

Review Article

Experiment Designs for the Assessment of Drug Combination Synergism

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2014; Published: November 05, 2014**Abstract**

We review the concepts and the processes leading to the formation of a Combination Index to quantitatively depict combination effects in the evaluation of combination chemotherapies. We raise the important possibility that synergistic results found and published in hundreds, even thousands, of articles on drug combinations maybe over-assessed; many or most results could simply be false positives. We then propose the formation of two Combination Indices to quantitatively depict combination effects in the evaluation of combination chemotherapies; one for the Lowe additive model and one for the Bliss multiplicative model. Within that modeling framework, we will focus on the experiment designs for the assessment of drug combination synergism preceding the formation of a proper combination index.

Keywords: Additivity; Antagonism; Combination index; Synergism

Forming Combination Indices to Measure Synergism

Drug combinations are widely used in treating the most dreadful diseases, including cancers. There are a number of reasons, but the main aim is to increase tumor response, ideally to achieve synergistic therapeutic effect. When the addition of one agent apparently increases the effect of the other, so that the effect of a combination appears to be greater than would be expected; the term “synergism” is used to describe these situations with enhancement of tumor response. The term “antagonism” is used when the effect of the combination is less effective than the sum of the individual effects; without synergism and antagonism, the two individual effects are “additive”. The problems are:

(1) In order to understand and characterize drug interactions, we need to define or model “additive effects” of drug combinations. It is not simple; Chou claimed that it took him “about 10 years to figure out what an additive effect is” [1]. Unfortunately, we believe, the issue is still unresolved; and we like to clarify the issue and hoping to strengthen it.

(2) Even with some concept of additivity, “synergism” is still not a well-defined term. In one review article by Goldin and Mantel in [2], 7 definitions of “synergism” were given and in a more recent review by Greco *et al.* [3], 13 different methods for determining synergism were listed and not even two methods agree with each other and a few more might have been added since that time.

In 1984 [4], Chou and Talalay jointly introduced a concept called the “Combination Index” (CI) to quantitatively depict synergism ($CI < 1$), additive effect ($CI = 1$), and antagonism ($CI > 1$). Users of the index (CI) claim that the Chou-Talalay Combination Index is based on the median-effect principle which is derived from the “mass-action law” principle. Chou [1] declared that “synergism is basically a physiochemical mass-action issue, not a statistical issue; determine synergism with CI values, not with p-values”. The Chou-Talalay

Combination Index has become vastly popular. According to Chou [1] CI has been cited in 3970 scientific articles globally – based on a Thompson ISI Web of Science search; and, by early 2010, it has been used in more than 1900 scientific articles published in over 400 biomedical journals internationally.

We review the development and the process leading to the Combination Index (CI) by Chou and Talalay, then raises the important possibility that in many – maybe even the majority - of those more than 1900 published papers, synergism has been over-assessed – simply because of inappropriate use of an “additivity model”. But first to avoid further confusion on the concept and the issue, we propose to replace the term “additive” by “additive neutral” (or additively neutral) and to define synergism ($CI < 1$), neutral effect ($CI = 1$), and antagonism ($CI > 1$) accordingly. We will soon introduce another term, “multiplicative neutral”; together the two terms, additive neutral and multiplicative neutral, will complete the description of neutral effects in drug combinations.

The evaluation process for drug combinations consists of two steps: (1) to establish potencies of both agents, and (2) to characterize or prove possible existence of synergistic or antagonistic effects.

Determination of drug potencies

The main first step here is to conduct two “Dose-ranging Experiments” to determine, say, the ED50 for both agents. The Dose-ranging Experiment is like a Quantal Bioassay [5,6]. The differences are minor and subtle: (a) Quantal bio assays are often used *in vivo* whereas dose-ranging experiments could be *in vivo* or *in vitro* (tumor-derived cells); and (b) The endpoint/outcome in quantal bioassays is binary (often the death of the animal) whereas endpoint/outcome of a dose-ranging experiment could be binary or on a continuous scale - such as tumor volume as used in the following illustration.

Experiment design: The primary endpoint is “tumor volume”. Suppose you have a group of mice with induced tumors, say n ; n_0 mice are selected and sacrificed to measure baseline tumor volumes.

The other $(n-n_0)$ mice are randomized into k groups of mice treated with k different doses of a test compound; with doses are spread over a wide range from very low to very high.

Data Summaries: Let

“ d ” be one of the doses; $x = \log(d)$

v_0 = average tumor volume of control group (baseline data)

v_x = average tumor volume of group treated with dose “ d ”; and

$p_x = v_x/v_0$; $(1-p_x)$ is the percent tumor reduction in response to treatment with dose d ; these proportions are statistics to measure efficacies.

The model: We fit the following linear model (with drug’s dose on log scale):

$$\ln \frac{p_x}{1-p_x} = \alpha + \beta x + \varepsilon$$

This model is a mathematical version to describe the “Median Effect Principle” of Pharmacology which is expressed as follows. When a dose d of an agent is applied to a pharmacological system, the fractions f_a and f_u of the system affected and unaffected satisfy [7]:

$$\frac{f_a}{f_u} = \left\{ \frac{d}{ED_{50}} \right\}^m$$

Where ED_{50} is the “median effective dose” and “ m ” is a Hill-type coefficient; $m = 1$ for first-degree or Michaelis-Menten linear system (first order). If we set “ $p = f_a$ ”, the median effect principle and the logistic regression model

Calculation of the ED_{50} : After estimating intercept and slope, α and β by “ a ” and “ b ” respectively using, for example, the method of “Least Squares”, we can calculate the median effective dose (ED_{50}) by setting $p_x = 0.5$ to obtain the dose for 50% reduction:

$$ED_{50} = \exp(-a/b)$$

Other measures of efficacy, say ED_{25} or ED_{40} , if desired, can be calculated similarly. If one ED_{50} does not exist (slope $b = 0$), it’s the case of sensitization; the term sensitization (or enhancement) is used for a one-sided situation. For example, one drug (say A) is ineffective (so, its ED_{50} does not exist) but it helps to improve the effect of the other drug (say B) (e.g. lower the dose of B needed for 50% effects); agent A is a sensitizer – for example, some drug could be sensitizer for radiation.

Forming combination index

To establish sensitization, the one-sided situation, is simple: we carry a dose-ranging experiment with a combination of both agents to estimate its ED_{50} ; all we need to prove is showing that this ED_{50} of B used in combination with agent A is smaller than the ED_{50} of agent B when used alone. To establish synergism, the two-sided situation and the main focus of most studies on drug combinations, the process is as follows.

Experiment design: Experiments are usually done in one of two ways: (1) Non-constant drug ratio, and (2) Constant drug ratio. In designing experiments with a non-constant drug ratio, data in each series carry different levels of synergistic effects; they may not fit the model well. Even if data still fit the model, the resulting CI

value would have a larger standard error. In a review article [1], Chou recommended only experiments with constant-ratio drug combinations which can be summarized as follows. Agents are pre-mixed at a certain ratio, say p -to- q in large quantities (say, p units of Agent A to q units of Agent B) before dispensing into k combined doses from low to high in a Dose-ranging experiment; the ratio in all k doses is the same, p -to- q .

Calculation of the combination index: Chou and Talalay defined a “combination index” CI as follows: Using a combined dose, d_1 of agent A mixed with d_2 of agent B so as to achieve 50% response, then calculating

$$CI = \left[\frac{d_1}{ED_{50}} \right]$$

If $CI < 1$, then the combination can be described as synergistic, if $CI > 1$, antagonistic, and if $CI = 1$, then it is additive (our newly proposed term for “additive” is “additive neutral”; Chou and Talalay, *Advances in Enzyme Regulation*, [4]). After the ED_{50} of the combination is obtained, the needed amount of each drug and the CI is calculated as follows:

$$d_1 = \left(\frac{p}{p+q} \right) ED_{50_c}$$

$$d_2 = \left(\frac{q}{p+q} \right) ED_{50_c}$$

$$CI = \frac{d_1}{ED_{50_1}} + \frac{d_2}{ED_{50_2}}$$

$$= \left\{ \frac{p}{(p+q)ED_{50_1}} + \frac{q}{(p+q)ED_{50_2}} \right\} ED_{50_c}$$

The model: Chou and Talalay claimed that their Index has been proven by Mass Action Law, but in their paper they derived it from what they called the “Median Effect Principle for drug combinations”:

$$\left[\frac{f_a}{f_u} \right]_{A,B}^{\frac{1}{m}} = \left[\frac{f_a}{f_u} \right]_A^{\frac{1}{m}} + \left[\frac{f_a}{f_u} \right]_B^{\frac{1}{m}}$$

Unlike the popular Median Effect Principle for single drugs, this is an additive model; again, we will comment on this issue in following sub-section.

The issue of additivity

As we mentioned earlier, the “additive effect” of drug combination (the result without synergism or antagonism) is not a simple concept and we believe that the issue is still unresolved; we proposed to use the term “additive neutral”. According to Lee et al. [3,8], it narrows down to two models:

(1) The Loewe Additive Model: The combination effect is neutral (our new term for additive) if $E(d_1, d_2) = E(d_1) + E(d_2)$, where $E(d)$ is the effect of d ; and

(2) The Bliss Multiplicative Model: The combination effect is neutral (our new term for additive) if $E(d_1, d_2) = E(d_1)E(d_2)$, where $E(d)$ is the effect of d .

According to Lee et al., the literature indicates that the

Loewe additive model works in the setting of mutually exclusive drugs while the Bliss multiplicative model works in the setting of mutually nonexclusive drugs. However, the true mechanism of drug interactions often remains unknown and “there is no generally accepted agreement as to which of the two models is more appropriate”. Obviously, the Chou-Talalay Combination Index (CI) is based on the Loewe Additive Model.

We support the concept of two models, one is additive and the other is multiplicative. We maintain that, according to experts in bioassays [5,6] there are two main categories of agents:

(a) Those agents, say in category “M” (“M” for multiplicative), for which the response is linearly related to the log of the dose; these agents lead to parallel-line bioassays and which follow the Median Effect Principle; and

(b) Those agents, say in category “A” (“A” for additive), for which the response is linearly related to the dose itself; these agents lead to slope-ratio bioassays.

Perhaps there are more agents in category M but not all agents follow the Median Effect Principle. We can easily find many examples of agents in category A; for example, see section 3.13 of Hubert’s book [6].

When two agents in Category M are combined, the combination effect follows the Bliss Multiplicative Model, and when two agents in Category A are combined, the combination effect follows the Loewe Additive Model.

It is true that the underlying mechanism of drug interactions often remains unknown and there is no general theoretical agreement as to which of the two models is more appropriate. But given the data, it is possible to establish empirical evidence as to which category an agent belongs as we could tell if the drug response is linearly related to the dose or to the log of the dose. And if we could tell which category the drugs belong to we could tell whether the Loewe Additive Model or the Bliss Multiplicative Model applies.

Proposed combination indices

To get out of a possible contradiction in the use of the Chou-Talalay Combination Index (CI), we propose not one but two combination indices:

a) For agents in category M where the response is linearly related to the log of the dose, the combination effect follows the Bliss Multiplicative Model and we use the term “Multiplicative Synergism” with a Combination Index defined by:

$$CIM = \left(\frac{\log(d_1)}{\log(ED50_1)} \right) + \left(\frac{\log(d_2)}{\log(ED50_2)} \right)$$

b) For agents in category A where the response is linearly related to the dose itself, the combination effect follows the Loewe Additive Model and we use the term “Additive Synergism” with a Combination Index defined by:

$$CIA = \left[\frac{d_1}{ED50_1} \right] + \left[\frac{d_2}{ED50_2} \right]$$

Could a method as popular as the Chou-Talalay Combination Index

(CI) be misleading? And if so, why? We strongly believe that Chou and Talalay contradicted themselves [9].

(1) First, the Median Effect Principle is not a “law”, so that response is always related to log of the dose (our Category M). The Median Effect Principle only provides empirical evidence; perhaps more agents belong to category M so we see them more often but agents in category A do exist. Secondly, if the Median Effect Principle is a chemical “law” so that response is always related to log of the dose, then a “Combination Index” should be expressed as CIM – with all drug quantities on the log scale.

Why it should be concerned?

Let consider a simple example, $(ED50)_1 = 50$, $(ED50)_2 = 100$, and we used 3.23 units of agent A mixed with 25.12 units of agent B to obtain 50% response ($ED50_c = 3.23 + 25.12 = 28.35$),

$$CIM = \frac{\log(3.23)}{\log(50)} + \frac{\log(25.12)}{\log(100)} = 1.0$$

$$CIA = \frac{3.23}{50} + \frac{25.12}{100} = 0.316$$

It is obvious that the combination under investigation is multiplicatively neutral but additively synergistic. An appropriate conclusion would depend on the nature of the response: (a) if the response is linearly related to the dose, a conclusion of synergistic effects would be justified, but (a) if the response is linearly related to the log of the dose, i.e. the Median Effect Principle applies, then synergistic effects should not be implicated. In fact, most or all multiplicative neutral combinations can be proven as being additively synergistic. This suggests that many of the 1900 publications using the Chou-Talalay Combination Index (CI; our CIA) method over-estimated synergistic effects; many or most results could simply be false positives.

We recommend investigating the nature of the response before using the appropriate index presented here to determine if the combination is synergistic; if data do not fit a linear model with dose on the log scale, it does not necessarily mean data were not good to apply a combination index. On the other hand, if data fit a linear model with dose on the log scale, the use of the Chou-Talalay Combination Index (CI) would not be appropriate – regardless of the result.

The standard error of the combination index CI by Chou and Talalay (which our CIA) has been derived using various approaches such as simulations to create “pseudo” values/samples [8]. Our CIM is newly proposed but its analysis could be handled similarly. Therefore, we are now turning to the other area, experiment design and hypothesis testing, which have not been explored thoroughly.

Designing Experiments to Assess Synergism

We will consider both additive and multiplicative models. If, for both agents, the response is linearly related to the log of the dose, the combination effect follows the Loewe Additive Model. If, for both agents, the response is linearly related to the dose, the combination effect follows the Bliss Multiplicative Model.

In addition, since the evaluation process for drug combinations is

usually in the pre-clinical stage; for the purpose of illustration, we will put the designs in the context of both *in vitro* and *in vivo* experiments.

Experiment designs under the lowe additive model

The form of the pre-clinical experiments would depend on the nature of the treatment’s response or “tumor response” in the case of cancers [10,11]:

(1) If the effect of the treatment is expressed by its ability to kill cancer cells, the outcome variable is binary and experiments are *in vitro*;

(2) If the effect of the treatment is expressed by its ability to shrink the tumors, the outcome variable is continuous and experiments are *in vivo*.

For a typical evaluation of drug combinations, an *in vitro* experiment would be designed as follows. Cells from a tumor-derived cell line are deposited in wells of cell culture dishes in complete growth medium. After log phase growth is established, one well serves as vehicle controls (control well) and for the other experimental wells, each is treated with (θ) $(ED50)_A$ of Treatment A mixed with $(1-\theta)$ $(ED50)_B$ of Treatment B, $0 < \theta < 1$, maybe for several values of θ . If the combined effect is additive neutral, we would expect 50% effects; if more than 50% effects are observed, the combined effect is likely synergistic; if less than 50% effects are observed, the combined effect is likely antagonistic.

Data summary

The endpoint is “cell survival”; for cells in a well. Some die, some survive and we want to summarize data into “survival rate” or survival proportion for each well. However, even when we assume that all wells were deposited with same number of cells, the number of cells at the beginning of the experiment in each well, prior to drug exposure, may be large but unknown; that is why a control (i.e. vehicle) well is needed. Using the count from the control well, data are summarized as follows. Let

“x” be one of the doses

n_0 = the number of surviving/viable cells from the control well

n_x = the number of surviving/viable cells from the well treated with dose “x”;

$p_x = n_x/n_0$ the survival rate or proportion for the well treated with dose “x”.

In recent years, many laboratories stopped counting cells because it could be too time-consuming. Instead, some substrate is incubated in the plate wells - mixed with an indicator dye - which binds into the cells similar to capturing “proteins” in ELISA (see, for example, Cell Proliferation Assay by Promega; Promega Technical Bulletin No. 169, 2001). The extent of the color change read in a spectrophotometer results in an “optical density” (also called “absorbance”) which correlates linearly to the number of viable cells present in the well. After subtracting for the “background absorbance”, the ratio of numbers of cells – the proportion n_x/n_0 - is equal to the ratio of the absorbance’s or optical densities; in other words, we have no cell counts but we still have numerical values for those proportions.

Loewe additive model and design justification

We assume that the treatment response is linearly related to the

dose; both agents and treatments A and B belong to our category A; and when two agents in Category A are combined, the combination effect follows the Loewe Additive Model. When the drug combination is additively neutral, that is no synergism, the models under the Null Hypothesis is:

$$\ln \frac{P_A}{1 - P_A} = \alpha + \beta_A X_A$$

$$\ln \frac{P_B}{1 - P_B} = \alpha + \beta_B X_B$$

$$\ln \frac{P_{AB}}{1 - P_{AB}} = \alpha + \beta_A X_A + \beta_B X_B$$

The first two equations are for single drugs and the third for drug combination. This set of three equations defines “additive neutrality”. In this neutral state, there is no chemical interaction; adding one drug does not change the effect of the other. In statistical terms, this is a complete lack of statistical interaction plus a complete lack of multicollinearity; note the equality of the three intercepts and two pairs of slopes. Under this Null Hypothesis, expected response could be derived and the proposed test is justified as follows:

(1) From the first equation:

$$ED50_A = -\frac{\alpha}{\beta_A}$$

(2) From the second equation:

$$ED50_B = -\frac{\alpha}{\beta_B}$$

Instead of performing three separate experiments then testing to compare the two pairs of slopes (if any difference between the three intercepts observed, it could be just “noise” in the data), we propose the above simple experiment with only several wells. When a well is treated with a combination of $(\theta)(ED50)_A$ of Treatment A mixed with $(1-\theta)(ED50)_B$ of Treatment B, $0 < \theta < 1$, we would have under the Null Hypothesis:

$$\ln \frac{P_{AB}}{1 - P_{AB}} = \alpha + \beta_A [(\theta)(ED50_A)] + \beta_B [(1-\theta)(ED50_B)]$$

$$\ln \frac{P_{AB}}{1 - P_{AB}} = \alpha + \beta_A \left[\left(\theta \right) \left(-\frac{\alpha}{\beta_A} \right) \right] + \beta_B \left[\left(1 - \theta \right) \left(-\frac{\alpha}{\beta_B} \right) \right] = 0$$

In other words, we would have 50% effect (50% cell survival rate) if a well is treated with a combination of $(\theta)(ED50)_A$ of Treatment A mixed with $(1-\theta)(ED50)_B$ of Treatment B, $0 < \theta < 1$, and the combination effects are additive neutral. If the combination effect is synergistic, the drug combination is more lethal. To prove synergism is simply to prove that the resulting survival rate is less than 50%. One possibility is to calculate the weighted average of survival rates from all wells and its 95% confidence interval, and to show that its upper endpoint is below 50%.

For *in vivo* experiments, the method and its justification remain the same provided that one could design studies and summarize data into those “proportions” measuring treatment effects. An *in vivo* experiment would be designed as follows. Mice are randomized into subgroups; one untreated (to establish baseline tumor volume) and

for the other experimental group, each is treated with $(\theta)(ED50)_A$ of Treatment A mixed with $(1-\theta)(ED50)_B$ of Treatment B, $0 < \theta < 1$, maybe for several values of θ .

When the effect of the treatment is expressed by its ability to shrink the tumors; the endpoint is “tumor volume” – on the continuous scale. Since the tumor volumes of mice at the beginning of the experiment, prior to drug exposure, are unknown; a control (i.e. untreated) group is needed. Using the measurements from the control group, data are summarized as follows. Let

“d” be one of the doses; $x = \log(d)$

v_0 = average tumor volume of control group

v_x = average tumor volume treated with dose “d”;

$p_x = v_x/v_0$; $(1-p_x)$ is the (estimated) percent of tumor volume reduction for dose d;

If the combination effect is synergistic, the drug combination is more effective and the average remaining percent of tumor volumes is less than 50%. One possibility is to calculate the weighted average of the percents of tumor volumes from all groups, form the 95% confidence interval, and show that its upper endpoint is below 50%.

Experiment designs under the bliss multiplicative model

Again, the form of the experiments would depend on the nature of the treatment’s “tumor response”; If the effect of the treatment is expressed by its ability to kill cancer cells, the outcome variable is binary and experiments are *in vitro*, and if the effect of the treatment is expressed by its ability to shrink the tumors, the outcome variable is continuous and experiments are *in vivo*. To avoid duplication with parts of section 2.1, we only present here the design and the method justification for *in vitro* data.

Design: An *in vitro* experiment would be designed as follows. Cells from a tumor-derived cell line are deposited in wells of cell culture dishes in complete growth medium. After log phase growth is established, one well serves as vehicle controls and for the other wells, each is treated with $(ED50)_A^\theta$ of Treatment A mixed with $(ED50)_B^{1-\theta}$ of Treatment B, $0 < \theta < 1$, maybe for various values of θ . For example, one well is treated with $(ED50)_A^{\theta_1}$ mixed with $(ED50)_B^{1-\theta_1}$.

Bliss multiplicative model and design justification: Under this multiplicative model, we assume that the treatment response is linearly related to the log of the dose; both agents and treatments A and B belong to our category M [12] and when two agents in Category M are combined, the combination effect follows the Bliss Additive Model. When the drug combination is additively neutral, that is no synergism, the model under the Null Hypothesis is:

$$\ln \frac{P_A}{1-P_A} = \alpha + \beta_A X_A$$

$$\ln \frac{P_B}{1-P_B} = \alpha + \beta_B X_B$$

$$\ln \frac{P_{AB}}{1-P_{AB}} = \alpha + \beta_A X_A + \beta_B X_B$$

We have, with “x” being the log of the dose:

(1) From the first equation:

$$ED50_A = \exp\left(\frac{\alpha}{\beta_A}\right)$$

(2) From the second equation:

$$ED50_B = \exp\left(\frac{\alpha}{\beta_B}\right)$$

When a well is treated with a combination of $(ED50)_A^\theta$ mixed with $(ED50)_B^{1-\theta}$, $0 < \theta < 1$, we would have under the Null Hypothesis:

$$\ln \frac{P_{AB}}{1-P_{AB}} = \alpha + \beta_A \left[\log(ED50_A^\theta) \right] + \beta_B \left[\log(ED50_B^{1-\theta}) \right]$$

$$\ln \frac{P_{AB}}{1-P_{AB}} = \alpha + \beta_A \left[(\theta) \left(-\frac{\alpha}{\beta_A} \right) \right] + \beta_B \left[(1-\theta) \left(-\frac{\alpha}{\beta_B} \right) \right] = 0$$

In other words, we would have 50% effect if a well is treated with a combination of $(ED50)_A^\theta$ mixed with $(ED50)_B^{1-\theta}$, $0 < \theta < 1$, and the combination effects are multiplicative neutral. If the combination effect is synergistic, the drug combination is more lethal. To prove synergism is simply to prove that the resulting survival rate is less than 50%. One possibility is to calculate the weighted average of survival rates from all wells and its 95% confidence interval, and to show that its upper endpoint is below 50%.

Concluding Remarks

After forming the test we propose, a comparison of average response to its hypothesized value of 50% - say forming a ratio – could be even more informative than a combination index. We previously published [12] the design and the test but, like everybody at the time, we only considered Lowe additive model; its justification was very informal before realizing that the lack of synergism has more to do with lack of multi-collinearity. The evaluation of drug combinations could be very important because it has been argued that “in the absence of a clear definition (and assessment) for synergism, government agencies (would) have no basis to regulate the drug combination synergy claims”. Over this background, our main concern is that when the combination of two drugs is really multiplicative neutral, it could be over-assessed or claimed as synergistic because they show additive synergism – a wrong model. This over-assessment is particularly problematic if, just as the case of response, their toxicity is also linearly related to the log of the dose [13,14]. In that case, their combination could drive up toxicity to an unsafe level.

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