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Development and validation of a general approach to predict and quantify the synergism of anti-cancer drugs using experimental design and artificial neural networks

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ABSTRACT

The combination of two or more drugs using *multidrug mixtures* is a trend in the treatment of cancer. The goal is to search for a synergistic effect and thereby reduce the required dose and inhibit the development of resistance. An advanced *model-free* approach for data exploration and analysis, based on artificial neural networks (ANN) and experimental design is proposed to predict and quantify the synergism of drugs. The proposed method non-linearly correlates the concentrations of drugs with the cytotoxicity of the mixture, providing the possibility of choosing the optimal drug combination that gives the maximum synergism. The use of ANN allows for the prediction of the cytotoxicity of each combination of drugs in the chosen concentration interval. The method was validated by preparing and experimentally testing the combinations with the predicted highest synergistic effect. In all cases, the data predicted by the network were experimentally confirmed.

The method was applied to several binary mixtures of cisplatin and [Cu(1,10-orthophenanthroline)$_2$(H$_2$O)](ClO$_4$)$_2$, Cu(1,10-orthophenanthroline)(H$_2$O)$_2$(ClO$_4$)$_2$ or [Cu(1,10-orthophenanthroline)$_2$(imidazolidine-2-thione)](ClO$_4$)$_2$. The cytotoxicity of the two drugs, alone and in combination, was determined against human acute T-lymphoblastic leukemia cells (CCRF-CEM). For all systems, a synergistic effect was found for selected combinations.

1. Introduction

Nowadays, a trend in the treatment of cancer is to combine the effects of several drugs using *multidrug mixtures* in order to reduce the required dose and to slow the onset of drug resistance. A combination of two or more drugs might show different effects with respect to those of the individual drugs. When no difference occurs, it means that the action of one drug is not influenced by the action of the other drugs, and the global effect is due to their individual activities. Otherwise, when a difference occurs, this can be either negative or positive; in the former case, the drugs act antagonistically, while in the latter synergistically. Then, the definition of the pure additive effect of the combined drugs is the reference point to understand if the biological activity of a multidrug mixture is due to synergism or antagonism. This definition has been proposed and interpreted by various authors [1–7] with different methods to search for synergy. The most common definitions are based on the combination index (CI) or isobologram (IB) methods. In particular, today, CI is the most commonly used method. In our opinion, this approach has limited applicability because it is based on the assumption that the action of the drugs is due only to the inhibition of enzyme kinetics [4]. In fact, a model introduced in 1984 [8] and based on this idea is still proposed for the interpretation of experimental data. However, it is now well-known that in addition to enzymatic inhibition, drug-receptor and non-specific interactions are also involved. Therefore, a modern model should consider the entire complexity of the phenomena occurring within the cell. Cisplatin (**CDDP**) is an example of drug that does not act directly on enzyme kinetics. In fact, it kills cancer cells by binding to their nuclear DNA [9,10], distorting its structure and triggering cellular processes that result in apoptosis [11]. Therefore, experimental data should be analyzed using approaches that are more general than CI or IB.

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Models based on the physico-chemical description of the processes occurring in a system are usually called *hard*. However, because of the lack of knowledge on all the possible interactions among drugs and the constituents and/or the biochemical processes involved within the cell, this kind of model is incomplete and therefore limited. In fact, the CI and IB methods are neither able to quantify nor to predict the cytotoxicity of a given combination of studied drugs. Therefore, the features of such models limit their general applicability and sometimes lead to contradictory results. For all these reasons, we suggest a different way of thinking.

Let us consider the concentrations of the drugs in the mixture as *input* variables while cytotoxicity as a response or output one. The values of the response variable for all the possible combinations of the *input* variables form the response surface. The value of the response depends upon all the processes or phenomena that occur in the system and are reflected in the shape of the response surface. Therefore, building a *model* of the response surface means to build a *model* of the whole system's behavior. As a consequence, the response for all the possible values of the input variables can be estimated or *predicted*. The model can be built by *soft* methods which do not require any assumptions. Polynomial fitting, multiple linear regression (MLR) and partial least squares are examples of common *soft* methods. However, several of them suffer of some limitations [12].

Artificial neural networks (ANN) represent a powerful mathematical tool able to accomplish a *learning* process using a set of experimental data (training set) and to generalize this *knowledge* to *predict* the response. Then, the ANN can *understand* the unknown relationship existing among the input and the response variables. The network *learns* by extracting the information hidden in the experimental data of the training set. The choice of the experimental data is crucial for good results, and, for practical reasons, the number of experiments should be as low as possible while providing a high content of information. This can be achieved by using experimental design (ED) techniques [13].

The aim of this work is to: (i) generalize in a rigorous mathematical form the definition of the pure additive effect of an arbitrary number of drugs, (ii) develop an original approach for the evaluation of cytotoxicity data with which to search for the optimal drug combination that gives the maximum synergism and (iii) apply the proposed approach to evaluate the occurrence of synergism in a series of binary mixtures of **CDDP** and three different complexes of copper(II) with 1,10-ortho-phenanthroline (phen) against a human acute T-lymphoblastic leukemia cell line (CCRF-CEM). The chosen complexes, Cu(1,10-orthophenanthroline)$(H_2O)_2(ClO_4)_2$ (**1**), [Cu(1,10-orthophenanthroline)$_2(H_2O)](ClO_4)_2$ (**2**) and [Cu(1,10-orthophenanthroline)$_2$(imidazolidine-2-thione)]$(ClO_4)_2$ (**C1**), show antitumor effects both *in vitro* and *in vivo* [14] and high cytotoxic activity against mouse neuroblastoma, human hematologic and also solid tumor-derived cell lines [15,16]. The proposed approach was validated and tested. The results were achieved using an advanced approach of data exploration and analysis, based on ANN and ED. A comparison with the traditional methods, IB and CI, is also given. The results obtained with the proposed method are compared with those obtained by MLR. The results of the mass spectrometric analysis of the studied mixtures are also given and discussed.

2. Theoretical aspects of the proposed approach

2.1. Generalized definition of additive effect

Starting from the simple definition of the additive effect of two drugs proposed by Webb and Bliss [17,18], we developed a general definition for any number of drugs. The additive effect of drugs is not the algebraic summation of their cytotoxic activities. For this reason, the expression "non-algebraic additive effect" (*NAAE*) will be used in this work in place of "additive effect".

Given n drugs (with $n\geq 2$) with individual percentage of mortality values (number of dead cells with respect to the controls) $a_1, a_2, \ldots, a_n$, (with $a_i\geq 0$), the *NAAE* is expressed for each drug combination in generalized form as:

$$NAAE = \sum_{i=1}^{n} a_i + \sum_{k=2}^{n} \left[(-1)^{k-1} \cdot \frac{C_{n,k}\{a_1, a_2, \ldots, a_n\}}{100^{k-1}} \right] \quad (1)$$

where $C_{n,k}\{a_1, a_2, \ldots, a_n\}$ are the simple combinations without repetition of the cytotoxicity values of the n drugs taken k at a time (with $k\geq 2$). The set of the values given by Eq. (1) has 0% and 100% as the lower and upper limits, respectively, because no mixture of drugs can have an effect greater than 100% and/or lower than 0%. The *NAAE* as expressed by Eq. (1) represents a new operational definition of the additive effect. It is calculated according to a sequential action of the drugs and does not need to be justified on a biochemical basis.

The expanded form of Eq. (1) is given by Eq. (2), where each term in square brackets accounts for $n!/[k!(n-k)!]$ elements.

$$NAAE = \sum_{i=1}^{n} a_i - \frac{1}{100}\left[\sum_{i\neq j} a_i a_j\right] + \frac{1}{100^2}\left[\sum_{i\neq j\neq l} a_i a_j a_l\right] - \ldots + (-1)^{k-1} \cdot \frac{1}{100^{n-1}}\left[\prod a_i\right] \quad (2)$$

For two drugs, Eq. (2) assumes the same form (Eq. (3)) of the equation proposed by Webb and Bliss [17,18].

$$NAAE = a_1 + a_2 - \frac{a_1 a_2}{100} \quad (3)$$

For three drugs, Eq. (2) becomes Eq. (4).

$$NAAE = a_1 + a_2 + a_3 - \frac{a_1 a_2 + a_1 a_3 + a_2 a_3}{100} + \frac{a_1 a_2 a_3}{100^2} \quad (4)$$

The definition of *NAAE* given by Eq. (1) is the reference point which allows us to define the occurrence of synergism or antagonism of drugs. This is done by calculating the net multi-drug effect index (*NMDEI*) as in Eq. (5), where $E_{exp.}$ is the experimental antiproliferative effect of a given drug combination.

$$NMDEI = E_{exp.} - NAAE \quad (5)$$

NMDEI gives a quantitative account of the net multidrug effect and can assume either positive or negative values that indicate synergism or antagonism, respectively. When *NMDEI* is equal to zero, then the experimental activity of the multidrug mixture is equal to *NAAE* (Eq. (5)). Since the cytotoxic activity of an individual drug and/or that of a drug combination is expressed as a percentage, *NAAE* and *NMDEI* are also expressed as a percentage.

Using the proposed ED–ANN combined approach, the cytotoxicity of individual drugs and that of their combinations at concentrations not experimentally tested can be predicted on the whole working space using a grid with desired dimensions. The values estimated for each point of the chosen grid in place of the experimental values ($E_{exp.}$) can be used in Eq. (5). The cytotoxicity values of individual drugs (a_i) estimated by the network compose their related dose–response curves. Using such curves, the *NAAE* can be calculated for each point of the grid. Then, the *NMDEI* can be calculated according to Eq. (5) for each point of the chosen grid.

2.2. Artificial neural networks

An ANN is a formal object that emulates the structure of the brain and its *learning* ability [13]. A series of logic units, called *neurons*, is organized in layers: input, hidden and output. An