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The application of drug dose equivalence in the quantitative analysis of receptor occupation and drug combinations

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Abstract

In this review we show that the concept of dose equivalence for two drugs, the theoretical basis of the isobologram, has a wider use in the analysis of pharmacological data derived from single and combination drug use. In both its application to drug combination analysis with isoboles and certain other actions, listed below, the determination of doses, or receptor occupancies, that yield equal effects provide useful metrics that can be used to obtain quantitative information on drug actions without postulating any intimate mechanism of action. These other drug actions discussed here include (1) combinations of agonists that produce opposite effects, (2) analysis of inverted U-shaped dose effect curves of single agents, (3) analysis on the effect scale as an alternative to isoboles and (4) the use of occupation isoboles to examine competitive antagonism in the dual receptor case. New formulas derived to assess the statistical variance for additive combinations are included, and the more detailed mathematical topics are included in the appendix.

Keywords

isobole; additivity; synergism; sub-additivity; inverted U-shaped dose-effect curves; receptor occupancy; competitive antagonism; combinations with opposite effects

1. Introduction

Dose-effect relations of drugs provide important quantitative information and are useful guides for clinical use and preclinical studies of mechanism. When drugs produce overtly similar agonistic effects, e.g., treating an infection, lowering blood pressure, relieving pain, etc., it can also be common to use the drugs in combination. In situations in which combinations are employed we often find that the combined drug effect is quantitatively predictable based on the individual drugs' potencies and efficacy. We call that kind of interaction "additive" (for reasons discussed subsequently). In other situations, however, the combination effect may exceed the predicted value or, alternatively, may be a value that is

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less than the expected magnitude. The former is described as synergism whereas the latter is sub-additivity or antagonism. When we refer to the predicted effect in this discussion we mean additivity, i.e., the effect magnitude that follows from the individual dose-effect relations (and which is not a simple addition of effect magnitudes as we will show). Besides possible mechanistic information an interaction between agonists has clinical relevance because additive effects (and, better yet, synergistic effects) can allow the use of limited doses and possible avoidance or reduction in side effects while still achieving the required effectiveness. There are certain situations in which the mechanism of the interaction is known, or reasonably close to known. For example, intrathecal administration of the alpha-2 adrenergic agonist clonidine produces dose-dependent antinociception in the tail-flick test in mice as shown by Fairbanks & Wilcox (1999), and it is well known that morphine is antinociceptive in this test. Further, it has been shown that morphine given supraspinally (i.c.v.) results in the release of norepinephrine at the level of the spinal cord (Kuraishi *et al.*, 1978; Yaksh, 1979). Hence one might expect that clonidine and morphine given together would produce an enhanced analgesic effect because of these mechanisms. But showing that such an enhancement is actually synergistic (Ossipov *et al.*, 1990 a,b; Fairbanks & Wilcox, 1999) required measurements derived from a quantitative comparison of expected and actual effects with isobolograms, a procedure that is rooted in the concept of dose equivalence which is discussed below. Another quantitative example is afforded by a well-known early study of alcohol and chloral hydrate, both known to be hypnotic and studied by Gessner and Cabana (1970). Here also one expects an enhanced effect (hypnosis) when the two drugs are given together; but that demonstration required a quantitative procedure (also with isoboles) to show that the interaction was synergistic. Thus, even when the mechanisms are known, we still need measures to document departures from the expected. The terms “expected effect,” or “expected dose” needed to get a specified effect, depend only partially on the state of knowledge. Something more is needed to classify an interaction as synergistic or antagonistic. That something more is an analysis that leads to metrics to distinguish between additivity and non-additivity.

As mentioned above the quantitative analysis of combinations involving two or more drugs or chemicals is often accomplished with isoboles. These are graphs of dose combinations that produce an effect of specified magnitude. This graphical procedure, introduced and applied by Loewe (1927; 1928; 1953, 1957), has been applied to numerous combinations of agonists that yield overtly similar effects, and is used to determine whether the combination is super-additive (synergistic), sub-additive or simply additive. The usual isobolographic procedure leading to linear isoboles of additivity is applicable only to drugs having log dose-effect curves with parallel slopes (a constant potency ratio) as we show in the next section. This graphical procedure has been examined theoretically (Tallarida, 2000; 2001; 2006; 2007) and extended to apply to cases in which the individual drugs have dissimilar slopes (a variable potency ratio) (Grabovsky & Tallarida, 2004), as well as to a new application that is based on receptor occupation of the constituent compounds (Braverman *et al.*, 2008). The theory that underlies the use of isobolographic analysis is rooted in the concept of dose equivalence. By that we mean doses of each drug that yield the same magnitude of effect when each is used alone. That concept is central to the main topic of the present paper in which we show how dose equivalence leads to applications that include (1) alternatives to

isobolographic methods, (2) analysis of combinations of drugs producing opposite effects, (3) the analysis of inverted U-shaped dose-effect curves, (4) isoboles based on receptor occupation and (5) further insights into the quantitative analysis of competitive antagonism. No mechanisms of drug receptor interaction need be assumed, and the entire presentation is derived from the consequences of dose equivalency. This discussion begins with a brief review of linear and nonlinear isoboles and how these are derived from dose equivalence. We follow with presentations dealing with the further applications mentioned above.

2. Dose Equivalence and the Linear Isobole

Dose equivalence is a key concept in the analysis of drug combinations, and our quantitative discussion begins with a demonstration of how that concept is used to yield isoboles. The analysis of drug combination data most commonly begins with the dose-effect data of the individual constituents, from which their individual potencies and efficacies are determined. Although we use the term “drug” throughout this discussion the term also applies to endogenous or other chemicals of interest. For two drugs with overtly similar action (e.g., analgesics in which both are effective in the same antinociceptive test) the individual dose-effect curves allow an assessment of doses that produce the same effect. Commonly these dose-effect (D - E) curves are fitted to hyperbolic relations of the form that relates dose D to effect E according to

$$E = E_{max} D / (D + C),$$

where E_{max} is the maximum effect and C is a constant that describes the drug's potency. For two drugs, denoted here as drug A and drug B, with respective maximum effects E_A and E_B , these hyperbolic relations are:

$$E = E_A A / (A + C_A) \text{ and } E = E_B B / (B + C_B),$$

for respective doses A and B , where the constants C_A and C_B represent doses for their respective half maximal effects. When the maximum effects are equal, then the dose of one that is equivalent to the other follows as:

$$A = B C_A / C_B = B R,$$

where we have denoted by R the dose ratio which equals C_A / C_B in this hyperbolic model. For any effect level the equivalent doses a and b are related as $b = a/R$. Thus, for an effect that requires B of drug B acting alone (or A of drug A alone) all combinations (a, b) are such that the dose b and the drug B-equivalent of dose a must add to B ; i.e., $b + a/R = B$. Division by B yields: $b/B + a/RB = 1$, or

$$\frac{b}{B} + \frac{a}{A} = 1 \quad [1]$$

The straight line relation of equation [1], with intercepts A and B , is the *isobole of additivity* and represents all dose combinations (a, b) that give the effect magnitude that is attained by

A alone or B alone. The term “additive” is used because of the addition of dose b and its drug-A equivalent. It is notable that no particular mechanism is indicated here. Instead this additive relation follows from the concept that in a drug combination each constituent contributes to the effect according to its potency. In the above we added b to its equivalent of a : $b + b_{eq}(a) = B$. The result is the same if we add dose a and the a -equivalent of dose b , i.e., $a + a_{eq}(b) = A$, which also leads to equation [1].

The concept of dose equivalence led to equation [1] and its straight line graph that was introduced and applied by Loewe (1927, 1928, 1953) to distinguish synergism and sub-additivity from simple additivity. When an experiment yields points $((a,b)$ pairs) that are not on the isobole we interpret this to mean that some interaction exists between the drugs. An experimental point above the isobole indicates sub-additivity, whereas a point below indicates super-additivity (synergism). No particular mechanism is derived from such findings but, as discussed subsequently, the analysis can be an important first step in determining if some mechanism is to be posited i.e., the quantitative assessments of drug combinations can be a critical tool for the empirical studies of mechanism. Further, a non-additive interaction is obviously important in clinical applications concerned with both toxic and beneficial effects.

3. Dose equivalence and the nonlinear isobole

When the individual maximum effects differ the relative potency is necessarily not constant over the effect range. This situation was examined by Grabovsky and Tallarida (2004) using the common hyperbolic model (described above) with different maximum effects, E_A and E_B , respectively for two drugs denoted drug A and drug B. In this case it is easily seen that there is a dose of drug B that is equally effective to dose a , and this is given by $b_{eq}(a)$ in the relation shown below with variables as previously defined:

$$b_{eq}(a) = \frac{C_B}{\frac{E_B}{E_A} \left(1 + \frac{C_A}{a}\right) - 1}$$

Therefore, an effect magnitude elicited by drug B that requires dose B (acting alone) must now be achieved by the sum, $b + b_{eq}(a) = B$. Thus,

$$b + \frac{C_B}{\frac{E_B}{E_A} \left(1 + \frac{C_A}{a}\right) - 1} = B \quad [2]$$

equation [2] is the additive isobole for this case and is seen to be nonlinear. If the specified effect magnitude is not attained by drug A alone, then there is no intercept on the abscissa, while for effects that are common to both drugs there is an intercept on both axes. Whether the additive isobole is linear or nonlinear, it still serves the same purpose in distinguishing synergistic and sub-additive interactions from simple additivity. We have applied this analysis in several combination studies (Parry et al, 2006; Tallarida, 2006), and a discussion of specific examples leading to curved isoboles is given in section 4.2 of this review.

Additional information on the isobolographic method and its many applications may be found in the monograph (Tallarida, 2000) and in recent reviews (Tallarida, 2006; 2007).

4. Dose equivalence: Further applications

4.1 A view on the effect scale as an alternative to isobolographic analysis

While drug combination analysis often utilizes isobolographic methods, it may be desirable (and visually more clear) to examine the combination by deriving the expected (additive) effect of the dose combination (a, b). Intuitively one might conclude that the effect of the combination is the simple sum of the effects that each achieves alone, but that is not correct. For example, if the individual effects are, say, 60% and 70% of $E_{\max}$, the addition of these percentages is without meaning. Thus, we again employ the concept of dose equivalence to illustrate the computation for two drugs having the same maximum and a constant potency ratio (i.e., parallel log dose-effect curves). With reference to drug B the additive dose of the a, b combination is, as shown in section 2, $b + a/R$, which equals, after re-arrangement of

terms, $\frac{bC_A + aC_B}{C_A}$. Use of this quantity in drug B's dose-effect equation gives the additive effect E_{ab} as expressed in equation [3]:

$$E_{ab} = \frac{E_{\max}(bC_A + aC_B)}{bC_A + aC_B + C_A C_B} \quad [3]$$

This relation, if viewed graphically, is a surface of height E_{ab} above the a - b plane and, thus, the surface height gives the expected (additive) effect of the combination. An analysis based on this approach has been used in several of our combination studies and was recently employed in our examination of endogenous vasoconstricting agents (Lamarre & Tallarida, 2008) and in another recent study dealing with combinations of cocaine and other compounds that inhibit dopamine uptake (Tanda, et al, 2009).

The effect magnitude for the (a, b) combination derived experimentally is compared to E_{ab} calculated from [3] in a test that, of course, requires the statistical variance estimates of both the calculated and experimental effects. The variance (square of the standard error) of the experimental effect is obtained directly from the data set, whereas the variance of E_{ab} must be estimated from the relation given by equation [3]. The form of that equation does not permit a direct variance calculation and, thus, we used the approximating method known as the "delta-method" (See, for example, Cassella and Burger (2002) and a further discussion provided in the appendix to this paper). Doses a and b are controlled and are therefore assumed to be error free, whereas C_A and C_B are random variables. The estimated variance (square of standard error) of E_{ab} is the sum of terms that include the variances of C_A and C_B and this is given by equation [4] from which the standard error of E_{ab} follows as the square root.

$$Var(E_{ab}) = (bE_{\max})^2 \left(\frac{C_A}{T} \right)^4 Var(C_B) + (aE_{\max})^2 \left(\frac{C_B}{T} \right)^4 Var(C_A) \quad [4]$$

where $T = bC_A + aC_B + C_AC_B$ and the constants C_A and C_B are constants representing the doses for their respective half maximal effects.

As previously mentioned, the variance in equation [4] is the statistical variance, which is the square of the standard error. Thus, this calculated standard error accompanies the mean additive effect, derived from equation [3]. These values are needed in a comparison with the experimentally derived mean combination effect and its standard error. A test appropriate for that comparison uses the familiar *Student's t* distribution to examine the difference between the mean effect determined experimentally and the calculated additive E_{ab} from equation [3] with standard error derived from equation [4]. (Further computational details of this standard statistical procedure are given in Tallarida, (2000, pp 60–62). This methodology, applicable to effect levels, was employed in our study (Tanda *et al*, 2009) of combinations of cocaine and WIN 35,428 on basal dopamine levels in which it was found that the combination produced effects above the predicted value, but not significantly above that value. In another application (Lamarre and Tallarida, 2008) we employed this statistical computation in assessing the interaction between urotensin II and angiotensin II in constricting isolated rat aorta; in that case, the result was synergism for this combination. These examples denote the importance of equation [4] for assessing whether an experimental effect mean value differs significantly from the additive effect predicted from dose equivalence.

4.2 Examples of agonists having different efficacy

There are many examples of agonists that are effective but yield different maximum effects in some specified animal model. As an example that we previously analyzed (Grabovsky and Tallarida, 2004), we point to data obtained by Rawls *et al* (2002) on hypothermia (in rat) produced by dextromethorphan (DXM) and the cannabinoid agonist, WIN 55212-2. Each compound alone produced dose-dependent hypothermia, but with different maxima. DXM showed a maximum temperature decrease of 1.58° C whereas the WIN compound decreased temperature by 4.17° C. This combination therefore resulted in the curved isoboles shown in Grabovsky and Tallarida (2004) and are especially interesting since the isoboles for some effect levels are unbounded i.e., have no intercept on one axis. Another example is represented in our work with Parry *et al* (2006) that examined drug combinations on smooth muscle cell proliferation, a topic of importance in the prevention of restenosis following stent deployment. That study included sirolimus, an inhibitor of cell proliferation through a cytostatic G1-arrest mechanism, and topotecan, an inhibitor of the enzyme topoisomerase. Each agent is effective individually and inhibited vascular smooth muscle proliferation in a dose-dependent manner. The maximum effect (percent reduction) produced by sirolimus was 69.8 % whereas that of topotecan was 88.9 %. Combinations of the two led to curved isoboles of additivity that formed the basis of our isobolographic examination of actual combinations of the two agents and from which we determined the interaction to be simply additive.

Because the two drugs differ in efficacy, the additive effect of the combination again employs the dose equivalent of one and adds it to the other. For illustration we take drug B to be the higher efficacy drug and define its maximum effect to be 100%. Thus, E_B/E_A , here

denoted by γ , is a number > 1 . The dose equivalent of dose a is, as previously shown, and with γ inserted,

$$b_{eq}(a) = \frac{C_B}{\gamma \left(1 + \frac{C_A}{a}\right) - 1}$$

This quantity, when added to dose b , is the effective dose of the combination for use in drug B's dose-effect relation. This addition of doses leads to the additive effect of the combination as given in equation [5] for the combination dose (a, b) :

$$E_{ab} = (E_{\max}) \frac{ab(\gamma - 1) + \gamma b C_A + a C_B}{\gamma(a + C_A)(b + C_B) - ab} \quad [5]$$

It is seen that in the special case $\gamma = 1$, equation [5] reduces to equation [3] as it should. The complexity of equation [5] suggests that a display of a general formula for its statistical variance would be complicated and computationally awkward. Instead we illustrate in the appendix (again using the delta method) how to calculate the needed variance for any particular a, b combination.

4.3 Drug combination analysis and clinical development

The use of formulated drug combinations offers potential benefits over the use of the individual drugs. In addition to the likely increase in convenience and compliance, there is the potential for a mechanistic interaction, in which the clinical effect is greater than the simply additive effect of the two agents. There are examples of such beneficial drug combinations in several clinical areas, but some of the most well known examples are in the area of pain relief (Raffa *et al.*, 2003). By its very nature, pain is multi-faceted, so it is perhaps not surprising that no single analgesic agent has been found capable of relieving all pains, and that many pain conditions are better treated with a combination of drugs that have different mechanisms of action. It is surprising, however, when the pain relief provided by a combination of analgesics is greater than additive (*i.e.*, synergistic). Nevertheless, several examples of synergy in analgesic action are available: for racemic drugs with two active enantiomers (*e.g.*, tramadol), for single drugs with multiple sites of action (*e.g.*, acetaminophen), for two drugs with multiple mechanisms of action (*e.g.*, tramadol plus acetaminophen), and for combinations in which one component does not independently display analgesic action (*e.g.*, ibuprofen plus glucosamine).

4.3.1 Tramadol—Tramadol consists of two enantiomers that differ in their pharmacologic profile (Raffa *et al.*, 1992). One enantiomer has greater affinity for opioid receptors and potency for inhibition of 5-HT (serotonin) neuronal reuptake; the other enantiomer has greater potency for inhibition of norepinephrine neuronal reuptake. Thus the racemate is essentially a self-contained combination. Both of the enantiomers display an independent analgesic effect when administered individually and synergistic analgesic effect when administered together (Raffa *et al.*, 1993). Importantly, the enantiomers do not interact synergistically to produce side-effects such as respiratory depression or constipation (Raffa *et al.*, 1993).

4.3.2 Acetaminophen—Although the exact mechanism of analgesic action of acetaminophen (paracetamol) is not fully known, an action on the central nervous system is suspected (see Ashton, 2008). When administered into either the spinal cord (intrathecal infusion) or into the brain (intracerebroventricular infusion), acetaminophen produces an antinociceptive (analgesic) effect in mice. When it is administered into both sites simultaneously, a synergistic analgesic effect is observed, a phenomenon termed ‘site-site synergy’ (Raffa *et al.*, 2000).

4.3.4 Tramadol + acetaminophen—It is common for combinations of established analgesic drugs to be used in clinical practice. It is less common, however, to find published evidence that the combinations result in a synergistic analgesic interaction in both preclinical and clinical studies. One exception is the combination of tramadol plus acetaminophen, which has been shown to result in a synergistic analgesic effect in preclinical (Tallarida *et al.*, 1997) and in clinical evaluations (Filitz *et al.*, 2008). There is also evidence to suggest that the synergy in analgesic effects is accompanied by increased efficacy and a better side-effect profile (Fricke *et al.*, 2004).

4.3.5 Ibuprofen + glucosamine—Glucosamine, an amino derivative of glucose, is widely available for the self-treatment of sports-related sprains and strains and osteoarthritic conditions. The reduction of pain that has been associated with glucosamine sulfate (Creamer, 2000) and possibly glucosamine hydrochloride (Haupt *et al.*, 1999) is usually attributed to a disease-modifying action of the substance rather than to a direct analgesic action. We also found the lack of a direct analgesic (antinociceptive) effect of glucosamine sulfate in the mouse abdominal constriction model. However, the combination of glucosamine sulfate in certain fixed-ratios with certain NSAIDs (non-steroidal anti-inflammatory drugs) such as ibuprofen and ketoprofen synergistically enhanced their analgesic effects (Tallarida *et al.*, 2003).

5. Combinations of agonists that produce opposite effects

There are situations in which two agonists that produce opposite effects are present in the organism. The second compound might be a drug used for some other therapeutic condition, an endogenous chemical whose effect is opposite that of the therapeutic agent, or an illicit drug used recreationally. As a specific example we point to the effects of morphine and delta 9-THC on body temperature. Doses above (1.0 mg/kg) of delta 9-THC produce dose dependent hypothermia in rat (Taylor & Fennessy, 1977). In contrast morphine produces a dose-dependent rise in body temperature (Rudy & Yaksh, 1977). Temperature is a very suitable metric in quantifying drug action and, thus, combinations of the above (and other drugs) represent ongoing studies in our laboratories. In a previous review in this journal (Tallarida, 2007) we also mentioned drugs that produce opposite effects and provided as an example lipopolysaccharide (LPS) which induces fever whereas certain cannabinoids produce hypothermia (Benamar *et al.*, 2007). That study showed that the concept of dose equivalence is applicable to cases in which the effects are in the opposite direction, and that the particular interaction was consistent with simple additivity. Details of the mathematical analysis were not provided in the previous review; hence we provide the needed quantitative detail here for a topic that has not previously received much attention.

The opposite effect situation is amenable to the concept of dose equivalence that has been discussed and applied here. In illustrating this analysis, we denote by drug B the primary agent that produces the positive effect while drug A is the drug (or chemical) that produces effects in the opposite direction. The maximum effects of these compounds need not be equal in magnitude. For a dose a of drug A that produces a negative effect of magnitude E^* there is a nullifying equivalent of drug B, and we denote this as $b_{eq}(a)$, as in our previous discussion (see Figure 1). Therefore the presence of dose a subtracts this equivalent from the dose b of drug B. The concept is identical to that used in isobolographic and related analyses except that this is *addition* of a negative quantity. Accordingly the term “additive” is applicable for this case also and we use the dose-effect relation of drug B to calculate the expected additive effect which is given here as equation [6]:

$$E_{ab} = \frac{E_B [b - b_{eq}(a)]}{b - b_{eq}(a) + C_B} \quad [6]$$

This equation holds for $b > b_{eq}(a)$ and has a variance, $Var(E_{ab})$, estimated as

$$Var(E_{AB}) = \left[\frac{E_B C_B}{(b - b_{eq}(a) + C_B)^2} \right]^2 Var(b_{eq}(a)) + \left[\frac{E_B (b_{eq}(a) - b)}{(b - b_{eq}(a) + C_B)^2} \right]^2 Var(C_B) \quad [7]$$

where

$$Var(b_{eq}(a)) = \left[\frac{E_B C_B}{(E_B - E^*)^2} \right]^2 Var(E^*) + \left[\frac{E^*}{E_B - E^*} \right]^2 Var(C_B)$$

6. Occupation-effect relations

While combinations of drugs represent the main use of isobolographic analysis, a single agonist drug that combines with two receptors is amenable to the same kind of analysis, and one may ask whether there is an interaction between the occupied receptors. The drug's concentration leads to the fractional occupation (f_{occ}) of each receptor by application of the mass-action law: $f_{occ} = [A] / ([A] + K)$, where $[A]$ is the concentration of drug A, and K is its dissociation constant (reciprocal of affinity constant) for that receptor. Occupation-effect relations provide a more specific view of drug action than do concentration-effect relations. (See for example, Pineda *et al*, 1997; Tallarida *et al*, 2007; Braverman *et al*, 2008). The concept of dose-equivalence has its obvious counterpart in transformations in which concentration-effect data become receptor occupation-effect data for each of the receptors to which the agonist binds. In other words, if an agonist combines with two receptors that each contribute to its effect, and if these relations are known (say, from experiments in which the receptors are individually inactivated by genetic deletion or irreversible blockade) then the two occupation-effect relations allow the determination of an additive isobole that describes occupation pairs that give the same magnitude of effect. Increasing doses of the agonist lead to increasing receptor occupancies, thereby providing a path (occupation pairs for the receptors) on the isobologram. This topic was discussed in a previous review in this journal (Tallarida, 2007). We recently applied this methodology to muscarinic M_2 and M_3 receptors

that contribute to carbachol-induced contraction in stomach muscle (Braverman *et al*, 2008; 2009), and provided the mathematical details needed in the calculation. A key observation was that individual occupation of M₂ and M₃ receptors did not yield the same maxima and, thus, the isobole equation is the form of equation [2] rather than the straight line form of equation [1]. Specifically, the concentrations in equation [2] are replaced by each receptor's fractional occupation and the needed C_A and C_B constants are replaced by appropriate constants obtained from the fitted occupation-effect curves. We calculated fractional occupancies (f_{occ} defined above) using the values of carbachol concentration, with the appropriate K values which are 2.75 mM for the M₂ receptor and 62 mM for the M₃ receptor. (The K values of these and various other drug-receptor sets can be found in various data bases such as the National Institute of Mental Health Psychoactive Drug Screening Program; PDSP Contract NO1MH32004). The remaining analysis is identical to the two-drug case. The application described showed that there is no unusual interaction between M₂ and M₃ muscarinic receptors in mediating mouse stomach muscle contraction; that is, the interaction is simply additive.

7. Competitive antagonism

The use of occupation-effect relations and the accompanying isobole that results provides some new quantitative insights on the use of competitive antagonists that combine with each of the two receptor subtypes. It is well known in the single receptor case that a competitive antagonist in concentration [B] reduces the agonist binding (and consequent effect) from $[A][R] / ([A] + K_A)$ to the lesser quantity $[A][R] / ([A] + K_A (1 + [B]/K_B))$, a relation first introduced by Gaddum (1937). Hence restoration of binding to the original level requires the greater agonist concentration $[A']$ and this leads to

$$\frac{[A']}{[A]} - 1 = \frac{[B]}{K_B} \quad [8]$$

where K_B is the dissociation constant of the antagonist.

equation [8] is well known and often applied to experiments that yield $[A']$ from the rightward shifted dose-effect curve that results from the fixed antagonist concentration [B], and this relation is the basis of the Schild plot (Arunlakshana & Schild, 1959). The two-receptor case under consideration here requires a further analysis of the basis for equation [8]. Figure 2 shows the isobologram based on fractional occupancy y at receptor #1 and fractional occupancy x at receptor #2. The figure shows the additive isobole of occupation as a broken curve for a specified effect along with two solid curves emanating from the origin and labeled "path (a)" and "path (b)." Path (a) defines the receptor occupations that accompany the changing agonist doses when there is no antagonist present and its intersection with the isobole locates the occupancy pair applicable to the isobole's effect level. It is worthy of note that, in contrast to the usual dose isobologram in which dose combinations are under control of the experimenter, the occupation path is defined by the agonist concentration and the K_A values for each receptor. Path (b) in the figure is that which applies to increasing agonist in the presence of the fixed antagonist concentration [B]. In this situation the increasing agonist concentration for restoring the effect is defined by

path (b) whose intersection with the isobole gives the point (fractional occupation pair) and, thus the $[A']$ value that restores the effect to its unblocked value. In other words, restoration of the effect means a return to a point on the isobole, and this is denoted by the intersection of path (b) with the isobole. equation [8] no longer applies to this two-receptor situation. Instead, the following apply (details in the APPENDIX), where the antagonist K values are labeled with the appropriate subscripts, K_{B1} and K_{B2} :

- o When the antagonist affinity at receptor #1 is greater than at receptor #2

$$\frac{[B]}{K_{B2}} < \frac{[A']}{[A]} - 1 < \frac{[B]}{K_{B1}} \quad [9]$$

- o When the antagonist affinity at receptor #2 is greater than at receptor #1

$$\frac{[B]}{K_{B1}} < \frac{[A']}{[A]} - 1 < \frac{[B]}{K_{B2}} \quad [10]$$

These inequalities demonstrate that equation [8] is not applicable to the case in which there are two receptors to which both the agonist and antagonist combine.

The methodology that employs occupation isoboles with (and without) antagonists was employed in our studies of muscarinic M_2/M_3 receptors (Braverman *et al*, 2008). That study used mice with genetically deleted M_2 receptors (M_2 knockouts) and mice with genetically deleted M_3 receptors (M_3 knockouts). In each we measured the isometric tension developed in response to graded doses of the agonist carbachol in an isolated stomach muscle preparation. From these experiments we obtained the occupation-effect relations for each receptor subtype and the consequent isobole of additivity for a specified effect magnitude (40% of KCl maximal contraction was used). The fractional occupancy of carbachol was calculated (using the law of mass action) from the administered concentrations and the carbachol dissociation constants (K values) which are 2.75 μM for M_2 and 62 μM for M_3 . The occupation isobole represents all occupation pairs that give the specified effect magnitude, a concept identical to the familiar dose-based isobole which represents all dose pairs that give the specified effect magnitude. Thus, as mentioned previously, when both receptors are present (wild type preparation) the restoration of this effect in the presence of an antagonist must be to a point (occupation pair) on the isobole if the interaction is additive. The competitive antagonist atropine was used in a fixed concentration, and the carbachol concentration-effect curves with this fixed dose of atropine revealed the expected rightward shift, thereby yielding the experimental occupation pair that restored the effect. Four different atropine fixed doses were used and, in each of these cases, the experimentally derived point was found to be only slightly below the occupation isobole; i.e., the experimental value did not differ significantly from the value on the additive isobole. These results indicate a simply additive interaction between the two muscarinic receptor sub-types when they are occupied by carbachol. Further computational and graphical details are given in Braverman *et al* (2008).

The above findings prompted a second study, this time in porcine stomach muscle (Vegesna *et al*, 2010). In this study gastric sling fibers were contracted with carbachol and subsequently with carbachol plus a fixed dose of the antagonist methoctramine, which is relatively selective for the M_2 muscarinic receptor. In this species both M_2 and M_3 also contribute to carbachol induced tension development. In this species a genetically altered (knockout) is not available and, therefore, the use of occupation isoboles, as in the previously described mouse study, was not feasible. However, an interesting finding, which we now describe, provides new insights on possible M_2 – M_3 interactions in this preparation. This finding refers to the dextrally shifted carbachol dose-effect curve that accompanied the administration of a fixed concentration ($1E-5$ M) of the antagonist methoctramine. The shift, approximately by one log unit of carbachol in this case, is not surprising for a competitive antagonist. However, the presence of this antagonist also increased the carbachol efficacy significantly, a finding that is rather unusual for a pure antagonist that competes with this agonist. Specifically, the mean value of the maximum went from 94.7 to 131.5 (% values based a previously established scale and $n = 8$). Our tentative conclusion is that in this porcine preparation there is a sub-additive interaction between M_2 and M_3 receptors and, thus, the block with methoctramine (a pure antagonist) nullifies this negative interaction. More studies are necessary to precisely determine the mechanism responsible for this unusual finding. But this finding also suggests the possibility that in preparations in which two receptors are synergistic, a selective block of one of them might produce enhanced antagonism, i.e., a greater rightward shift.

8. Dose equivalence and “Inverted U-shaped” dose-effect curves

Dose-effect curves are, for the most part, monotone non-decreasing functions with some upper limit. However, there are some tests of drug action in which the higher doses produce a decrease in effect (“inverted U”) that is dose related. The phenomenon of a biphasic dose-effect curve has been termed ‘hormesis.’ As demonstrated in the recent comprehensive review by Calabrese (2008), there are many examples of such curves in pharmacology. They are found for central nervous system drugs (*e.g.*, anti-anxiety, anti-seizure, and learning and memory enhancers), anti-tumor drugs, chemoprevention of stroke and traumatic brain injuries, cardiac glycosides, statins (for cardiovascular disease or cancer), bisphosphonates, and many others. A very notable additional example of hormesis is the stimulation of locomotor activity by cocaine (see, for example, Ferrario *et al*, 2005; Flagel and Robinson, 2007). Of special interest to us is the analgesic buprenorphine, a drug studied in our laboratories which also displays a biphasic dose-response relation in some preclinical models. The analysis of inverted U-shaped dose-effect curves (method described below) was therefore applied to buprenorphine (Tallarida *et al*, 2010). The quantitative information from such a dose-response relation has intrinsic interest and may also confer beneficial clinical use.

Without postulating any particular mechanism, we illustrate here (Figure 3) how the concept of dose equivalence can be applied to give useful new insights on the form of this dose related effect reduction. In that figure we illustrate the decrease in effect and show a typical drop denoted by E at dose b^* . A drop of that magnitude is associated with a decrease in dose denoted by b . One now examines the smooth curve (broken) to find the effect level

that corresponds to the quantity b , and we see that it occurs at the effect level E^* . Therefore, the point with coordinates (b^*, E^*) is located and E^* represents the magnitude of the negative component of drug B's action for dose b^* . All other points of decreased effect (small squares) are analyzed in the same way in order to produce the smooth (solid) curve. Further detail is given in the figure legend. The reduction in effect could be due to a second receptor that mediates an effect opposite to that of the first receptor, or it could be due to release of a substance or activation of a new pathway that negates the positive effect of the agonist. Regardless of the mechanism, it is possible to use the reduced effect data to determine the dose-effect relation of this negative component of action. The methodology for this determination is based on dose equivalence and is another demonstration of the utility of this concept in providing quantitative information that is not initially tied to any particular mechanism.

Our previously mentioned study with buprenorphine (Tallarida *et al*, 2010) applied the method of analysis described here to derive the dose-effect relation of the second component that reduces the effect of this analgesic. While we do not know the intimate mechanism the most commonly held view is that this second component is mediated through an orphanin/nociceptin-FQ pathway (NOP/ORL1 receptors) as discussed in the cited publication.

9. Interaction Studies and Exploration of Mechanism

As previously mentioned, the quantitative analysis of an interaction as described here does not postulate (or require) a mechanism; i.e., departures from additivity such as synergism use only the concept of dose equivalence. But the finding of a non-additive interaction can be a first step in exploring mechanism. An example is afforded in a study in our laboratories (Tallarida *et al*, 2003) that examined ibuprofen in combination with various analgesics in a standard rodent model of antinociception (mouse abdominal constriction). That study showed that ibuprofen was effective with an $ED_{50} = 26.1 \pm 3.4$ mg/kg, (p.o) whereas glucosamine sulfate alone lacked efficacy. The combination, however, achieved the 50% effect with a much lower ibuprofen dose (11.0 ± 2.1), thereby indicating synergism for the fixed ratio combination tested. Similar results were obtained with several other dose ratios. This unexpected result, with no known mechanism, prompted further studies (still under way) and, in one of these, it was found that a chemical complex containing both moieties was also synergistic. Further, this complex is quite soluble in saline (in contrast to ibuprofen which is not very soluble), thereby suggesting that the increased solubility might also enhance the binding of ibuprofen to cyclooxygenase with the consequent decrease in PGE_2 formation. This is still speculative. Further exploration of this possible mechanism is underway and this is mentioned here merely as one example demonstrating how the finding of synergism can lead to further studies of mechanism. It prompts the question of whether other agents might enhance the binding of NSAIDs to cyclooxygenase.

Another example is afforded by our studies of antagonists of neurokinin 1 (NK1) and gabapentin. One NK1 antagonist, CI-1021, in combination with gabapentin showed synergism in two rat models of neuropathic pain (Field *et al*, 2002). The finding that one NK1 antagonist gave synergism with gabapentin prompted a further examination with another structurally different NK1 antagonist, and this, too, showed synergism. Further, the

degree of synergism was found to depend on the ratio of the constituents while the duration of action of each fixed dose ratio remained the same. Together these findings suggest a pharmacodynamic interaction (rather than a pharmacokinetic interaction), although the precise mechanism is still not clear. Further studies examining plasma and central nervous system levels of each compound after administration of the combinations are required to more fully evaluate this phenomenon. This study and the previously mentioned synergism between ibuprofen and glucosamine are merely representative examples to illustrate how the finding of synergism, besides its quantitative importance, can lead to studies that might reveal mechanism.

10. Summary

The analysis of drug combinations very often uses isobolographic methods, a graphical procedure that is rooted in the concept of dose equivalence. No intimate mechanism of action is assumed, yet finding a departure from simple additivity (synergism or sub-additivity) from this method indicates an interaction between the drugs and this may serve as an important first step in exploring mechanisms. In this review we have summarized the connection of dose equivalence to the isobole and also demonstrated how dose equivalence can be used to quantitatively examine other actions such as inverted-U dose-effect curves and combinations of agonists that produce opposite effects. We also showed that interactions can be viewed on the effect scale as an alternative to isobolographic analysis and, further, demonstrated that the use of receptor occupation equivalence provides insights for understanding competitive antagonism in the dual receptor case. The method applied in that case is a logical extension of dose equivalence and employs only the well known theory that follows from the law of mass action.

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APPENDIX

List of symbols

a or A , dose of drug A; b or B , dose of drug B; $[A]$, concentration in mass-action law; $[A']$, agonist concentration in the presence of an antagonist; $b_{eq}(a)$, the drug B equivalent of dose a ; C_A and C_B , terms representing potency of drug A and drug B, respectively; D , dose; E , effect; E_{max} , maximum effect; E_A , maximum effect of drug A; E_B , maximum effect of drug B; E^* or E^* , negative drug effect; E_{ab} , the additive effect of dose combination (a,b) ; f_{occ} , fractional receptor occupation; $\gamma = E_B/E_A$; K , dissociation constant; K_A , dissociation constant for drug A; K_B , dissociation constant for antagonist drug B; $R = C_A/C_B$; $Var(E_{ab})$, variance of E_{ab} (square of standard error).

A-1. The Delta Method

The delta method of variance approximation is derived from Taylor series and is such that the variance of a function f containing random variables x and y is approximated by

$$\text{Var}(f(x, y)) \cong \left(\frac{\delta f}{\delta x}\right)^2 \text{Var}(x) + \left(\frac{\delta f}{\delta y}\right)^2 \text{Var}(y) + 2 \left(\frac{\delta f}{\delta x} \frac{\delta f}{\delta y}\right) \text{Cov}(x, y) \quad [\text{A-1}]$$

In our application the function is E_{ab} and x and y are C_A and C_B , respectively. Because C_A and C_B are independent the covariance term is zero and, thus

$$\text{Var}(E_{ab}) \cong \left(\frac{\delta E_{ab}}{\delta C_A}\right)^2 \text{Var}(C_A) + \left(\frac{\delta E_{ab}}{\delta C_B}\right)^2 \text{Var}(C_B) \quad [\text{A-2}]$$

This method, applied to equation [3], led to equation [4] which expresses the variance formula explicitly. To demonstrate its application to equation [5] we illustrate with a numerical example to calculate E_{ab} from equation [5] and its variance from equation [A-2].

Example. Drug B: $E_B = 100$, $C_B = 10$, $\text{Var}(C_B) = 4$

Drug A: $E_A = 50$, $C_A = 40$, $\text{Var}(C_A) = 36$

Thus $\gamma = 2$. We consider the combination $a = 20$, $b = 5$.

Substitution in equation [5] leads to

$$E_{ab} = (100) \left(\frac{(100 + 10C_A + 20C_B)}{100 + 10C_A + 40C_B + 2C_A C_B} \right)$$

and, for the chosen a, b is seen to yield the additive effect $E_{ab} = 41.18\%$.

From the above we get the partial derivative at this (a, b) point:

$$\frac{\partial E_{ab}}{\partial C_B} = \frac{(100)(-2000 - 400C_A - 20C_A^2)}{(100 + 10C_A + 40C_B + 2C_A C_B)^2}$$

Inserting $C_A = 40$ and $C_B = 10$, yields

$$\frac{\partial E_{ab}}{\partial C_B} = \frac{(100)(-50000)}{(1700)^2} = -1.73.$$

Similarly, the partial derivative with respect to C_A follows as

$$\frac{\partial E_{ab}}{\partial C_A} = \frac{(100)(-40C_B^2)}{(100 + 10C_A + 40C_B + 2C_A C_B)^2}$$

and insertion of the C_A and C_B values yields

$$\frac{\partial E_{ab}}{\partial C_A} = \frac{(100)(-40)(10)^2}{(1700)^2} = - \frac{(4000)(10)^2}{(1700)^2} = -0.138$$

With these partial derivatives of E_{ab} , evaluated for the indicated C_A and C_B and calculated as shown above, we get the statistical variance from equation [A-2] for the values in this example:

$\text{Var}(E_{ab}) = (-0.138)^2(36) + (-1.730)^2(4) = 12.66$, from which the standard error = 3.56 follows as the square root for the additive effect = 41.18.

A-2. Restoration of the agonist effect in the presence of a competitive antagonist

Figure A-1 illustrates the isobole of occupation (curved) for a specified effect and is seen to be typically decreasing because occupation of one receptor means that less occupation of the other receptor is needed to maintain this constant effect level. If the agonist has equal affinity at both receptors then the occupation due to changing agonist concentration follows some path as shown by the solid radial line (not necessarily linear). The intersection of this radial line with the isobole gives the occupation pair needed for the specified effect. We now consider the situation in the presence of a fixed antagonist concentration. Here, instead of the solid curve path we have either path 1 or path 2 depending on the affinity of the antagonist at each receptor.

When the antagonist affinity is high at receptor #2 and low at receptor #1, an increase in its concentration $[B]$ means *agonist* occupancy paths to the right of the solid line (greater receptor #1 occupancy) as shown below in Figure A-1 for a representative path labeled (1). The opposite applies when the antagonist affinity is greater at receptor #1 and this is shown by the representative path labeled (2), i.e., increasing $[B]$ moves the agonist binding path progressively to the left. The intersections of these paths with the contour curve give the agonist occupation values that lead to the relations between $[A']$ for effect recovery in the blocked cases and $[A]$ in the unblocked case. The coordinates of these intersections lead easily to mathematical expressions of inequality. For example, consider path (2) and observe from its intersection that agonist binding to receptor #2 is greater in the presence of blocker

than in its absence; thus, $\frac{[A']}{[A'] + K_A(1 + [B]/K_{B2})} > \frac{[A]}{[A] + K_A}$ which leads to $\frac{[A']}{[A]} - 1 > \frac{[B]}{K_{B2}}$,

while binding x gives $\frac{[A']}{[A'] + K_A(1 + [B]/K_{B1})} < \frac{[A]}{[A] + K_A}$, thereby leading to

$$\frac{[A']}{[A]} - 1 < \frac{[B]}{K_{B1}}.$$

Combining these, we get the relation below which is the inequality labeled [9] in the text.

$$\frac{[B]}{K_{B2}} < \frac{[A']}{[A]} - 1 < \frac{[B]}{K_{B1}}$$

A similar analysis applies to path (1),

$$\frac{[A']}{[A'] + K_A(1 + [B]/K_{B2})} < \frac{[A]}{[A] + K_A},$$

leading to

$$\frac{[A']}{[A]} - 1 < \frac{[B]}{K_{B2}},$$

$$\text{while } \frac{[A']}{[A'] + K_A(1 + [B]/K_{B1})} > \frac{[A]}{[A] + K_A}, \text{ from which } \frac{[A']}{[A]} - 1 > \frac{[B]}{K_{B1}}.$$

Together these yield

$$\frac{[B]}{K_{B1}} < \frac{[A']}{[A]} - 1 < \frac{[B]}{K_{B2}}$$

which is labeled in the text as an inequality [10].

References

- Arunlakshana O, Schild HO. Some quantitative use of drug antagonists. *Br. J. Pharmacol.* 1959; 14:48–57.
- Ashton JC. Pro-drugs for indirect cannabinoids as therapeutic agents. *Current Drug Delivery.* 2008; 5(4):243–247. [PubMed: 18855592]
- Benamar K, Yondorf M, Meissler JJ, Geller EB, Tallarida RJ, Eisenstein TK, Adler MW. A Novel Role of Cannabinoids: Implication in the Fever Induced by Bacterial Lipopolysaccharide. *J. Pharmacol Exp. Ther.* 2007; 320:1127–1133. [PubMed: 17194800]
- Braverman AS, Tallarida RJ, Ruggieri MR Sr. The use of occupation isoboles for analysis of a response mediated by two receptors: M₂ and M₃ muscarinic receptor subtype-induced mouse stomach contraction. *J. Pharmacol. Exp. Ther.* 2008; 325:954–960. [PubMed: 18339971]
- Braverman AS, Miller LS, Vegesna AK, Tiwana MI, Tallarida RJ, Ruggieri MR Sr. Quantitation of the Contractile Response Mediated by Two Receptors: M₂ and M₃ Muscarinic Receptor-Mediated Contractions of Human Gastroesophageal Smooth Muscle. *J. Pharmacol Exp Ther.* 2009 In press.
- Calabrese EJ. Hormesis and medicine. *Brit. J. Clin. Pharmacol.* 2008; 66:594–617. [PubMed: 18662293]
- Casella, G.; Burger, RL. *Statistical Inference.* 2nd ed.. Pacific, Grove, CA: Duxbury; 2002.
- Creamer P. Osteoarthritis pain and its treatment. *Curr. Opin. Rheumatol.* 2000; 12:450–455. [PubMed: 10990186]
- Ferrario CR, Gorny G, Crombag HS, Li Y, Kolb B, Robinson TE. Neural and behavioral plasticity associated with the transition from controlled to escalated cocaine use. *Biol. Psychiatry.* 2005; 58:751–759. [PubMed: 16098484]
- Field MK, Gonzalez MI, Tallarida RJ, Singh L. Gabapentin and the NK1 antagonist CI-1021 act synergistically in two rat models of neuropathic pain. *J. Pharmacol. Exp. Ther.* 2002; 303:730–735. [PubMed: 12388658]
- Filitz J, Ihmsen H, Günther W, Tröster A, Schwilden H, Schüttler J, Koppert W. Supra-additive effects of tramadol and acetaminophen in a human pain model. *Pain.* 2008; 136:262–270. [PubMed: 17709207]
- Flagel SB, Robinson TE. Quantifying the psychomotor activating effects of cocaine in the rat. *Behav. Pharmacol.* 2007; 18:297–302. [PubMed: 17551322]

- Fricke JR Jr, Hewitt DJ, Jordan DM, Fisher A, Rosenthal NR. A double-blind placebo-controlled comparison of tramadol/acetaminophen and tramadol in patients with postoperative dental pain. *Pain*. 2004; 109:250–257. [PubMed: 15157685]
- Gaddum J. The quantitative effects of antagonistic drugs. *J. Physiol (London)*. 1937; 89:7P–9P.
- Gessner PK, Cabana BE. A study of the hypnotic effects and the toxic effects of chloral hydrate and alcohol. *J. Pharmacol. Exp. Ther.* 1970; 174:247–259. [PubMed: 5451362]
- Grabovsky Y, Tallarida RJ. Isobolographic analysis for combinations of full and partial agonist: curved isoboles. *J. Pharmacol. Exp. Ther.* 2004; 310:981–986. [PubMed: 15175417]
- Houpt JB, McMillan R, Wein C, Paget-Dellio SD. Effect of glucosamine hydrochloride in the treatment of pain of osteoarthritis of the knee. *J. Rheumatol.* 1999; 26:2294–2297. [PubMed: 10555879]
- Keith RA, Donahue JY, Salama AI. The effect of varying carbachol concentration on the slope of the Schild plots of selective beta-adrenoceptor antagonists in carbachol-contracted guinea-pig trachea. *Pharm Pharmacol.* 1986; 38(2):107–112.
- Kuraishi Y, Fukui K, Shiomi H, Akaike A, Takagi H. Microinjections of opioids into the nucleus reticularis gigantocellularis of the rat: Analgesia and increase in the normetanephrine level in the spinal cord. *Biochem Pharmacol.* 1978; 27:2756–2758. [PubMed: 728230]
- Loewe S. Die Mischiamei. *Klin Wochenschr.* 1927; 6:1077–1085.
- Loewe S. Die quantitativen Probleme der Pharmakologie. *Ergeb Physiol.* 1928; 27:47–187.
- Loewe S. The problem of synergism and antagonism of combined drugs. *Arzneimittelforschung.* 1953; 3:285–290. [PubMed: 13081480]
- Loewe S. Antagonisms and antagonists. *Pharmacol Rev.* 1957; 9:237–242. [PubMed: 13465303]
- National Institute of Mental Health Psychoactive Drug Screening Program's (NIMH-PDSP). K_i Database. <<http://pdsp.med.unc.edu>>.
- Parry TJ, Thyagarajan R, Argentieri D, Falotico R, Sickierka J, Tallarida RJ. Effects of drug combinations on smooth muscle cell proliferation: An isobolographic analysis. *Eur. J. Pharmacol.* 2006; 532:38–43. [PubMed: 16448648]
- Pineda J, Ruiz-Ortega JA, Uged L. Receptor Reserve and Turnover of *Alpha*-2 Adrenoceptors that Mediate the Clonidine-Induced Inhibition of Rat Locus Coeruleus Neurons *In Vivo*. *J. Pharmacol. Exp. Ther.* 1997; 281(Issue 2):690–698. [PubMed: 9152374]
- Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL. Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an "atypical" opioid analgesic. *J. Pharmacol. Exp. Ther.* 1992; 260:275–285. [PubMed: 1309873]
- Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL, Jacoby HI, Selve N. Complementary and synergistic antinociceptive interaction between the enantiomers of tramadol. *J. Pharmacol. Exp. Ther.* 1993; 267:331–340. [PubMed: 8229760]
- Raffa RB, Stone DJ Jr, Tallarida RJ. Discovery of self-synergistic spinal/supra-spinal antinociception produced by acetaminophen (paracetamol). *J. Pharmacol. Exp. Ther.* 2000; 295:291–294. [PubMed: 10991992]
- Raffa RB, Clark-Vetri R, Tallarida RJ, Wertheimer A. Combination strategies for pain management. *Expert Opin. Pharmacother.* 2003; 4:1697–1708. [PubMed: 14521480]
- Rawls SM, Cowan A, Tallarida RJ, Geller EB, Adler MW. NMDA antagonists and WIN 55212-2, a cannabinoid antagonist, interact to produce synergistic hypothermia. *J. Pharmacol. Exp. Ther.* 2002; 303:395–402. [PubMed: 12235276]
- Rudy TA, Yaksh TL. Hyperthermic effects of morphine: set point manipulation by a direct spinal action. *Br. J. Pharmacol.* 1977; 61(1):91–96. [PubMed: 912212]
- Tallarida RJ, Stone DJ Jr, Raffa RB. Efficient designs for studying synergistic drug combinations. *Life Sci.* 1997; 61:417–425.
- Tallarida, RJ. *Drug Synergism and Dose-Effect Data Analysis* (monograph). Boca Raton: CRC/Chapman-Hall; 2000.
- Tallarida RJ. *Drug Synergism: Its Detection and Applications. Perspectives in Pharmacology.* J. Pharmacol. Exp. Ther. 2001; 298:865–872. [PubMed: 11504778]

- Tallarida RJ, Cowan A, Raffa RB. Antinociceptive synergy, additivity, and subadditivity with combinations of oral glucosamine plus nonopioid analgesics in mice. *J. Pharmacol. Exp. Ther.* 2003; 307:699–704. [PubMed: 12966152]
- Tallarida RJ. An overview of drug combination analysis with isobolograms. *Perspectives in Pharmacology. J. Pharmacol Exp Ther.* 2006; 319(1):1–7. [PubMed: 16670349]
- Tallarida RJ. Interactions between drugs and occupied receptors. *Pharmacology & Ther.* 2007; 113:197–209.
- Tanda G, Newman AH, Ebbs AL, Tronci V, Green JL, Tallarida RJ, Katz JL. Combinations of Cocaine with other Dopamine Uptake Inhibitors: Assessment of Additivity. *J. Pharmacol. Exp. Ther.* 2009; 330:802–809. [PubMed: 19483071]
- Taylor DA, Fennessy MR. Biphasic nature of the effects of delta9-tetrahydrocannabinol on body temperature and brain amines of the rat. *Eur. J. Pharmacol.* 1977; 46(2):93–99. [PubMed: 590330]
- Vegesna AK, Braverman AS, Miller LS, Tallarida RJ, Tiwana MI, Khayyam U, Ruggieri M Sr. Comparison of human and porcine gastric clasp and sling fiber contraction by M₂ and M₃ muscarinic receptors. *Am. J. Physiol.* 2010 In press.
- Yaksh TL. Direct evidence that spinal serotonin and noradrenaline terminals mediate the spinal antinociceptive effects of morphine in the periaqueductal gray. *Brain Res.* 1979; 160:180–185. [PubMed: 581478]

Figure 1.

Dose a alone produces a negative effect of magnitude E^* which nullifies a dose of drug B that is shown here as $b_{eq}(a)$.

Figure 2.

The broken curve is the isobole for a specified effect. Also shown are paths (a) and (b), not necessarily linear, that represent the relation between the agonist's fractional occupations x at receptor #1 and y at receptor #2. Path (a) represents the bound agonist when it is the sole agent, while path (b) shows the agonist binding when there is an antagonist with fixed concentration $[B]$ with higher affinity for receptor #2. The high antagonist affinity for receptor #2 means that the agonist binding for receptor #1 denoted by x is increased; hence it shows as path (b) on the right. Each intersection is on the isobole curve and each gives the agonist fractional occupation values of the two receptors that produce the specified effect magnitude for the two cases. These occupation values, in turn, allow a calculation of the agonist concentration for this effect in the blocked and un-blocked cases. These agonist concentrations are denoted by $[A']$ and $[A]$ respectively in expression (8), (9) and (10). Path (b) is to the right of path (a), as in this illustration, because $K_{B1} > K_{B2}$ but would be to the

left of curve (a) if $K_{B2} < K_{B1}$. If the experiment yields termination points that are off the contour, there is an interaction between the agonist-occupied receptors as described in the text.

Fig.3.

(*upper*) Inverted U-shaped dose-effect curves (black squares) refer to situations in which the monotone increasing dose-effect curve (shown broken) shows decreasing effects (dark squares) as the dose b is increased above a certain value. (*lower*) The trend in the monotone increasing curve allows approximate values of the decrease E which, in turn, corresponds to a decrease b in the dose of drug B at some value b^* . When the decrease b is referred to drug B's dose-effect curve it is seen to occur at an effect level E^* , thereby locating the point (b^*, E^*) . This point represents the magnitude of the negative effect component of drug B's

action. This point is shown (for visual convenience) as a function with a positive slope, but it should be noted that its height indicates the magnitude of the negative effect. Points corresponding to other effect drops are similarly analyzed and together these (shown as diamond shape) give the solid curve for this negative component.

Fig. A-1.

A competitive antagonist in concentration $[B]$ diminishes agonist binding at both receptors with consequent reduction in effect. The restoration of the effect requires a greater agonist concentration and consequent increase in binding (shown as one of the radial lines) that must terminate on the isobole (smooth curve) to restore the specified effect. Each radial line represents the fractional agonist occupation at receptor #1 (denoted x) and at receptor #2 (denoted y) that accompany the increase in agonist concentration. (See Gaddum equation, text, section 7.) For purposes of illustration we show this x - y relation (as a solid radial line) for the special (and unlikely) case in which the antagonist affinity is the same at each receptor. Two other cases are illustrated. In case (1) the antagonist affinity is greater for receptor #2, whereas in case (2) the antagonist affinity is greater for receptor #1. The arrows indicate for each case how these radial lines would be displaced if $[B]$ were increased.

(Although shown as radial lines, the actual x - y binding curve, determined from mass action, could be curvilinear.)