the Sigmoidal E_{max} model as well as the Operational Model. In both cases we will use a popular graphing and analysis package (GraphPad Prism).

FIGURE 2.5 Example
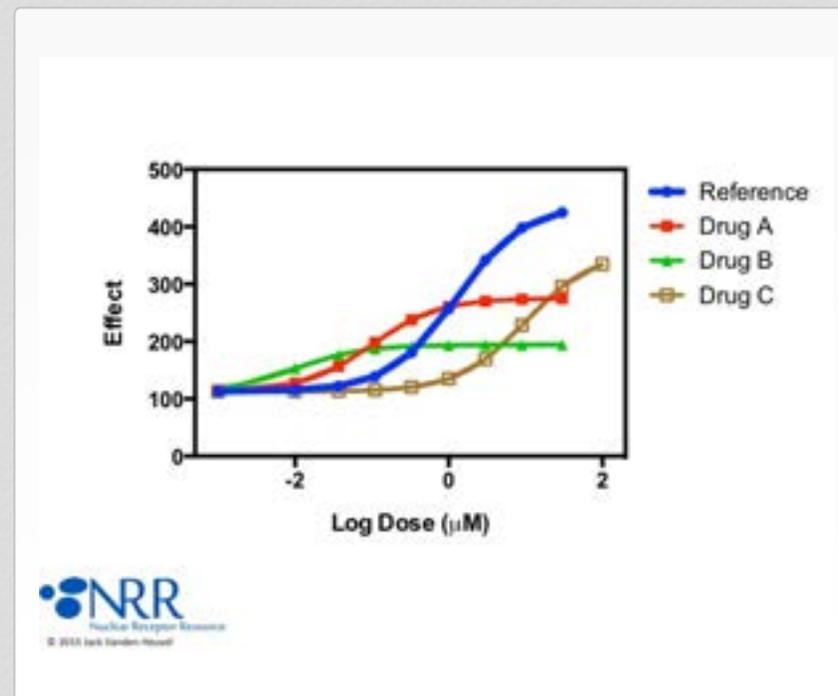
In the Sigmoidal E_{max} model, the interpretation is fairly straightforward. The relative potency of shows Drug A is 13 times and Drug B is 150 times more potent and Drug C is 10 times less potent than the reference compound. The relative efficacy of the drugs show that Drug A, B and C are 0.5, 0.3 and 0.8 times the Span (fold-change) of the reference compound. The relative efficiency (Span/EC_{50}), in this case follows the potency trends. Note, that the values for EC_{50} and Span are experimental vari-

	Reference	Drug A	Drug B	Drug C
Bottom	113	112	103	113
Top	438	275	194	357
EC_{50}	1.258	0.098	0.008	9.999
Span	325	163	91	244
Relative Potency $EC_{50}Ref/EC_{50}Drug$	1	12.81	153.3	0.13
Relative Efficacy $SpanDrug/SpanRef$	1	0.5	0.28	0.75
Relative Efficiency $(Span/EC_{50})$	1	6	43	0.09

ables and may vary from experiment to experiment, even for the reference compound. Parameters such as receptor number as well as pharmacokinetic variables are affected by the state of the system being studied. However, the relative potency and efficacy should be more stable due to accounting for some of these factors by comparing to a reference compound. It is this reason that it is advisable to run a compound of known effect (a full agonist is preferred) and to report the data relative to this reference.

The operational model defines the Δ_{max} of the reference compound as the $\Delta_{Maxsystem.}$ and uses other aspects of the reference dose-response to define the experimental system. As shown below, the Tau (τ) for the three partial agonists, show Drug C have three times the intrinsic efficacy than Drug A. The affinity (K_A) is similar, but not identical to the EC_{50} values. Both these measures would be considered less variable than those of the E_{max} model from experiment to experiment. Note, that in this case the Tau for the reference compound would be very large, and the K_A would be approximately its EC_{50} value.

	Reference	Drug A	Drug B	Drug C
n	0.9942	0.9942	0.9942	0.9942
Basal	112.2	112.2	112.2	112.2
Δ_{max}	438.3	438.3	438.3	438.3
EC_{50}	1.258	0.098	0.008	9.999
Tau	(not used)	1.003	0.3354	3.012
K_A	(not used)	0.1997	0.01427	39.98

Comparison of Dose-Response Curves of Different Responses: Therapeutic Index and Margin of Safety

The question arises from the previous example, which of these compounds would make the best drug? Of course, the compound's efficacy and potency are important issues when it comes to evaluating the drug's ability to elicit the desired effects. However, many other issues must be taken into account including pharmacokinetics (i.e. absorption, distribution, metabolism and excretion), compound stability, and market-place prominence. One key issue that can be addressed is comparison to a reference compound, which is presumably a drug already on the market or one that has been studied extensively for its on-target (effects on the intended receptor). From the previous example, none of the compounds achieved the same maximal effect as the reference, although Drug C had the highest efficacy (highest τ and span). However, decisions on best drug candidates cannot be made on a single endpoint and multiple endpoints must be considered. In this context, we will discuss using an dose-response relationships for a toxic or unwanted response to evaluate overall drug prioritization.

On-target versus off-target toxicity

In general, drugs can cause adverse or toxic effects by acting on the primary pharmacological target to elicit **on-target toxicity** (often referred to as mechanistic toxicity) or by acting on a known or unknown **off-target** (also referred to as an anti-target) to elicit off-target pharmacology or toxicity. Recognizing the underlying mechanism of toxicity is essential for any kind of safety-related decision that needs to be taken during drug development. On-target toxicity may include both exaggerated pharmacology in the therapeutic target tissue(s) as well as on-target pharmacology (secondary pharmacology) in tissues other than the therapeutic target tissue(s). By contrast, off-target toxicity is a frequent mode of toxicity for small-molecule drugs, which is often due to their pharmacological promiscuity and/or their chemical reactivity with biomolecules (either reactivity of the parent drug or its metabolite(s)).

On-target toxicity of drug candidates against (novel) targets can often be predicted on the basis of genetic and/or pharmacological knowledge of the target and/or its biological signaling pathway. Strategies that can be used to improve the therapeutic window for on-target toxicities may include the following: selection of drug candidates that are partial antagonists or partial agonists of the target, selection of drug candidates that have more selective distribution into the tissue of intended pharmacological action; or optimizing the dosing regimen. The off-target pharma-

cology or toxicity profile of a compound can be optimized by modifying the chemical structure while retaining its on-target activity. In vitro off-target receptor profiling should be considered for both parent and major metabolites as early as the lead optimization stage of small-molecule drugs, with hits in radioligand binding assays being confirmed in functional assays to distinguish agonism from antagonism.

Therapeutic Index

A widely used concept in making such decisions is the therapeutic index (TI) of drug candidates, which is a quantitative relationship between their efficacy (pharmacology) and safety (toxicology) that can be calculated using various pairs of pharmacological and toxicological end points. The classical definition of the TI is the ratio of the dose of the drug that causes adverse effects at an

incidence and/or severity not compatible with the targeted indication .

Therapeutic Index (TI)= TD_{50}/ED_{50}.

The TD_{50} (toxic dose 50) is the dose that leads to an unwanted effect to 50% of the test subjects, or dose that results in 50% of the maximal unintended response; Similarly, the ED_{50} (effective dose) is the dose required to cause the intended pharmacologic response

A high TI is preferable in order for a drug to be viewed as having a favorable safety profile, whereas lower TIs may be acceptable for the treatment of life-threatening diseases that have limited treatment options. An alternative, more conservative measure of a therapeutic window is the Margin of Safety (MS). Since the MS is examining the TD_1 (dose that leads to 1% toxicity) relative to ED_{99} (dose that leads to 99% therapeutic effect), the

FIGURE 2.6 Therapeutic Index
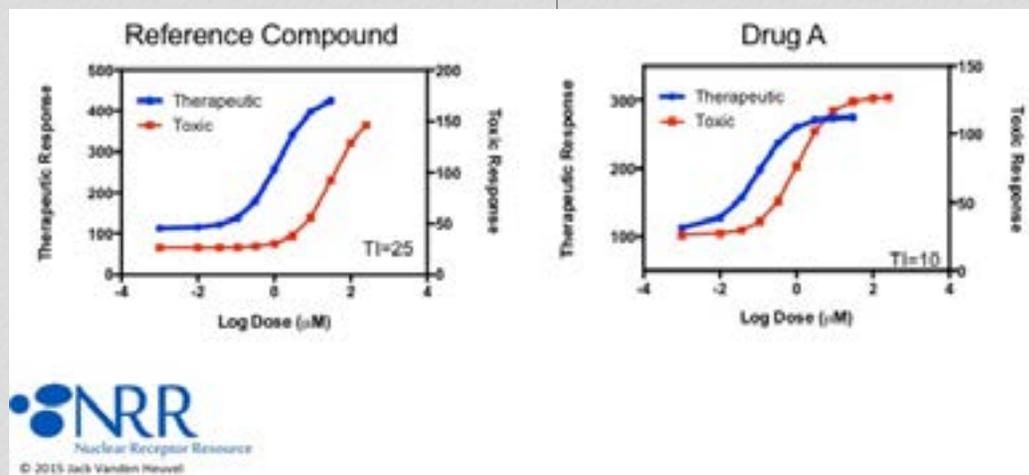
Example of determining a therapeutic index for two compounds (reference, left; Drug A, right). Reference compound: TD_{50}, 31.6 µM, ED_{50}, 1.3 µM; TI=25. Drug A: TD_{50}, 0.99 µM, ED_{50}, 0.1 µM; TI=10.

slope of the curves is less important.

Margin of Safety (MS)= TD_{50}/ED_{50}.

In the comparison shown in <u>Interactive 2.1</u>, although Drug A is more potent than the Reference Compound (EC_{50} 0.1 versus 1.3 µM, respectively), the TI for Drug A is less than than of the Reference. This means that the therapeutic window of Drug A is smaller than that of the Reference and from a safety standpoint would be less attractive. This approach of comparing dose-response relationships for multiple endpoints is being utilized more extensively in the evaluation of compounds and prioritizaiton of compounds for future research. That is, in addition to examining potency and efficacy for a series of compounds, the TIs, perhaps for multiple toxic responses, can be used to rank compounds.

References

3

Included are some of the key references and resources for the previous Chapters

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