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TRANSLATIONAL PERSPECTIVE OF MATHEMATICAL MODELS USED IN THE ASSESSMENT OF CELLULAR SYNERGY IN CYTOTOXICITY ASSAYS

Layka ABBASİ ASBAGH[1], Aslı DENİZ[2], Selim UZUNOĞLU[1]*, Rüçhan USLU[3]

[1]Celal Bayar University, Faculty of Science and Arts, Department of Biology, Manisa, TÜRKİYE
[2]Izmir Institute of Technology, Faculty of Science, Department of Mathematics, Izmir, TÜRKİYE
[3]Ege University, School of Medicine, Department of Medical Oncology, Izmir, TÜRKİYE

Abstract: Drug combinations are currently a choice of cancer treatment. For the accurate assessment of cellular synergy, there is much need for a communication bridge between specialized researchers and physicians. Our objective is to enlighten the critical aspects of cellular synergism and the experimental design. Chou's median-effect equation and combination index are the most popular methods in this area. Since the effect of drug combination on cancer cells is determined by their own potency and concentration, the potency of drug combination (variable ratio design) and dose effect (constant ratio) should be taken into account during the experimental design. The experiment should be designed according to validity of chosen mathematical model.

Key words: *cancer, drug combination, experimental design, median effect equation, combination index*

HÜCRESEL SİNERJİNİN BELİRLENMESİNDE KULLANILAN MATEMATİKSEL MODELLERİN SİTOTOKSİSİTE DENEYLERİNE YANSITILMASI

Özet: Günümüzde ilaç kombinasyonları kanser tedavisinde önemli bir seçenektir. İlaç kombinasyonuna dayalı hücresel sinerjisinin doğru belirlenmesi için uzman araştırmacılarla klinisyenler arasında köprü kuracak yansıtıcı çalışmalara ihtiyaç duyulmaktadır. Bu derlemenin amacı hücresel sinerjinin kritik yönlerini ve tercih edilen matematiksel modele dayalı olarak kurgulanması gereken deney tasarımlarını anlaşılır kılmaktır. Chou' nun medyan etki denklemi ve kombinasyon indeksi bu alanda kullanılan en yaygın modeldir. Bir ilacın kanser hücrelerine etkisi onun potansiyeli ve konsantrasyonu ile belirlendiğinden hücresel sinerjinin belirlenmesinde ilaç kombinasyonlarının potansiyeli (değişen oran) ve dozu (sabit oran) deney kurgulanırken dikkate alınmalıdır. Deney seçilen matematiksel modelin geçerlilik şartlarına uygun şekilde tasarlanmalıdır.

Anahtar Kelimeler: *kanser, ilaç kombinasyonu, deney tasarımı, medyan etki denklemi, kombinasyon indeksi*

***Sorumlu Yazar**

selim@bayar.edu.tr

1. INTRODUCTION

Drug combinations are currently a choice of cancer treatment since one of the biggest problem is multiple drug resistance phenomena by which cancer cells become resistant to various anticancer agents [1]. There are numerous different types of anticancer agents in clinical use and various combinations of anticancer agents are investigated in order to find out the best drug synergism.

The investigation of combination chemotherapy in clinical trials is very expensive and time-consuming. Therefore, the search for drug combinations in preclinical studies is widely recognized as being of the utmost importance in the field of oncology [2]. At present there are many approaches for designing the drug combination experiments and data analysis for the assessment of synergy. Unfortunately, there is still no consensus on the definition of the concepts and mathematical models in this area [3,4]. Moreover, there is no clear and easily applicable experimental guidance for drug combination studies in cellular level since both the design of the experiment as well as the analysis of the generated data need to be fast, easy and robust.

The preferred mathematical model defines how to design experiments and data collection. For this reason there is much need for translational research which provides a bridge of communication between highly specialized research scientists and physicians or researchers since they are speaking very different languages. Simplifying the specialized knowledge from related articles will give us a better chance to understand how to design experiments and to analyze the dose-response data for cellular synergy assessment, since standardization and validation of preclinical assays accurately is very important to put the experimental data into clinical use.

1.1 What is synergy?

Every research group has its own definition of synergism and they use different mathematical models to analyze data [5]. There is still ongoing conflict in defining the synergy concept [4]. Moreover, many research groups prefer using different terminologies for the same phenomena. In a practical manner, inhibition concentration (IC), effective concentration (EC), effective dose (ED) has the same meanings.

One of the main reasons is that the biological system has hierarchical organizational level which requires different definitions of synergy [6]. To understand synergy phenomena, a unified definition of synergy is urgently needed for each level.

Synergistic effect is defined as the effect of the drugs in combination is multiplied when compared with the effect of each drug alone in a given concentrations. Additive effect is equal to sum of individual drug effects when they are used in combination. Antagonistic effect is defined as the individual effects of drugs found in combination is suppressed or decreased by others [7].

1.2 What is Cellular Synergy?

The synergism can be defined in three main levels; pharmacological (biomolecular), cellular and therapeutic (clinical) synergy. Among these, the cellular synergy describes the data obtained in cell culture experiments by using several mathematical models such as isobologram analysis, combination index method derived from median-effect principle [8].
Our objective is to enlighten the critical aspects of cellular synergism and the experimental design based on given mathematical models in a translational perspective.

2. A SUMMARY OF MATHEMATICAL MODELS USED IN DRUG SYNERGY ASSESSMENT

It is important to discuss dose-effect relationships for single drug in order to understand the philosophy of drug interaction. Power Law, Probit, Logit and Median-Effect Equation of Mass-Action Law are some of the equations for understanding dose-effect relationship [3]. Chou's median-effect equation and combination index are the most popular ones and calculated by two softwares CalcuSyn (Biosoft Inc.,1997)and CompuSyn (Combosyn, 2005).General dose-response function $f(C)$ for a single drug related to the concentration C can be generalized to;

$$f_a(C) = \frac{E_{\max}\left(\frac{C}{IC_{50}}\right)^m}{1+\left(\frac{C}{IC_{50}}\right)^m} + h \qquad (1)$$

It is remarkable that $\lim_{C\to 0} f_a(C) = h$ and $\lim_{C\to\infty} f_a(C) = E_{\max}$. Here, $E_{\max}$ is the predicted response of an infinite drug dose, in which toxicity is ignored and can be taken equal to 1. h is the predicted response at zero concentration and can be taken equal to 0. Concentration value IC_{50} (Inhibitory Concentration) is the parameter for median effective dose which yields the response $\frac{E_{\max}+h}{2}$, with our assumption $\frac{1}{2}$. m is Hill coefficient signifying the shape of the dose-effect relationship as $m=1$, $m>1$, $m<1$ indicate hyperbolic, sigmoidal and flat sigmoidal dose effect curves, respectively [3, 4]. Then the equation of fraction affected by dose C turns into the equation as follows:

$$f_a(C) = \frac{\left(\frac{C}{IC_{50}}\right)^m}{1+\left(\frac{C}{IC_{50}}\right)^m} \qquad (2)$$

Considering $f_u = 1 - f_a$, where $f_u(C)$ denotes the unaffected fraction by C, we get the following equality;

$$f_u(C) = \frac{1}{1+\left(\frac{C}{IC_{50}}\right)^m} \qquad (3)$$

which is so called Hill equation. Thus, we can conclude that fraction affected by concentration increases from 0 to 1, conversely, fraction unaffected by dose decreases from 1 to 0, taking into account that relationship between dose and effect is not linear generally, which changes depending on the value of m.

Dividing $f_a(C)$ by $f_u(C)$,

$$\frac{f_a(C)}{f_u(C)} = \left(\frac{C}{IC_{50}}\right)^m \qquad (4)$$

which is equal to Chou-Talalay's median-effect equation with the order m. It is important that, the median-effect equation describes dose-effect relationship in very simple form.

Taking the logarithm of both sides of the equation **(4)**, we obtain the median-effect plot as

$$\log\left(\frac{f_a(C)}{f_u(C)}\right) = m\log C - m\log IC_{50} \qquad (5)$$

which yields a straight line and independent from the value of m. The median-effect equation is called the general theory of dose and effect [3].

Therefore, fractional effect analysis is known as one of the most straightforward methods for assessing synergy where the effect of the drugs alone is multiplied and comparable with that of the combination at the same drug concentration [9].

This method can be formulized generally as;

$$Af_a + Bf_b = (A+B)f_{ab} \qquad \textbf{(additive)}$$

$$Af_a + Bf_b < (A+B)f_{ab} \qquad \textbf{(synergistic)}$$

$$Af_a + Bf_b > (A+B)f_{ab} \qquad \textbf{(antagonistic)}$$

where A is the concentration of drug a and f_a is the effect of drug a at a unit concentration, B is the concentration of drug b and f_b is the effect of drug b at the same concentration and f_{ab} is the effect of the two drug combination at the same concentration. The above equations have significant limitations assuming a linear concentration-effect relationship for each agent and the combination [9].

The concentrations of two drugs used in a combination experiment are independent variables and effect is the dependent variable so we can represent the dose-effect relationship in three dimension, where the concentrations are plotted as Cartesian coordinates in the first quadrant of the xy-plane and the effect is plotted as the distance in the nonnegative part of the z plane [10]. Dose-response relationship indicates a surface for two drug combination, and it is known as Response-Surface Model [11].

An isobol is a level curve on the surface of effect among two drugs, that is, the collection of all points on the surface which lie at a particular effect. Specifically, the isobol formulation is used to denote the additivity of multiple drugs in order to compare with any other concentration pairs and interprets any deviation as evidence of synergy or antagonism [12]. It can be shown that the isobologram is valid only for drugs whose effects are mutually exclusive (Figure 1), whereas the fractional product method is valid only for mutually nonexclusive drugs which have hyperbolic dose-effect curves (Figure 2) [13].

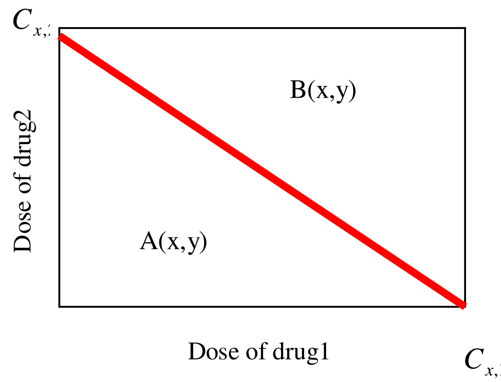

Figure 1. An example of Isobologram. $C_{x,1}$ is IC_{50} of drug1 and $C_{x,2}$ is IC_{50} of drug2 The line segment connecting two points $(C_{x,1},0)$ and $(0,C_{x,2})$ indicates additivity.

The straight line in Figure 1 connecting the points $(C_{x,1},0)$ and $(0,C_{x,2})$ has the equation:

$$\frac{x}{C_{x,1}} + \frac{y}{C_{x,2}} = 1 \qquad (6)$$

Here, (x, y) denotes the pair for concentration of the drug combinations. Moreover, $x \geq 0$ and $y \geq 0$. Thus, the line segment between the points $(C_{x,1},0)$ and $(0,C_{x,2})$ indicates the collection of concentration pairs which have no interaction, i.e., additivity. $A(x, y)$ is the dose pair which elicits the same effect with less dose in total, therefore synergetic, whereas $B(x, y)$ is the dose pair which elicit the same effect with more dose so, $B(x, y)$ is antagonistic.

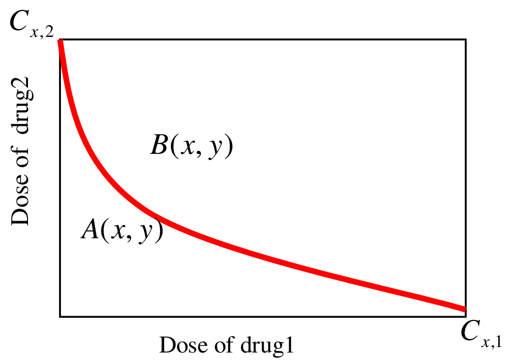

Figure 2. Isobologram for drugs with mutually nonexclusive mechanism of action.

For drugs with mutually nonexclusive mechanism of action (Figure 2), isobol turns into a curve with the following equation:

$$\frac{x}{C_{x,1}} + \frac{y}{C_{x,2}} + \frac{xy}{C_{x,1}C_{x,2}} = 1 \qquad (7)$$

or equivalently

$$y = \frac{2C_{x,1}C_{x,2}}{C_{x,1} + x} - C_{x,2} \qquad (8)$$

The red curve segment indicates the collection of points which yield additivity effect. Moreover, the point $A(x, y)$ yields synergy whereas $B(x, y)$ antagonism.

Median effect analysis overcomes the sigmodicity problem found with isobologrаms [13]. Combination-index (CI), is a model which serves as a quantitative measure of synergism. There are two formulas for combination index based on drug's mechanism of action. The first one is for mutually exclusive mechanism, that two drugs share similar modes of action which is formulated as follows;

$$CI = \frac{C_1}{C_{x,1}} + \frac{C_2}{C_{x,2}} \qquad (9)$$

where C_i, $i = 1,2$ denote the drug concentration in the mixture eliciting an effect x and $C_{i,x}$ denote the respective single agent doses of drugs that elicit the effect x. The second one is mutually nonexclusive mechanism which drugs uses the different pathways or targets of action. It is obtained as follows;

$$CI = \frac{C_1}{C_{x,1}} + \frac{C_2}{C_{x,2}} + \frac{C_1C_2}{C_{x,1}C_{x,2}} \qquad (10)$$

By using the equation $\left(\frac{f_a}{f_u}\right)^{1/m} = \frac{C_x}{IC_{50}}$ for each drug in order to find dose of a drug that inhibits x percent of cells, that is, C_x [14].

Table 1. The levels of drug interaction based on combination index (CI) [14].

CI	< *0.1*, very strong synergism
	0.1 - 0.3, strong synergism
	0.3 - 0.7, synergism
	0.7 - 0.9, moderate to slight synergism
	0.9 - 1.1 additive
	1.1 – 1.45 nearly antagonism
	1.45 - 3.3, antagonism
	> *3.3*, very strong antagonism.

According to Chou-Talalay median–effect analysis the drug mixture should be at constant concentration ratios so that the relative effects of each drug in the mixture are identical. The growth inhibitory effect of each drug, the sigmoidicity of the curves, and an estimated IC_{50} are used to calculate a combination index (CI) that serves as a quantitative measure of synergism [9]. This method has the advantage of allowing evaluation of drug combinations in cellular synergy.

3. BUILDING A MODEL FOR DRUG COMBINATION EXPERIMENTS IN PRECLINICAL CYTOTOXICITY STUDIES

It is suggested that equations derived from mass action law are the most available approach in the assessment of cellular drug synergy in the recent article [3]. This model was built on the understanding of Median effect equation and Combination Index Theory developed by Chou T.

The effect of drug on the cancer cell is determined by its potency and its concentration. Drug potency or its efficiency is mainly originated from unique molecular structure and its side functional groups. Since the measurable effect of drug potency is dependent on its concentration, dose-response curve could be obtained in the selected concentration range of each drug. According to mass action law, increasing drug concentration leads to increase the potency and the measurable dose-response and effect on cellular synergy. In order to find out the best possible synergistic combinations, we should design non-constant ratio experiments which the total concentration is kept the same in each well while the ratio in each well is changing continuously in certain range. This experiment is required for determining the drug mixture ratio giving the synergistic potency
interaction.

After only determined the best synergistic ratio, we can search for the drug synergy level in constant ratio experimental design by using CalcuSyn software based on combination index principle. There is simple mathematical reasoning for this approach. The maximum effect of a drug on viability of the cell could not be bigger than 1.

The combination of two drugs can be applied to the tumor cell population in only certain ratio and defined concentration range. The two drug mixture is assumed as if they are a single drug. The tumor cell population consisted of heterogeneous sensitivity to the potency and concentration of each drug and their combination effect [6]. This kind of experiments predicts the cellular synergy by measuring the degree of viability or cytotoxicity. Median effect equation assumes that the contribution of each drug to total effect is proportional to their ratio in the mixture and calculates this median effect by using the experimental data. For example, A and B drug mixture in 1:2 ratio were exposed to the cell population. The total effect was found to be 0.6. Here unaffected value is calculated indirectly (1-0.6= 0.4). Median effect equation implies that the 0.2 effect is from A drug, and 0.4 effect is from B drug based on their ratio assuming that there is additive effect. If the doses of drug combinations are prepared and applied to the cells in the constant ratio, the relative potency contribution of each drug is kept the same even if the mixture concentrations are increased or decreased. Only in those experimental conditions either synergy ($\leq$0.9) or antagonistic ($\geq$1.1) effect can be measured from the Combination Index (CI) values.

In cellular synergy, direct interaction of drugs is not necessary while it is required in pharmacological synergy. Since the synergy of drug combinations can be dependent on the increasing of their potency more than their additive effect, or the increasing of their efficiency rather than increasing their potency due to higher doses. Therefore, it is important to get the cellular synergy as possible as the lowest concentrations in experimentally defined constant ratio. The utility of preclinical data in clinical use depends on the ratio of drug mixture giving the best synergy in the lowest concentration in order to avoid toxic side effects in higher concentrations.

In order to find out the synergy of potency of two drugs, it is essential to carry out experiments in non-constant ratio but in a fixed concentration. What is more, determining the synergy of efficiency at constant ratio requires designing experiments which the increased and decreased concentrations are tested.

There are two alternatives for selecting the drugs to be combined in order to get the desired cytotoxic outcome. One is to combine drugs which target the same molecules or pathways. Second is to combine drugs which use totally different pathways or targets. It is more likely to discover the best synergy when drugs targeted different pathways are chosen.

There are process and effect perspectives in the assessment of drug synergy. Drug interaction is defined as synergistic, additive and antagonistic in terms of effect perspectives (see section1.1). On the other hand, in the process perspective, drugs can be exposed to the cell population either simultaneously or sequentially at certain time intervals (0-72 hours). Based on this theoretical approach, there should be two strategies of studying drug combinations *in vitro*. One is to get experimental data by applying both drugs at the same time and the other is to deliver each drug sequentially either before or after which is called sequential treatments of drugs. The second strategy is also known as modulation or augmentation of the effect of second drug. This method is very useful to find out if one of the drugs is able to sensitize the cells (Chemosensitivity) and increase the cytotoxic effect of second drug.

4. CONCLUSION

In this review, we have tried to understand the frequently used mathematical models in drug combination analysis and to translate them into experimental use in cellular synergy phenomena, since there is still ongoing discussion about synergy definition and its assessment methods.

The experimental model built in this study in translational perspective can be summarized as follow. Firstly determine the value of 50 percentage of inhibition concentration (IC_{50}) in each drug (A and B) in the wide range concentrations then test at least five different concentrations to obtain dose-response curve. Design a drug combination experiment by using non-constant ratio for each drug either in increasing or decreasing concentrations. Find out the best cytotoxic ratio in the lower concentration below IC_{50} by evaluating the previous data. Select it as the best combination ratio indicating the synergistic potency. Then test two drug combinations in increasing and decreasing fold concentrations (0,25X, 0,50X, 1X, 2X, 4X) by using defined constant ratio (X). At least obtain the experimental data in 5 different combination concentrations in constant ratio. Finally assess the degree of drug synergy by using Combination Index Model based CalcuSyn software.

We have also made clear that what type of experimental works should be done to assess it accurately. The experimental design and assay setting should be complied with the assumptions of the preferred mathematical model and software.

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