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Perspective

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Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method

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Abstract

This brief perspective article focuses on the most common errors and pitfalls, as well as the do's and don'ts in drug combination studies, in terms of experimental design, data acquisition, data interpretation, and computerized simulation. The Chou-Talalay method for drug combination is based on the median-effect equation, derived from the mass-action law principle, which is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. This general equation encompasses the Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations in biochemistry and biophysics. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. This theory also provides algorithms for automated computer simulation for synergism and/or antagonism at any effect and dose level, as shown in the CI plot and isobologram, respectively. *Cancer Res; 70(2); 440–6. ©2010 AACR.*

Introduction

Drug combination is most widely used in treating the most dreadful diseases, such as cancer and AIDS. The main aims are to achieve synergistic therapeutic effect, dose and toxicity reduction, and to minimize or delay the induction of drug resistance (1). Toxicity reduction and resistance minimization benefits could also be the outcomes of synergism. However, in one review article by Goldin and Mantel in 1957 (2), seven different definitions for synergism were given, and in a more recent review by Greco and colleagues in 1995 (3), 13 different methods for determining synergism were listed and none of them supported the others (1). The meaning of synergism has become an individual's preference. Faulty or unsubstantiated synergy claims are pervasive. This is serious because it is frequently referred to in patient therapy.

Without a standardized definition for synergism, it is argued that there will be a mess in making synergy claims, whether in publishing a scientific article, submitting a grant application, planning drug combination clinical trials for Food and Drug Administration approval, or asserting drug combination discovery to the Patent Office (4, 5). It is also argued that in the absence of a clear "definition for synergism", governmental agencies have no basis to regulate the drug combination synergy claims (4, 5).

I have devoted over four decades on this important fundamental issue. In all, more than 300 mechanism-specific equations have been derived and published (6–13). It took about 10 years to figure out what an additive effect is (1, 7, 12). This is important, because by definition, synergism is more than an additive effect and antagonism is less than an additive effect.

Along with Professor Paul Talalay of the Johns Hopkins University School of Medicine, in 1983 to 1984, we jointly introduced a scientific term "combination index" (CI) to quantitatively depict synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1; refs. 11, 12). Its applications were greatly facilitated by the help of Joseph Chou, who developed the first-generation computer software for dose-effect analysis based on the "mass-action law" (14). Due to fast changes in computer hardware and software, the second-generation "CalcuSyn" was written by Mike Hayball of Cambridge, United Kingdom in 1997 (15), and the third-generation "CompuSyn" was then written by Nick Martin of MIT, Cambridge, MA in 2005 (16).

The route from the unified theory to algorithms, to quantitative practical applications is shown in Fig. 1. The essence behind the mass-action law–based theory is that the "median" is the unified common link of single entity and multiple entities, and for the first-order and higher-order dynamics (1). The "median" in the median-effect equation also serves as the universal reference point, which evolves into the global positioning system (GPS) concept for bioinformatics. The features of this methodology are its simplicity and flexibility (e.g., mechanism- and unit-independent), its quantitative definition (e.g., numerically indexed conclusion), and its efficiency and economy (e.g., allows for a small number of data points of measurements and uses a small number of animals). More details have been described in refs. 1 and 17.

The Common Pitfalls and Errors in Drug Combination Studies

To avoid pitfalls in drug combination studies, the following fundamental, scientific concept, and practical issues need

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to be addressed. Below are my scientific views (1, 4, 5). Debates are welcome because there is an urgent need for synergy definition that requires a global consensus and standardization.

1. ***The nature of the problem.*** Synergism is basically a physicochemical mass-action law issue, not a statistical issue. Determine synergism with CI values, not with *P* values.
2. ***The P value?*** A combined effect greater than each drug alone does not necessarily indicate synergism. Sometimes this can be a result of additive effect or even a slight antagonism. A + B > A or A + B > B is a simple axiom which does not require elaborate proof, such as *P* values. Thus, if the combined effect is greater than each drug alone, it does not necessarily indicate synergism.
3. ***The arithmetic sum.*** The additive effect of two drugs is not the simple "arithmetic sum" of effects of two drugs. If $(D)_1$ and $(D)_2$ inhibits 30% and 40%, respectively, the additive effect is not 70%, because if they inhibit 60% and 70%, respectively, the additive effect cannot be 130%!
4. ***The fractional product concept.*** If $(D)_1$ and $(D)_2$ each inhibit 50%, then the combined additive effect is 75%. Is this correct? Answer: it is most likely wrong, because it is valid only when the dose-effect curves for both drugs are hyperbolic [i.e., follows the Michaelis-Menten (first order) kinetics/dynamics with $m = 1$; ref. (18)] and only if the effects of both drugs are mutually nonexclusive (1, 10). In simple enzyme or receptor systems, where m is frequently ≈ 1, we can calculate additive effect by $(1 - 0.5) \times (1 - 0.5) = 0.25$, and $(1 - 0.25) = 0.75$ as indicated by Webb (19). However, Chou and Talalay indicate that in cellular or animal systems, the dose-effect curves are most likely sigmoidal ($m > 1$) or flat sigmoidal ($m < 1$; refs. 1, 12, 13). When we talk about the dose-effect curve, we need to take into account both the potency (the D_m or IC_{50} value) and the shape of the dose-effect curve (the m value) simultaneously, not just pay attention to potency.
5. **In vitro *versus in animals.*** Does the determination of synergy *in vitro* and in animals follow the same principle? The answer is yes. The main practical differences in animal drug combinations are (*a*) it is more expensive, (*b*) it is more time-consuming, (*c*) there is more variability, and (*d*) the smaller population size (i.e., smaller n). For anticancer drug combination studies against xenograft tumors in nude mice under optimal therapeutic conditions, only 65 nude mice were used in Chou's laboratory to determine synergy with F_a-CI plot and isobologram (5, 20, 21). By contrast, using the empirical response surface method (3), one needs to use ~800 animals, and yet, the synergy conclusion may still be vague and not quantitative. The determined synergism or antagonism is obtained from the given experimental conditions. The extrapolation of results from *in vitro* to animals, or from animals to humans, is a general and separate biomedical problem which is not expected to be solved by the Chou-Talalay method.
6. ***Determining synergy in clinics?*** Is it possible to "determine" synergism in clinical trials or in clinics? The answer is generally no for the disease per se (e.g., cancer or AIDS). This is based on scientific, practical, and ethical reasons, as indicated in ref. 1 (pp. 641–642) and in ref. 5 (Table 1). Most clinical synergy claims thus far, to my knowledge, are not supported by the available data, especially when only a single dose for a single drug was used. This is why, prior to drug combination clinical trials, preclinical drug combination studies *in vitro* and/or in animals should be carried out to obtain the basis and rationale for studies in humans. To obtain the therapeutic benefits of drug combination in humans, the basis and rationale involving explorative details should be obtained from preclinical studies and should not be done entirely on human subjects. This is the principle that the Food and Drug Administration needs to enforce. When two drugs are combined, they can behave like a third drug with a lot of uncertainties. It should be noted that by using a surrogate marker and the repeated fractional-dose schedule of multiple doses, it is possible to determine synergy in a small-scale clinical trial. An elegant example is using P24 antigen or CD_4^+ as a marker, synergy with CI value can be obtained in AIDS clinical trials for AZT and IFNα using only 36 patients (22). In another AIDS clinical trial for AZT and 3TC (23), 366 patients were used, also using surrogate markers such as CD_4^+ and HIV-RNA; however, this study could not determine synergy because only a single dose of AZT was used. It only concluded that combined therapy was better than monotherapy alone, which is equivalent to A + B > A or A + B > B, which is not the definition for synergy.
7. ***Prediction by mechanisms?*** Is a knowledge of mechanisms required for determining synergism? Answer: no, because the mass-action law–based determination of synergism is mechanism-independent. Information about the mechanism of action is good to have for knowledge and for guesswork. In reality, there are many well-known drugs whose various mechanisms we know very little about, for example, aspirin which has been widely used for over a century. In most cases, we cannot predict synergy from mechanisms, e.g., taxol (microtubule stabilization) + cisplatin (DNA alkylation) + topotecan (DNA topoisomerase I inhibition; ref. 1; Table 10 and ref. 24, Fig. 1). Even if it is partially predictable, it is still not quantitative, e.g., taxol + MDR-reversing agent, such as ningalin (25) or anti-HIV agents, ribavirin + zidovudine (26). In addition, some drugs have several modes of actions (e.g., upregulate or downregulate a lot of gene expressions) and it is difficult to determine which mode of action contributed to the synergy and to what extent. Furthermore, there is an issue of efficiency of research that can be raised because the quantitative determination of synergism of two drugs *in vitro* usually takes 1 to 2 weeks, but to figure out how and why synergy occurs may take several years and the conclusion could

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still likely be a "might be", "maybe", "suggest", "imply", etc. (1, 5). Synergy or antagonism needs to be determined, not to be predicted. In this case, to determine is easy but to predict is difficult. If synergy is predictable, then there would be no need to conduct drug combination studies. Sometimes, the prediction might be correct by luck but it will not be quantitative. Frequently, predictions were done after the observed facts retrospectively, as can be seen in the biomedical literature. There has been no rigorous theory or method to predict synergy, except that "polygonogram", introduced by Chou and Chou (1, 27), can frequently project synergism or antagonism semiquantitatively (see Fig. 9 in ref. 1). This powerful and efficient projection, however, still needs a certain amount of experimental work (e.g., project the outcomes of three-drug or four-drug combinations based on two-drug combinations for the semiquantitative projection of outcomes).

8. ***Synergism versus enhancement***. Does synergism and enhancement have the same meaning? Answer: no. Synergism (or antagonism) is "mutual" whereas enhancement, potentiation, or augmentation is "one-sided" (1, 5). Synergism or antagonism needs to be determined with CI values, whereas for enhancement, potentiation, or augmentation, we simply just need to state *x*% potentiation or *y*-fold enhancement, etc. When a drug but itself has no effect, there will be no D_m and m values for calculating CI.
9. ***Efficiency and economy***. Based on Chou's theory, is it possible to draw a specific curve with only two data points? Answer: yes, if they are accurately determined (1, 5, 17). This statement defies the widely held belief that from two data points, we can only draw a straight line. Here, the two points is actually four points. The third point is the origin at dose zero, and the fourth point is the median, which is the common link and universal reference point for the first-order and higher order kinetics/dynamics, as well as for the single entity and multiple entities (1, 5, 17). When the mass-action law parameters (D_m for potency and m for shape) are determined by the median-effect plot (Chou plot), the entire dose-effect curve is automatically determined (e.g., using CompuSyn software simulation; ref. 16). This is the basis for the GPS concept for bioinformatics proposed by Chou. This concept should have far-reaching consequences in biomedical research, in terms of efficiency and economy, and in the conservation of human workforce, materials, and animal resources. This is exemplified in the CI method for drug combination studies, discussed in this brief perspective article. More details are given in refs. (1, 5, 17).

Frequently Asked Questions for the CI Method and "Do's and Don'ts"

Given below are frequently asked questions for the Chou-Talalay CI method, in terms of experimental design, data entry, data analysis with CompuSyn software, and other practical questions. I will try to give answers clearly, but briefly. For more details, please refer to refs. 1 and 5. For simplicity, the following discussions refer to two-drug combinations *in vitro*. The questions or issues are itemized below for easy references, in a random order.

1. ***Why do you recommend the constant-ratio drug combinations?*** The cells do not know it is a single drug or drug combination, nor its combination ratio, nor their mechanisms, etc. We just enter the "dose" and "effect" for each drug and combination's numerical information to the computer. When we make a mixture and dilute it serially (usually 2-fold serial dilution with several concentration points above and below its IC_{50} value), it will always stay at a constant ratio [at the $(IC_{50})_1/(IC_{50})_2$ ratio or other selected ratio; refs. (1, 5), if you have reason(s) to do so, such as solubility limit or intend to de-emphasize one drug over others to avoid bad toxicity or side effects; refs. (1, 5, 12, 28)]. This mixture, in fact, behaves like a third drug to the cells. In this way, we can obtain the parameters [$(D_m)_{1,2}$, $(m)_{1,2}$ and $(r)_{1,2}$ for the mixture, just like the single drugs $(D_m)_1$, $(m)_1$, $(r)_1$ and $(D_m)_2$, $(m)_2$, $(r)_2$] by using the automated median-effect plot (the Chou plot) with a computer software. The r value is the linear correlation coefficient of the median-effect plot, which signifies the conformity of the data to the mass-action law. For *in vitro* experiments, it usually yields $r > 0.97$. The advantage is that they allow the automated computer simulation for the F_a-CI plot (CI plot or Chou-Talalay plot), F_a-DRI plot (dose-reduction index plot or Chou-Martin plot), and the classic isobologram (and if there are more than two drugs, the polygonogram or Chou-Chou graphics), in addition to the dose-effect curves and the median-effect plot. If the experiment is carried out in non-constant ratio combinations (e.g., 1:1, 1:2, 1:5, and 1:10), the CI values can still be determined at their corresponding specified data points,

Figure 1. The route from theory to algorithms, to practical applications for drug combination studies. *A,* the median-effect equation as the unified theory. *B,* the merging of the median-effect equation with the CI equation leads to the quantitative definition for synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1), and provides the algorithms for their computer simulation. Rearrangement of the equation also provides algorithms for simulation of dose-reduction index (*DRI*) for each drug in their combination. The favorable DRI (>1) allows dose-reduction that leads to toxicity reduction in the therapeutic applications. *C,* the depiction of typical quantitative diagnostic graphics generated by the computer simulation. *a,* the F_a-CI plot (Chou-Talalay plot). *b,* the classic isobologram (for the constant ratio combination design). *c,* the normalized isobologram (Chou-Chou plot) for the non-constant ratio combination design. *d,* the F_a-DRI plot (Chou-Martin plot) for the constant ratio combination design. When all single drug parameters (m and D_m values) are available, the CI values can be calculated for the non-constant ratio combinations. But no computer simulation for F_a-CI plot or the F_a-DRI plot is possible due to changing ratios (*A* is reproduced from ref. 13 with permission from Elsevier. *B* and *C* are reproduced from ref. 1 with permission from the American Society of Pharmacology and Experimental Therapeutics).

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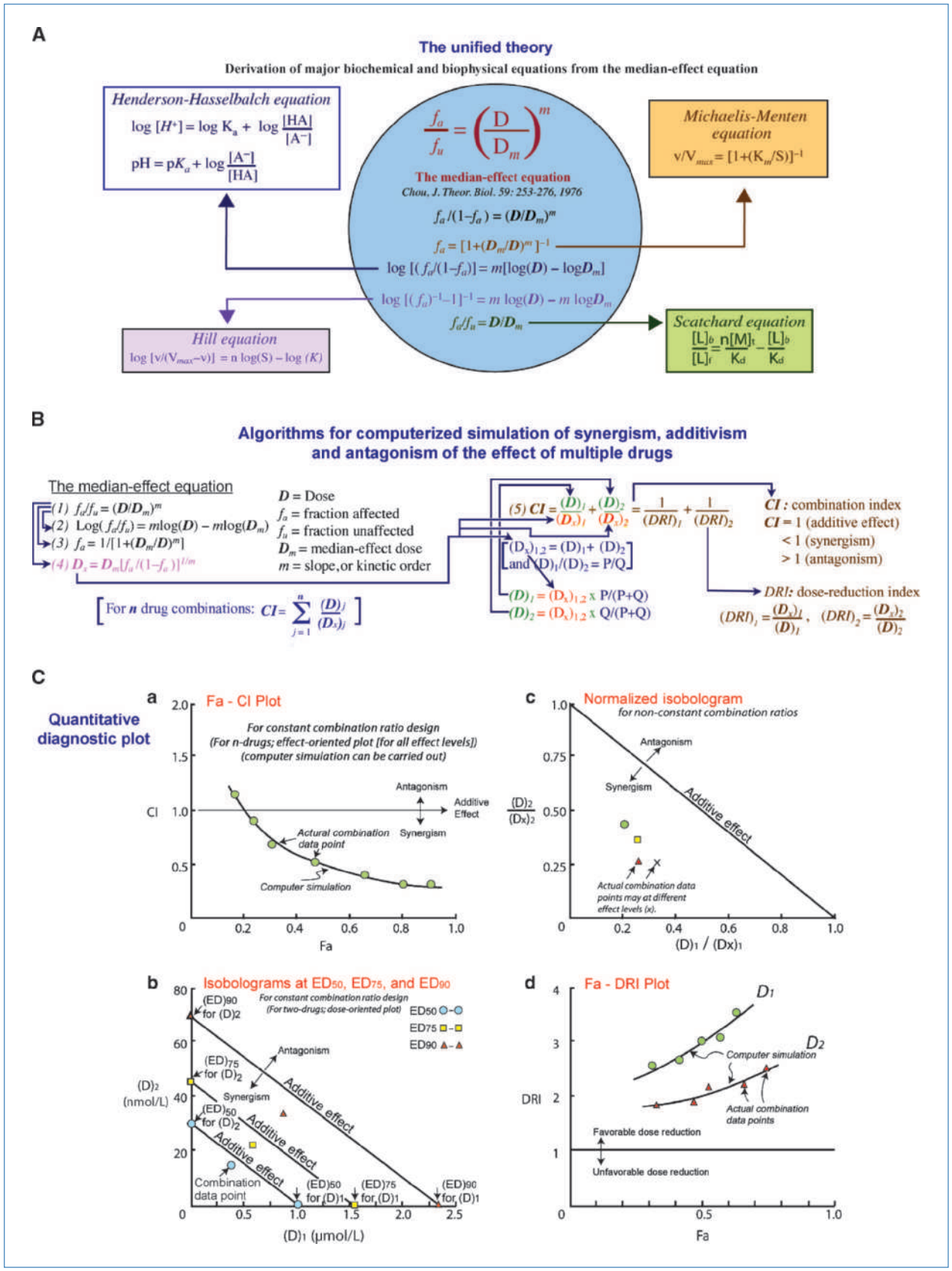

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if the $(m)_1$, $(D_m)_1$ and $(m)_2$, $(D_m)_2$ for single drugs are available, but no "simulation" for the CI plot. In nonconstant ratio design, the DRI plot can be done for specific data points but with no simulations, and the computer-generated classic isobologram (usually from the constant-ratio combinations) will be replaced with the computer-generated "normalized isobologram" (or the Chou-Chou conservative isobologram; refs. 1, 26). Typical representations of the classic and conservative isobolograms are shown in Fig. 1*C* (and in Fig. 9 of ref. 1 or Fig. 5 of ref. 5). If one prefers to have more information of more than one combination ratio, and if the experimental size and cost are of less concern (especially when facing animal studies), it is recommended to use a checkerboard or Latin square design, in which several constant-combination ratios are provided, as shown in Tables 5, 8, and 9 of ref. 1).

2. ***What is the prerequisite for all drug combination studies?*** Answer: the prerequisite is the dose-effect curves for each drug alone. Each drug not only has a different potency (the D_m value) but also a different shape of the dose-effect curve (the m value). For any determination of synergy, we need to know both the potency and the shape of the dose-effect curve of each drug (1, 5, 12). The D_m and m values can easily (and automatically) be obtained from the median-effect equation using computer software (e.g., CompuSyn) or by using a pocket calculator. For examples of manual calculations, see Table 10 in ref. 1. With these parameters [$(m)_1$, $(D_m)_1$ and $(m)_2$, $(D_m)_2$] available, we could determine whether there is synergism or antagonism, quantitatively, by using the CI equation, even for only a single combination data point (1). However, if multiple data points for constant-ratio combination are available [i.e., $(D_m)_{1,2}$ and $(m)_{1,2}$], then the entire spectrum of synergism or antagonism at all effect levels can be automatically simulated. Thus, the hypothetical minimum for a drug combination study is five data points, in which two are for $(D)_1$, two are for $(D)_2$, and one for the combination. For reasons of biological and technical variability, of course, we do not advocate the use of minimum data points. However, the median-effect principle (MEP) provides the legitimacy of using a small number of data points if variability is in a small magnitude (1, 5, 17).
3. ***Dose range and dose density questions.*** It is ideal to have several data points above IC_{50} and several below IC_{50} because this would make the assay more accurate. Because the unified theory of the median-effect equation and plot is general and versatile, it can easily handle screwed data points in which it's all above IC_{50} or all below IC_{50}, if the assays are accurate, and with a good r value (e.g., $r > 0.95$; refs. 1, 14–16). Although the dose range is very flexible for analytic purposes, it is important to realize whether the concentration range used *in vitro* is achievable *in vivo*, and whether these concentrations are within the tolerable toxicity *in vivo*. As a common rule, do not delete the data points in the middle of the dose-effect curve, unless there are specific reasons (e.g., inadvertent error or accident). However, at extremely high concentrations or at extremely low concentrations, where accurate assays are not possible (i.e., beyond the sensitivity of detection), the unreliable data points should be deleted (1). Otherwise, the computer software (based on the median-effect equation and plot) takes into account every data point equally significantly. Never enter 0% or 100% inhibition ($f_a = 0$ or $f_a = 1$) into the computer because log 0 = negative infinity (at infinitely low concentration) and log 1 = 0 (at infinitely high concentration) will lead the computer to crash (14–16).
4. ***What does extraordinarily high CI value mean?*** Sometimes the CI values are >3 or much greater, especially at low effect levels (i.e., low f_a level). Don't be surprised! Keep in mind that the synergy scale is from 1 to 0 and the antagonism scale is from 1 to infinity. Frequently, at high dose or high effect levels, the synergistic interaction is stronger than otherwise. For anticancer or antiviral agents, synergy at high effect levels (e.g., at $f_a > 0.8$) is more relevant to therapy than at low effect levels (e.g., at $f_a < 0.2$). A semiquantitative expression of CI ranges for synergism (or antagonism) in symbols, colors, and grades (slight, moderate, substantial, strong, and very strong) are recommended in Table 4 of ref. 1.
5. ***What is the scope of applications of the MEP and the CI method?*** The MEP of Chou (i.e., the median-effect equation and the median-effect plot; refs. 7 and 8) and the extended CI theorem of Chou-Talalay (i.e., the CI equation and plot; ref. 12) are derived from Nature's fundamental mass-action law in biophysics and biochemistry. The theory of MEP (for single entity) and of CI (for multiple entities) should be generally applicable in all dose-effect relationships that follow the mass-action law (i.e., with good r values; refs. 1, 17). The derivation of these equations is accomplished by using the mathematical induction/deduction from more than 300 mechanism-specific equations. As shown in refs. (7–10), the general equations are valid with different reaction mechanisms, different types of mechanisms of inhibition, and with different numbers of reactants.The broad application is attested by the fact that MEP and CI have been cited in more than 3,970 scientific articles globally, based on the Thompson ISI Web of Science search. It is of interest to note that one review article on drug combination by Chou and Talalay (12), which was published in a journal with an impact factor of 1.83, has now been cited in more than 1,915 scientific articles internationally from over 407 different biomedical journals. The CI method helps answer the following primary questions (1, 5): (*a*) Are there any synergisms? (*b*) How much synergism? (*c*) Synergism at what dose levels? (*d*) Synergism at what effect levels? (*e*) What did the exhibited CI plot, isobologram, and polygonogram look like? (*f*) How many folds of dose-reduction (for toxicity reduction) for each drug as a result of synergism? All of the above tasks could be accomplished with computerized simulation

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for experiments, which normally takes ~1 to 2 weeks for the *in vitro* experiment to complete. For a slight alteration in experimental design, one can also answer these other questions: (*g*) the optimal combination ratio for maximal synergy, (*h*) the schedule dependency of synergy, (*i*) the selectivity of synergy against the target versus the host, and (*j*) the condition-directed synergism, such as the influence of pH, temperature, radiation, and oxygen tension, etc., on the synergistic outcome. The major advantages of this theory are its sound theoretical basis, general validity for broad application, and the algorithm for computer simulation. The overwhelming significance of the theory is its features of simplicity, efficiency, and economy, which have influenced the face of biomedical research in a measurable way. The validity of the MEP is, without a doubt, because it is the unified form of Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations, which are the main theories of biochemistry and biophysics (Fig. 1*A*). As indicated in refs. 1 and 17, the CI concept and its application is just an extension of the MEP. There are many other utilities on the horizon that can be applied to the median-effect equation and its principles (1), which include (*a*) conducting small-sized experimentations; (*b*) conservation of use of laboratory animals; (*c*) low-dose risk assessment of carcinogens, radiation, or toxic substances; (*d*) calculation of K_i from IC_{50}; (*e*) agricultural control of pests; (*f*) topologic analysis of exclusivity and competitiveness; (*g*) calculation of therapeutic index and safety margin; and (*h*) epidemiologic applications, such as age-specific disease incidences and their projections. These are just to mention a few. The overall significance of the median-mediated unity theory is its economic enabling of increasing efficiency for biomedical research, and the conservation of human workforce, materials, and animal resources. It also benefits the humanistic side on improving health and well-being by defining and determining synergism to avoid faulty synergism claims and confusions (1), and quickening the new drug discovery process by using the efficient quantitative approach for drug evaluation and the GPS concept for bioinformatics (17).

Conclusions and Future Directions

The CI is the natural law–based general expression of pharmacologic drug interactions. It is shown to be the simplest possible way for quantifying synergism or antagonism. Its simplicity in equations, experimental designs, and data analysis features efficiency, economy, and reducing the experimental size of animals used or the number of patients needed for drug combination clinical trials. The general theory of the MEP of the mass-action law, its CI algorithm, and its computerized simulation have paved the way for future drug combination studies, as indicated by the broad acceptance in scientific applications and by the rapid increase in citation numbers.

Disclosure of Potential Conflicts of Interest

T-C. Chou is a principal in and holds the copyright to CompuSyn, the software used for the analysis performed in this article. His son, who contributes to the software's development, is in a position to receive royalties for its publication. T-C. Chou has also received honoraria from seminars and lectures, and consultation fees from universities and pharmaceutical and biotech companies. The author has not received any grant supports for his four decades of theoretical work. The software development was financed by personal funds.

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