PCTH404 - DOSE RESPONSE CURVE ANALYSIS

Simplest model is where:

\[ A + R = AR \]

\[ I + R = IR \]

where A is agonist and R the receptor while I + R = IR where I is a competitive antagonist. This really is a very simplistic model because the assumption is that dose-responses (dose-effects) are directly proportional to the drug-receptor occupancy curve and that the actual response is directly proportional to AR. Such assumptions may not be valid because response is a complex, non-linear function of R occupancy, dependent on signal transduction factors as second messengers and response mechanisms. The initial binding of drug to receptor is linked to response, often by being transduced, via second messengers, plus a pathway of events leading to a final response. Furthermore, the response itself can be modulated by other regulatory mechanisms that are independent of the drug response mechanisms, especially when in vivo responses are measured. In vivo the agonist concentration at the receptor is likely not always directly proportional to the dose administered to the animal. There are a wide variety of these and other reasons that means we cannot use estimates made from D/R curves as a true measure of drug affinity for receptors.

Complexities:

There is an increasing recognition that the classic view of drugs (e.g. agonists and competitive antagonists) binding to a very specific binding site (R) at a particular receptor bind is an incomplete view. Non-competitive and uncompetitive inhibition of agonist responses can be explained by a receptor having other binding sites for different classes drugs such that allosteric effects can be mediated by drugs binding to other than the classic receptor recognition site. Another discovery that weakened the classic view was inverse agonism. What was an initially surprising idea was that receptors, independent of agonists, can be in at least two states: activated and inactivated, hence the apparently paradoxical inverse agonism. The mathematics of dose response analysis is basically that of mass action which is good enough at the macro level since many of the complexities of what is actually going on at the molecular/cellular level collapse down at the mathematical level to some simple pharmacologically relevant equations. All stages from binding to receptor, change in receptor state, a chain of transduction and varying response mechanisms can be described by simple hyperbolic and/or logistic equations. These tend to collapse into one over-reaching equation although much more extensive models of the basic model are possible. The basic model is:

\[ A+R=AR \] for agonists and \[ I+R=IR \] for competitive antagonist.

This level of simplicity works surprisingly well even for a more complex model such as that seen with acetylcholine binding to nicotinic receptors where two agonist molecules have to bind, e.g. \[ A + R = AR + A = A2R. \]

With regard to the problem of transduction this is partly solved by the concept of efficacy (e) where the basic assumption is that response is proportional to AR such that Response = e [AR], where e varies for drugs and tissue. This helps to explain partial agonists, but it is still an incomplete explanation. The other factor that confounds Dose/Response curves analyses is that there are spare receptors since in many cases far more receptors are present than need to be occupied in order to produce a maximum response.

The special case of antagonists

One way of avoiding the complexities of transduction is to make comparisons at equal responses. This works well for antagonists with the Schild plot of \[ \log(Dose\ Ratio -1) = \log I + \log K_i \] where \( K_i \) can be remarkable close to the affinity constant found by binding studies, but it only works well when comparing full agonists.
The final conundrum:
The advent of thermodynamic binding curves of agonists and antagonist to isolated or ‘pure’
receptors provided more direct evidence of the validity of the A+R model which helps justify its use. In
the case of some receptors, and their drugs, Dose/Response curves relate reasonably to binding study
data, but in other cases this is not so. It works particularly well for some neurotransmitter receptors, but
not for others. Such binding studies provide good estimates of total number of receptors, but are not so
useful in all situations. Response data derived for antagonists can correspond very well to binding data
as discussed above.

In some senses, regardless of the clarity of mathematically modeling of dose response curves,
there remains a host of unknowns, even for the well characterized neurotransmitter receptors. While the
isolation of the pure receptors helps, it is not the complete answer since receptors do not exist in
isolation. They exist in particular environments that are dependent upon numerous factors.

Regardless of all of the above, the competent pharmacologist’s toolkit should include methods
for dealing with Dose/Response data, both in vitro and in vivo. Such data is critical for: comparing
drugs; comparing doses required to produce different responses (as in calculation of therapeutic ratios
for wanted versus unwanted effects), and for a better understanding of mechanisms of drug action.
These considerations apply to full agonists, partial agonist, inverse agonists, antagonists (competitive,
non competitive). All require a need for systematically understanding and analyzing Dose/Response
curves

How to analyze D/R curves:
The usual sigmoid-shaped D/R curve is analogous to other logistics curves that occur in nature
where low level stimuli produce limited responses that are not linearly proportional to the stimulus until
it reaches a stimulus level where the response is quasi-linearly proportional to the stimulus (or log
stimulus). This is followed by a flattening curve and an eventual plateau where any further stimulus
causes no further increase. The “linear” proportion occupies about 20-80% of the curve.

Three (four) parameters describe D/R curves:
1) **Locator**: potency (e.g. EC50) that relates to a drug’s affinity for the receptor binding site
2) **Slope**: can imply an underlying mechanism, its other value is in establishing parallelism between
dose response curves for different drug, responses or beneficial versus adverse effects
3) **Maximum**: is often not obtainable; it is function of agonist efficacy and the number of spare
receptors. It is dependent upon the ability of a drug, once bound, to elicit a maximal response
providing the response system is not maximized.
4) **Minimum response** which is the pre-drug value but is usefully set this to 0 when responses in
presence of different drug doses are normalized by subtracting pre-drug values from drug values.
In an idealized model the relationship between drug concentration (dose) and response is described by a hyperbolic curve. The hyperbolic curve has a linear x axis. It is usually used for simple systems, such as an enzyme or isolated receptor. A sigmoidal curve of response versus log dose is best used in complex systems, e.g., cellular, multicellular, or in vivo systems. For many drug studies the slope is 1.0 but higher values are characteristically found in lethality studies or other quantal dose-response curves.

Some derived equations include:

\[
\frac{E}{E_{\text{max}}} = \frac{A}{(A + EA_{50})} \text{ where } E = \text{effect; } E_{\text{max}}=\text{max. effect; } A = \text{conc., } EA_{50} = \text{conc. producing 50% of the maximal effect, with a slope of 1.0. If one divides the right hand side by } EC_{50} \text{ then } \frac{E}{E_{\text{max}}} = A/E_{50}/(1+A/E_{50})\text{ at equilibrium. This is a simple logistic function of the form } y = \frac{x^n}{1+x^n} \text{ where } x = A/K_A \text{ and } y \text{ is the proportion (0 to 1.0) of the maximum response. The value of } n \text{ is 1 for the model } A + R = AR \text{ in the simple mass action analogy. The curve therefore relies on a given affinity for the binding or association between two molecules. If } n=2 \text{ the slope of DR curve is steeper, i.e. } 2\times1.0=2.
\]

Drug-receptor binding can be described by the analogous \(B/B_{\text{max}} = C/(C + K_P)\) where \(B = \text{drug bound to receptors, } C = \text{unbound drug, } B_{\text{max}} = \text{total conc. of receptors, } K_P = \text{equilibrium dissociation constant (represents the concentration of free drug at which half-maximal binding is observed thus it characterizes the affinity for binding the drug with a low } K_P = \text{a high binding affinity). Note that } EC_{50} = K_D \text{ for a full agonist when there is no receptor reserve, but the } K_I \text{ for an antagonist regardless of receptor reserve.}

The use of the log transform for the concentration axis makes a hyperbolic curve assume a sigmoid shape. This form of graph gives a useful scale for the conc. axis (x) at intermediate concentrations where response is changing relatively rapidly with dose and compresses it at high and low concentrations where response is changing slowly with dose. Also, it allows for statistical tests that assume a normal distribution of response with dose*. Otherwise, there is no special biological or pharmacological significance to using the log axis form of plot.

* Note: both dose and response are in fact log-normally distributed variables but few workers actually log transform the response data because: a) it is not convenient, and b) many do not accept that it may be preferable. Many statistical tests are applied to doses (X) required to produce a defined response, but perhaps they should be reported as log X in an analogous manner to where concentration of hydrogen ions is expressed as pH (a log scale), or sound in decibels. Such power relationships are common in nature.

It is inappropriate to mean responses for D/R analyses when one has a defined maximum for the drug, but if this is not the case, responses can be averaged. The values that should theoretically be used are the log of response; this mathematical transform generally gives response data that are more normally (Gaussian) distributed. As noted above, this point is not normally considered important by many researchers. Again please note again that both responses and doses are log normally distributed.

Ideally, when comparing dose response curves in which full D/R curves have been estimated in equal number of samples (e.g. pieces of guinea pig ileum) the best procedure is to fit individual dose response curve for each sample and then obtain best estimates for EC50, slope and maximum from each of these curves. The values so obtained for each sample can then be averaged so as to give mean values for the different treatment groups and get better group estimates of potency, slope and maximum.

The software swamp

Software is readily available for “fitting” dose response data via a variety of algorithms. However, such software should be used with discretion, care and with some understanding of the principles underlying the different “fitting” processes. It is too easy to use software in an unthinking manner, and thereby make elementary mistakes.
Relationship between occupancy of receptors and response:

\[
\text{Response} = f \left( \frac{\varepsilon N_{\text{TOT}} \cdot x_A}{x_A + K_A} \right)
\]

Where \( f \) = “transducer function”, describes the characteristics of the responding system; \( \varepsilon \) = “intrinsic efficacy”, \( N \) = total number of receptors, \( x_A \) = concentration of agonist, \( K_A \) = binding coefficient. This does not include consideration of the Hill coefficient where more than 1 molecule of agonist is required to ‘activate’ a receptor.

\( \varepsilon \) and \( K_A \) are characteristics of the drug-receptor interaction whereas \( f \) and \( N_{\text{TOT}} \) are characteristics of the tissue.

This can explain findings such as: 1) with the same agonist and the same receptor a full agonist in one tissue can be a partial agonist in another; or 2) the relative potencies between two agonists may differ in different tissue types.

Approach to analyzing Dose Response obtained from multiple tissue samples

1) Plot full curve for each tissue sample and try to obtain a maximum.
2) Interpolate or extrapolate locator (EC\(_{50}\)) and maximum
3) Determine slope (i.e. Hill coefficient) from Hill plot although all software determines a Hill coefficient a paper and pencil Hill plot allows a more critical view of data and produces a straight line. It requires a maximum (Rmax). The slope, \( h \), is the Hill coefficient and can relate to the number of binding sites in some theoretical models.

A simple linear analysis technique for D/R curves:

A linear plot that is easy to use, and should give a straight line which everyone can easily recognize in these days of mass computing (see Figure below).

\[
\log \frac{R}{(R_{\text{max}} - R)} = y/x = \text{slope}
\]

Log \([A]\)

What is important about slope?

Slope is useful for comparing drug potencies. It gives us an idea of the “purity of the measured response” (i.e. are we measuring the true response, or are some other factors/responses mixed in together). If slope is too shallow, it suggests a greater chance of overlap between desired effects and undesired effects. If slope is too steep and the maximum response is not therapeutic (toxic) then it may be very hard to achieve a dose for a particular wanted response, and not also have an unwanted response.

A short-cut techniques for estimating slope, when scanning published or presented data

Consider the dose response curve below and the following equation:

\[\Delta \text{ fractional response (FR)} = 0.575 \ h \text{ where } h = \text{slope (Hill coefficient)}\]

Where the \( \Delta \text{ fractional response (FR)} \) is defined on the ‘linear’ portion of the dose response curve as the change in response resulting from a one decade (10x) change in concentration.
In the figure below $\Delta FR = 0.775 - 0.20 = 0.575$, therefore $h = 1.0$

This leads to a “rule of thumb” such that \textbf{when $h = 1.0$}, a 20\% to 75\% change in response (FR 0.20 to 0.75) should occur over a 10x change in concentration of agonist (on a log scale = 1.0 units). \textbf{When $h = 2.0$}; an approximately 20\% to 75\% change in response should occur over a 3-fold (3x) change in concentration of agonist (on a log scale = 0.5 units). This rule of thumb, makes it easy to assess D/R curves by eye almost.

![Graph showing fractional response vs log concentration](image)

\textbf{Cautions when analyzing slope:}

All steps between binding and response (there may be many physiological responses between drug and measured response) have their characteristic functions, many of which will be sigmoid curves. The sum of a series of sigmoid curves can be a sigmoid curve. A series of sigmoid curves of high slope will be smeared into one curve with a lower slope. It is for this reason that one should analyze data as individual dose response curves and then take the means of the parameters measured for each of these curves. This will give different value than if one simply puts all data into one global analysis of a multipoint dose response curve,

\textbf{Complexities in DR Analysis.}

1) \textbf{The no maximum problem} is where no maximal response data are available and one has to average responses; normally you would not do this but would average parameters for all of the curves obtained from individual samples.

2) \textbf{The receptor reserve problem}. The obvious answer is to block receptors with irreversible competitive blockers, applied for sufficient time for the maximum response to a full agonist to just begin to be reduced. This occurs when all of the receptor reserve has all been ‘used up’. Classical examples are alpha-bungarotoxin for the nicotinic skeletal muscle receptor, or phenoxybenzamine for alpha adrenoceptors.

3) \textbf{The pseudo equilibrium problem} occurs when there is an uptake or metabolism system present for the agonist being tested, and whose presence results in a false equilibrium concentration. For example, the actual concentration of acetylcholine at a nicotinic receptor site will be much lower than the bath concentration when there is acetylcholinesterase present in the preparation since the enzyme acts to “protect” the receptors from ACh. Another example is uptake systems for monoamines such as norepinephrine, or 5HT

4) \textbf{The non-linearities or saturation of transduction (second messenger and response) mechanisms}
More on Receptor Reserve:

As an example, myocardial cells contain large numbers of beta-receptors, up to 90% occupancy by antagonist can still allow a maximum cardiac response. How can we explain this biologically?

Spare Receptors in Time

The effect of receptor activation may greatly outlast the agonist-receptor interaction such that there are “free receptors” available for binding even after the response has reached maximum. Spareness for receptors can thus be temporal (e.g. second messengers).

Spare Receptors in Number

If the concentration of a cellular component limits the 1:1 coupling of drug binding to response then maximum response occurs at limits of that component, not the number of receptors. Thus, sensitivity of tissue or cells to a particular concentration of agonist depends not only on the affinity of the receptor for agonist (K_D) but also on the “degree of spareness”, which is the total number of receptors needed for maximum response. K_D determines what fraction of total receptors will be occupied at a given concentration of agonist: B/Bmax = C / (C + K_D)

E.g. A cell with 4 receptors and 4 effectors has no spare receptors; if agonist concentration C = K_D then you get a 50% response (occupies 50% of receptors, activates 50% of effectors) or the half maximum. However, if the number of receptors = 40, but we still have only 4 effectors, a much smaller conc. of agonist is enough to occupy only 2 receptors to give a half-maximal response because there are only 4 effectors. Thus tissue sensitivity is changed by changing receptor concentration such that there are spare receptors.

QUANTAL DR CURVES

Responses in individuals or tissue samples are most often graded with stimulus. Responses in populations may be quantal in that the drug response in a given individual is either present, or absent, e.g. death. In the quantal D/R (% response), slope represents the response variability in a population, rather than a concentration range of effects in the individual. The classic quantal dose response curve is that for estimation of a quantal ED50. Much of the old toxicity data used LD50 values estimated from the quantal lethality curve. The slope coefficient for such curves is much higher than 1.0. If the response is receptor dependent, but the response is dependent upon a restricted system, then the slope is steep. For example death often occur when a critical “tipping point” is reached (e.g. >95% of skeletal muscle nicotinic receptors are blocked) and this makes for a lethality D/R curve which is steep, especially in acute experiments.

A common method that was used for analyzing quantal D/R was a linearizing technique known as probit analysis. Probit (“probability units”) designates the deviation of a value from the median (median effective dose = ED50, median lethal dose = LD50). A probit value of 5 is the value given for a 50% (mean) response and one probit is 1 standard deviation which is added (> mean) or subtracted (< mean) from that mean. For example a response that is 1.0 standard deviation below the mean has the probit value of 4, while one 2 SDs below would be 3 etc. and vice versa, 6 and 7 for responses greater 1 and 2 SDs above the mean. From knowledge of the Gaussian curve a nomogram gives percentage responses in probit units and the probits are plotted against log dose. Obviously this can be accomplished with suitable software.

Uses of Dose Response curves

D/R curves have an intrinsic intellectual value and place pharmacology on a mathematical footing while on a practical level they are essential for making estimates of:

1) Potency
2) Efficacy (a word wrongly used by non-pharmacologists to indicate effectiveness)
3) Slopes for comparing potencies since D/R curves with different slopes do not allow for easy comparison of potencies, i.e. the potency ratio varies with the level of response

4) Therapeutic ratios of ED$_{50}$, for good or wanted effects, versus ED$_{50}$ for bad or unwanted effects, but only if the slopes of the respective DR curves have parallel slopes. If slopes are not parallel a useful measure is ED$_{95}$ for good effects versus ED$_{5}$ for bad effects, or whatever best clarifies the separation of wanted from unwanted effects.

5) Can be used as tool for investigating mechanisms of drug action and Structure Activity Relationships.

ANALYSES TECHNIQUES FOR INTERACTIONS BETWEEN TWO OR MORE DRUGS

**Additivity, synergism and antagonisms between drugs**

Interactions between two or more different drugs is a source of difficulty in terms of analyses.

An interaction can be ADDITIVE where addition of a second drug is equivalent to addition of the first drug:

An interaction can be SYNERGISTIC where the addition of a second drug potentiates the actions of the first drug, or of each other, such that the response to the combination of the two drugs is greater than additive.

An interaction can be ANTAGONISTIC where the addition of a second drug appears to prevent the first drug having an action.

When analyzing the action of two drugs under equilibrium conditions various analytical approaches can be used. The easiest approach is to consider two drugs given in each other’s presence. If various combinations of drugs are tested the resulting data can be expressed graphically as an isobologram (see later).

**ADDITIVITY**

This is relatively easy to appreciate since addition of responses occur when a second drug (B) is added to the first drug (A) since the addition of B is equivalent to addition of more of A, allowing for differences in potency. In terms of isobolograms, a straight line connecting doses of A and B in any combination producing a particular level of response, e.g. 50% of maximum, is seen with additive drugs.

**SYNERGISM**

This is when the addition of a second drug to a first drug produces a greater than additive response. The classic graphical representation of additivity, synergy and antagonism is easy to see in an ISOBOLICRAM where the doses of two drugs in combination required to produce a particular response is plotted as doses of one drug on the ‘x’ axis against the other drug on the ‘y’ axis. If there is no synergy the dose producing a defined response (say 50% for the ED50) to X, when Y is absent, will form a straight line to the ED50 for Y in the absence of X. All points along this straight line represent combinations of X and Y that will produce a 50% response, or any other level of response.

Isobolograms are extensively discussed by Chou TC *Pharmacol Rev* 58:621–681, 2006 in a review of analyses of drug interactions. This review provides a link for a free down-load of software.
The left hand diagram shows an isobologram for two drugs X and Y. The dose of X required to produce a 50% maximum response (ED\textsubscript{50}) is 16nM and for Y it is 8nM. A line (the ED\textsubscript{50} isobole) can then be drawn connecting the ED\textsubscript{50} values for each drug in the absence of the other drug. From this line any combination of doses of X and Y producing a 50% response can be interpolated. An example is shown (in dotted lines and arrowheads) from which one can see that solution of the equation \( \frac{x}{ED_{50}^X} + \frac{y}{ED_{50}^Y} = 1.0 \), e.g. \( \frac{6}{8} + \frac{4}{16} = \frac{12}{16} + \frac{4}{16} = 1 \) so any combinations that are additive the 50% isobole gives an answer of 1. Solving the equation for 33% isobole the solution is \( \frac{2}{4} + \frac{4}{8} = 1 \). Thus for additivity between two drugs at different levels of percentage maximum responses, one can have a series of parallel isoboles. If one tests combinations of X and Y and they lie on the straight line isobole (dotted line in right hand diagram) the combination is additive. However if the result lies below the dotted line the combination is synergistic while above the line, antagonistic. Below (to the left) of the line the combinations of X and Y required to produce a 50% response is less than that required for additivity (dotted line). Conversely with combinations required to produce a 50% response that are displaced above (to the right) are antagonistic to each other.

**ANTAGONISM**

Dose response curves for antagonists can be complex since the nature and mechanisms of antagonism vary. CHEMICAL, PHYSIOLOGICAL and PHARMACOLOGICAL antagonism are all possible.

**Chemical antagonism** involves a chemical reaction of a second drug with a first drug such as to inactivate the first drug (and vice versa). While this is not a common occurrence it is possible.

**Physiological antagonism** involves a second drug having an opposing physiological action to the first drug, and that this antagonistic action is mediated through an entirely different receptor system so nullifying the pharmacological actions of the first drug.

**PHARMACOLOGICAL antagonism** is where the action of a first drug is antagonized by a second drug at the receptor common to both. This sort of antagonism implies an action at the agonist receptor although not necessarily at identical binding sites. It can be classified as being COMPETITIVE, UNCOMPETITIVE and NON COMPETITIVE on various bases of observation and kinetic modelling. What results from such forms of antagonism assumes that the antagonist is reversible in its action.
Some antagonists bind **IRREVERSIBLY** to the receptor and thus, in a time dependent manner, reduce the total number of available receptors.

The above simplified picture of antagonisms does not really take into account the whole spectrum of possible antagonist (undefined) actions since it basically considers antagonism as occurring at the receptor level. The situation becomes complex if we consider pharmacological antagonism at the level of the second messenger, and/or the effector systems, for the agonist action.

Competitive Antagonism is relatively easy to model if certain assumptions are made. The simplest model, often substantiated in many pharmacological experiments, is based upon $A + R = AR$ where $A$ is agonist, and $I + R = IR$, where $I$ is the antagonist. It assumes that $A$ and $I$ compete for access to $R$. By implication it may mean they do so by binding at the same site. However, such a view is probably naïve. Many competitive antagonists are larger and more lipophilic than agonists. This can imply interaction with ancillary ‘sites’, at or near the receptor, that are not involved with agonist binding.

The most basic observation compatible with competitive antagonism is the parallel shift of an agonist dose-response curve to the right with increasing doses of a competitive antagonist. Providing the shift is parallel and the same maximum response maintained, a Schild plot can be constructed and the $K_i$ for the antagonist calculated. If the dose ratio (DR) equals the ratio of the ED$_{50}$ with drug to the control ED$_{50}$ in the absence of drug then $\log (DR-1) = \log[I] - \log K_i$ which is an equation for a straight line. Where $DR = 2$, $\log (DR-1) = 0$ and therefore $\log[I] = \log K_i$. This relationship works surprisingly well, especially for larger DR values. However Log versus Log plots are notoriously forgiving and the above equation often deviates from a straight line at low DR values. Analogous models exist for other forms of antagonism where the basic assumption is that the antagonism occurs “at” the receptor site for the agonist.

While with competitive antagonists increasing doses shift the dose response curve to the right, i.e. increasing doses of agonists are required to overcome the effect of increasing doses of antagonists in a parallel manner, there are other forms of antagonism where the shift of the dose response curve in the presence of antagonists are not so clear. For irreversible competitive antagonists in the presence of spare receptors (receptor reserve) the agonist curve is shifted to the right with exposure-time to the antagonist to an extent that depends on receptor reserve (up to 1.0 log units for a 90% reserve) before the maximum is reduced.