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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_2O)](ClO_4)$_2$, Cu(1,10-orthophenanthroline)(H_2O)$_2$(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

example of the ANN architecture used in this work is given in the supplementary materials (Fig. S1).

As for the biological brain, an ANN needs to be trained before it can be used for *prediction*. This is done using a *training set* formed by known input-response data. The inputs are iteratively processed by the network according to a suitable *training algorithm* in order to estimate the actual output of the training set. Several algorithms are available [19], for example standard back-propagation [20], R-propagation [21] and quick-propagation [22]. The root mean square error (*RMSE*) is calculated according to Eq. (6), where N, M, o_{pk}, and $o_{pk}^{(*)}$ are the number of experiments used for training, the number of response variables, the estimated and the actual output value, respectively.

$$RMSE = \sqrt{\frac{\sum_{p=1}^{N}\sum_{k=1}^{M}(o_{pk} - o_{pk}^{*})^2}{N \times M}} \quad (6)$$

The available data set is usually divided into three subsets: (i) training, (ii) verification and (iii) the test set. The verification set is used during training to monitor the generalization ability of the network. Training is stopped when the minimum *RMSE* for the verification set is reached. Once the network is trained and verified, it should be validated. This is done in the so-called *test step* by comparing the estimated and experimental response for selected input data.

2.3. Response surface and experimental design

The *working space* is the plane delimited by the minimum and maximum values of the chosen variables. For example, let us consider the concentration of drug A (C_A) as the x-axis, while that of drug B (C_B) as the y-axis. Supposing $p \le C_A \le q$ and $r \le C_B \le s$, the working space is given by all the points w belonging to the set W defined by Eq. (7).

$$W = \{w / w = (C_A, C_B) \in R / p \le C_A \le q \wedge r \le C_B \le s\} \quad (7)$$

Therefore, each point w belonging to W represents the conditions of one experiment. The response variable z (e.g. cytotoxicity) is a function of the chosen C_A and C_B variables (Eq. (8)).

$$z = f(w) = f(C_A, C_B) \quad (8)$$

The set Z given by Eq. (9) defines the *response surface*.

$$Z = \{z / z = f(w), w \in W\} \quad (9)$$

The aim of *hard* modeling is finding out the analytical expression of the function f according to a rigorous physical description of the studied system. However, f is often very complex or even unknown. Therefore, the function f can be approximated employing *soft* modeling. Each point w provides information about the function f. A greater number of experiments is associated with a better model. However, the duration and the cost of the experiments are often crucial. Then, a compromise to obtain an acceptable model with the lowest number of experiments is needed. ED is a chemometrical tool used to distribute n experiments on the working space in such a way that the information content for that n is the highest [23]. For the study of drug combinations, as a first approach, the concentrations of the drugs should be chosen in the range within 0 and twice the CC_{50} (the dose of the drug which inhibits 50% of cell proliferation). Therefore, knowledge of a simple estimation of CC_{50} is needed.

For the evaluation of the cytotoxic activity of two or more drugs, the following information is necessary:

- a sufficient number of experiments to model the dose–response curve of each drug;
- a sufficient number of experiments distributed in the studied space to model the cytotoxicity response surface.

Due to the assay techniques of the cytotoxic activity, a non-symmetrical experimental design was used here. However, the lack of symmetry of the experimental design does not affect the performance of the chosen method that has been shown to properly describe the studied systems.

The chosen experimental designs are shown in Fig. 1a, c and e. The points used in the training, validation and test sets are highlighted in Figs. 1–4 and S3–S6.

2.4. Structure of the proposed approach

The proposed ED–ANN approach consists of the following steps:

(1) Set-up of the ED.
(2) Experimental determination of the cytotoxicity of the drugs alone and those of the multidrug mixtures prepared according to the chosen ED.
(3) Training and verification of the artificial neural network.
(4) Prediction of the response using the network, according to a suitable grid that covers the entire working space.
(5) Calculation of the *NAAE* for each point of the grid.
(6) Calculation and plot as a function of the drug concentrations of *NMDEI* (*NMDEI* surface).
(7) Testing the obtained results.

3. Materials and methods

3.1. Reagents

Copper(II) carbonate basic ($Cu_2(CO_3)(OH)_2$), 1,10-phenanthroline monohydrate, cisplatin (**CDDP**), perchloric acid, ethanol, ethyl ether, dimethyl sulfoxide (DMSO), imidazolidine-2-thione, 3-hydroxy picolinic acid, acetonitrile (CH_3CN), trypan blue and 6-mercaptopurine were purchased from Sigma-Aldrich (Milan, Italy) and used without any further purification. Fetal calf serum (FCS), penicillin G sodium and streptomycin sulfate were purchased from Gibco-Invitrogen (Milan, Italy) and used without any further purification. Red phosphorus was purchased from Riedel de Haën (Hannover, Germany). Water was double distilled from a quartz apparatus from Heraeus Quarzschmelze (Hanau, Germany).

3.2. Synthesis

The compounds **1**, **2** and **C1** were prepared as previously reported [15,16]. Briefly, copper perchlorate was prepared *in situ* by the reaction of $Cu_2(CO_3)_2(OH)_2$ with $HClO_4$ in a 1:2 M ratio in ethanol solution; phen was added in a Cu(II):phen molar ratio of 1:1 or 1:2 to obtain **1** or **2**, respectively. **C1** was prepared by the reaction of **2** and imidazolidine-2-thione in a 1:1 M ratio in CH_3CN solution.

3.3. Cell lines

The CCRF-CEM (acute T-lymphoblastic leukemia) human cell line was purchased from the American Type Culture Collection (ATCC-LGC; Milan, Italy). The cell line was grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium according to instructions from ATCC in the presence of 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cell line was maintained in

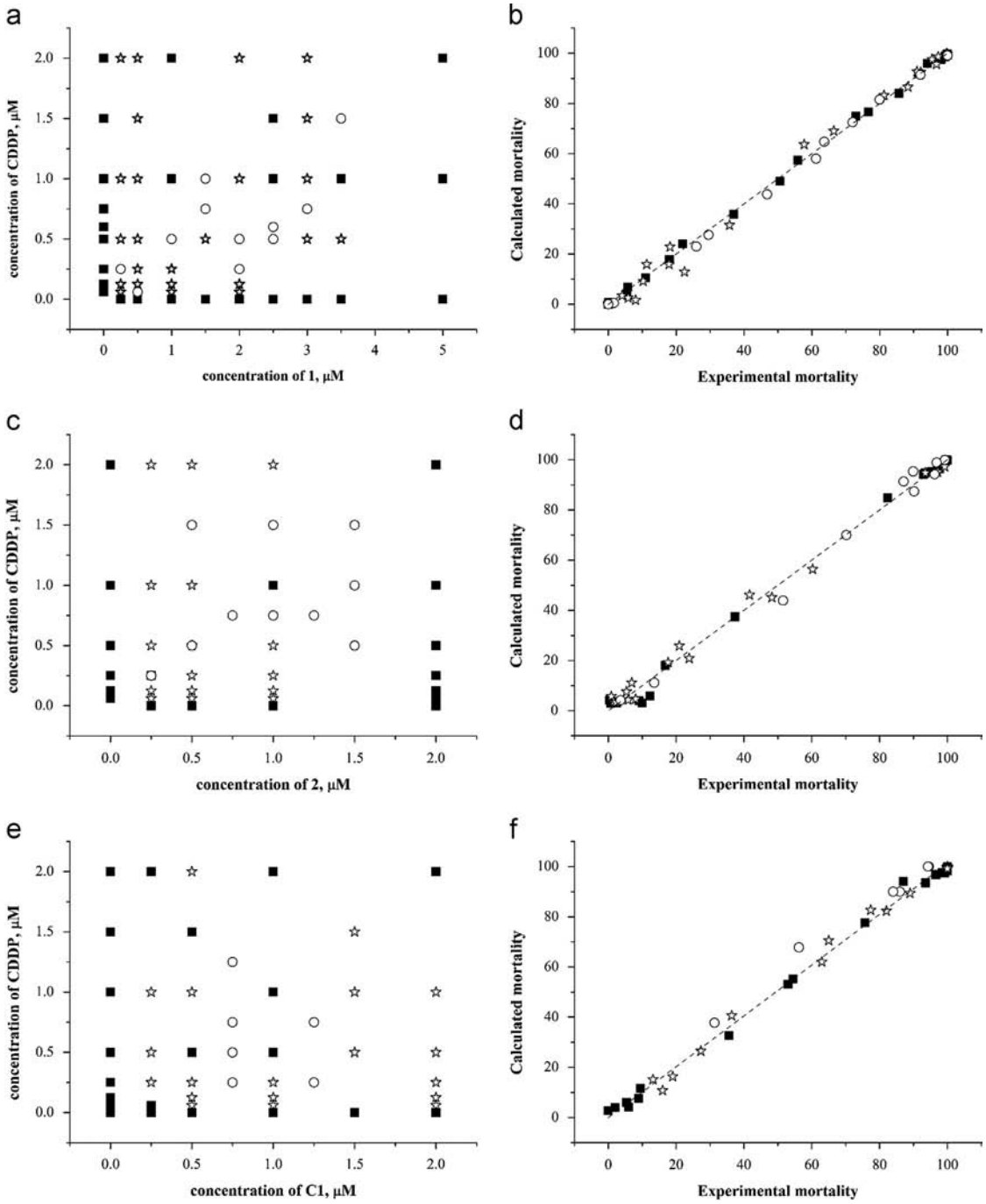

Fig. 1. Experimental design (■ training set, ☆ validation set and ○ test set) for the systems (a) **1**-**CDDP**, (c) **2**-**CDDP** and (e) **C1**-**CDDP**; comparison between experimental (■ training set, ☆ validation set and ○ test set) and calculated mortality values for the systems (b) **1**-**CDDP**, (d) **2**-**CDDP** and (f) **C1**-**CDDP** (linear fitting parameters for the equation $y=mX$ are $m=0.999(3)$ with $r=0.9999$ for the training set, $m=1.00(1)$ and $r=0.9986$ for the validation set, $m=0.990(1)$ and $r=0.9995$ for the test set, $m=1.00(1)$ and $r=0.9993$ for all the data values for **1**-**CDDP**; $m=1.00(1)$ with $r=0.9991$ for the training set, $m=0.99(1)$ and $r=0.9978$ for the validation set, $m=1.00(1)$ and $r=0.9988$ for the test set, $m=1.00(1)$ and $r=0.9988$ for all the data values for **2**-**CDDP**; $m=1.00(1)$ with $r=0.9991$ for the training set, $m=1.00(1)$ and $r=0.9995$ for the validation set, $m=1.08(2)$ and $r=0.9977$ for the test set, $m=1.02(1)$ and $r=0.9988$ for all the data values for **C1**-**CDDP**.

exponential growth by periodically splitting high density suspension cultures (*i.e.* 10^6/mL). The cell culture was periodically tested for the absence of mycoplasma contamination.

3.4. Cytotoxic assay

The cytotoxic effect of test compounds was evaluated in exponentially growing cell cultures. Stock solutions of test compounds were made at 1 mM in DMSO and stored at 4 °C in the dark. For the evaluation of cytotoxicity, each compound was serially diluted in specific growth medium so that the concentration of DMSO was never higher than 0.1%. Cell growth in the absence and in the presence of the test compounds was determined after 96 h of incubation, corresponding to three duplication rounds of untreated cells, by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [24]. Numbers of viable cells were also determined by the trypan blue dye exclusion method. Cell growth at each drug concentration was expressed as the percentage of untreated controls. Each compound was tested in at least three independent assays. Statistical analysis was performed with

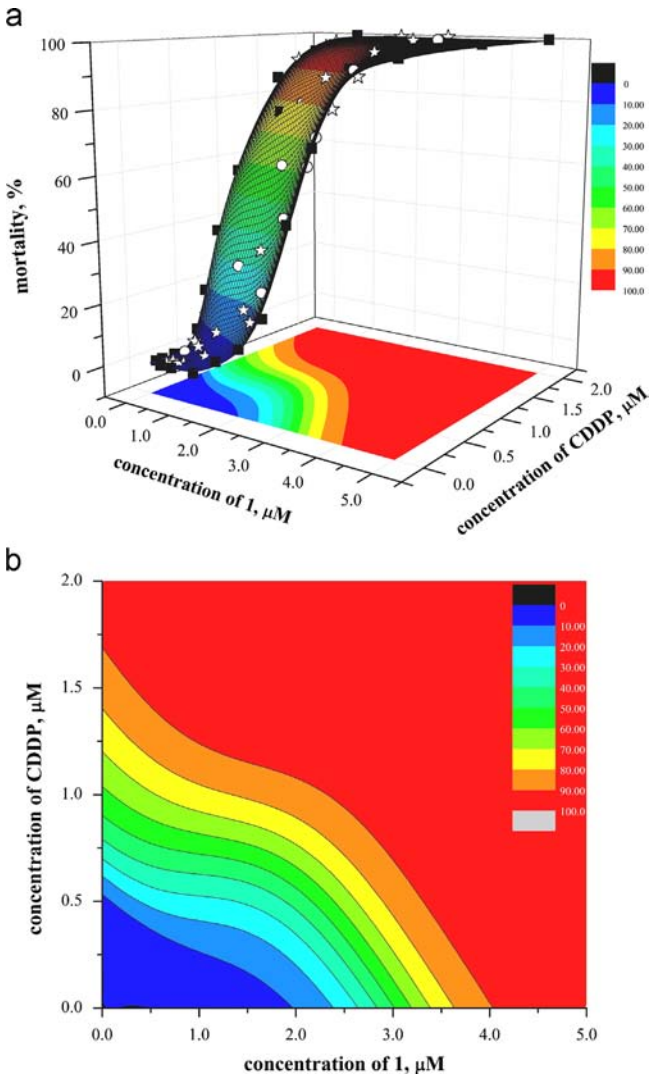

Fig. 2. Response surface with experimental data (■ training set, ☆ validation set and ○ test set) (a) and contour plot of cytotoxicity iso-values (b) for the system **1**-**CDDP**.

Student's t-test, and significance was set at $p \leq 0.05$. The antitumor agent 6-mercaptopurine was used as a reference compound. Because of the variability in cell reactivity (correlated to the growing time and the final density of the suspension cultures) slight differences were observed in the cytotoxicity values of **CDDP** obtained in these experiments.

3.5. Mass spectrometry

Mass spectra were recorded using AXIMA Resonance mass spectrometer from Kratos Analytical (Manchester, UK), equipped with a quadruple ion trap (QIT) and a reflectron time-of-flight analyzer (RTOF). The instrument for drying samples was from Kratos Analytical. Calibration was done using red phosphorus clusters as described in [25].

Laser desorption ionization (LDI) and matrix-assisted laser-desorption ionization (MALDI) were used. From several matrices examined, 3-hydroxo picolinic acid was found to be the most suitable. It was found that all the species detected both in LDI and MALDI analyses were singly charged. The mixtures of **1**, **2** and **C1** complexes with **CDDP** (5:1 molar ratio) were prepared in 50% acetonitrile/water. For LDI analysis, 5 μL of the mixture were deposited on the metallic target of the instrument and, after drying, the mass spectra were recorded. For MALDI analysis, first 5 μL of saturated solution of matrix were deposited and, after drying, 5 μL of the mixture were deposited on the matrix layer and dried again.

Mass spectra were recorded in both reflectron positive and negative ion mode after the pressure was below 10^{-4} Torr. Mass spectra recorded in negative ion mode were found to be not suitable, therefore only mass spectra recorded in positive ion mode were used.

3.6. Software

ANN computation was performed using the Trajan Neural Network Simulator (release 3.0 D, Trajan Software, Horncastle, UK) and EasyNN-plus (Neural Planner Software Ltd, Cheadle Hulme, UK) programs. All computations were performed on a standard PC computer with Microsoft Windows XP Professional as operating system. Other mathematical calculations were done using GNU Octave ver. 3.2.4 (Free Software Foundation, Inc. http://fsf.org/) configured for i686-pc-linux-gnu on a standard PC x86 running Ubuntu Linux ver. 12.04.

4. Results and discussion

4.1. Set up of the ED

For each binary mixture, the experimental design was built choosing the concentration interval of the drugs in the range 0 −~2 × CC_{50}. Following the experimental design reported in Fig. 1a, 42 combinations of **1** and **CDDP**, nine solutions of **CDDP** alone and nine solutions of **1** alone were prepared. Among the 60 solutions, 25 were used as the training set, 24 as the validating set and 11 as the test set. Following the experimental design reported in Fig. 1c, 34 combinations of **2** and **CDDP**, six solutions of **CDDP** alone and four solutions of **2** alone were prepared. Among the 44 solutions, 18 were used as the training set, 16 as the validating set and 10 as the test set. Following the experimental design reported in Fig. 1e, 33 combinations of **C1** and **CDDP**, seven solutions of **CDDP** alone and five solutions of **C1** alone were prepared. Among the 46 solutions, 21 were used as the training set, 19 as the validating set and six as the test set. The test sets for all the systems were chosen after the inspection of the results obtained by the network.

4.2. Experimental determination of the cytotoxicity of individual drugs and multidrug mixtures prepared according to the chosen ED

Three different sets of experiments were carried out, each one in three replicates, for the **1**-**CDDP**, **2**-**CDDP** and **C1**-**CDDP** combinations. The concentration of **1** was in the range of 0–5 μM, while the concentrations of **2**, **C1** and **CDDP** were in the range of 0–2 μM. The vitality (% of living cells) after the treatment with the drugs was measured for each solution with respect to the control (untreated cells) and converted into mortality (100% minus vitality).

4.3. Training and verification of the artificial neural network

The concentrations of the drugs and the experimental cytotoxicity values were used as the input and output data. As the training algorithm, standard back-propagation [20] was used.

At first, the optimal neural network architecture was searched for using the criteria of lowest *RMSE*. The number of hidden layers and the number of neurons therein were investigated. The value of *RMSE* for the training and verification sets were plotted against the number of neurons in the hidden layers. The optimal number of

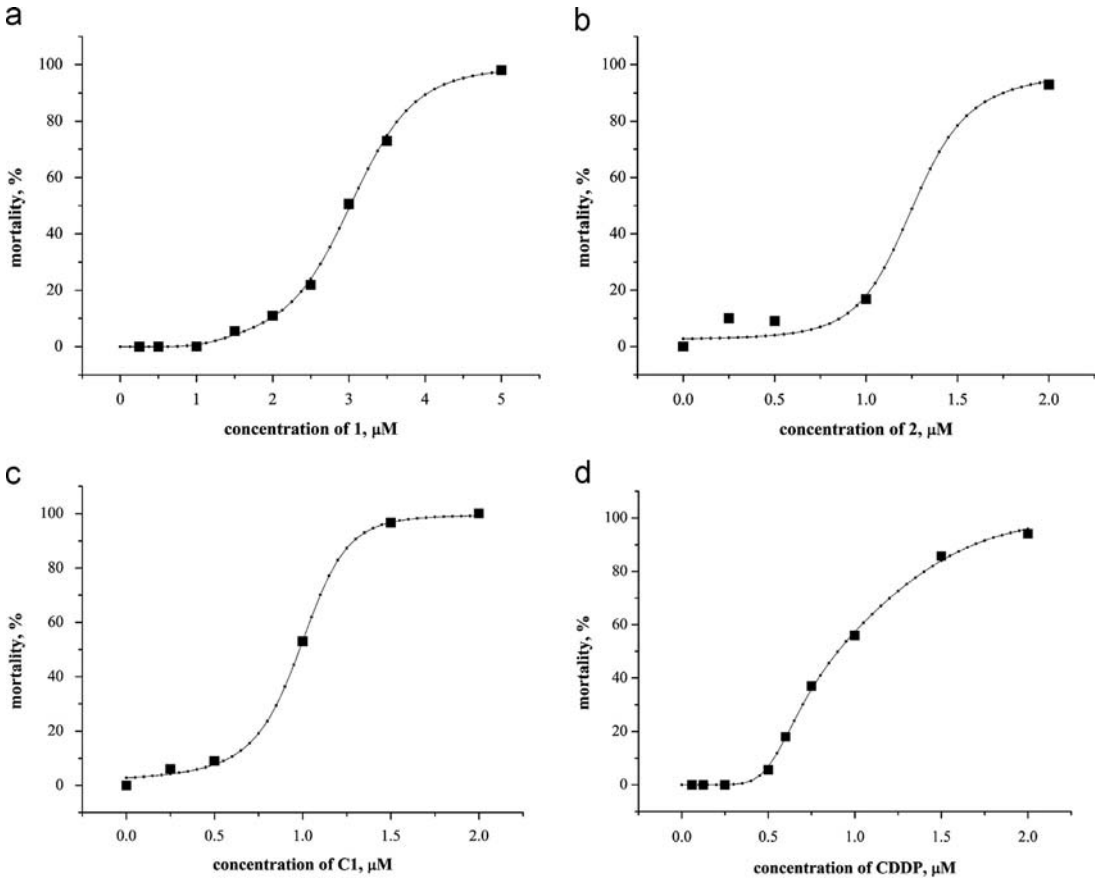

Fig. 3. Calculated (-●-) and experimental (■) dose–response data for (a) **1**, (b) **2**, (c) **C1** and (d) **CDDP** against the CCRF-CEM cell line.

neurons is found when the minimum *RMSE* for the verification set is reached. It was found that a three-layer structure, *i.e.* one input, one hidden and one output, with four (for **1-CDDP** and **C1-CDDP**) or five (for **2-CDDP**) neurons in the hidden layer was sufficient. The network's parameters were re-initialized several times, randomly changing their initial values to prove the robustness of the model and the reproducibility of the results. The resulting architecture of the network for the **C1-CDDP** system is shown in Fig. S1 as an example.

For each system, the network was trained and verified using the proper training and validation sets. All points lying on the borders of the experimental design were included in the training set. Validation points were chosen randomly on the working space. The training was stopped when the minimum *RMSE* for verification step was reached (the plot of *RMSE vs.* number of epochs for the system **1-CDDP** is shown as an example in Fig. S2). The agreement between the values calculated by the network and the experimental ones is shown in Fig. 1b, d, f, where the calculated and experimental data are plotted against each other.

Once trained and verified, the network was used to predict the cytotoxicity on the whole working space according to a grid with dimensions 40×40.

4.4. Exploring the response surface

The plot of the calculated cytotoxicity values as a function of the concentration of the drugs represents the response surface. The response surface and related contour plot for **1-CDDP** system is shown in Fig. 2. The results for **2-CDDP** and **C1-CDDP** systems are shown in Figs. S3 and S4. The mortality of the mixtures increases proportionally with the concentrations of the two drugs (Fig. 2a, S3a and S4a). The resulting surface shows similar trend to that of the dose–response curve.

Using the contour plot (Fig. 2b, S3b and S4b), the areas of cytotoxicity iso-values for the various mixtures can be explored. In this way, the mixture with the desired cytotoxicity and the related lowest dose of both drugs can be found. In fact, for a toxicity of 70%, instead of choosing **1** alone at a concentration of ≈3.5 μM, **2** alone at ≈1.5 μM, **C1** alone at ≈1.4 μM or **CDDP** alone at a ≈1.2 μM, it is possible to select appropriate combinations such as:

- **1** at 2.0 μM and **CDDP** at 0.78 μM (Fig. 2b)
- **2** at 0.9 μM and **CDDP** at 0.95 μM (Fig. S3b)
- **C1** at 0.65 μM and **CDDP** at 0.60 μM (Fig. S4b)

For a toxicity of 50%, instead of choosing **1** alone at a concentration of ≈3 μM, **2** alone at ≈1.25 μM, **C1** alone at ≈1 μM or **CDDP** alone at ≈1 μM, it is possible to select:

- **1** at 1.88 μM and **CDDP** at 0.60 μM (Fig. 2b)
- **2** at 0.75 μM and **CDDP** at 0.80 μM (Fig. S3b)
- **C1** at 0.50 μM and **CDDP** at 0.45 μM (Fig. S4b).

The use of a lower concentration of each drug minimizes the side effects related to the doses. The reliability of the network and of the obtained results is shown by the good agreement between the calculated and experimental values, as can be seen in Fig. 2a, S3a and S4a, where the experimental points are superimposed on the calculated surfaces.

From the calculated cytotoxicity values of the bottom and left borders of the ED (*i.e.* for a single drug at time) the dose–response curves for **1**, **2**, **C1** and **CDDP** is obtained. In Fig. 3a–d, the calculated curves are shown together with the experimental

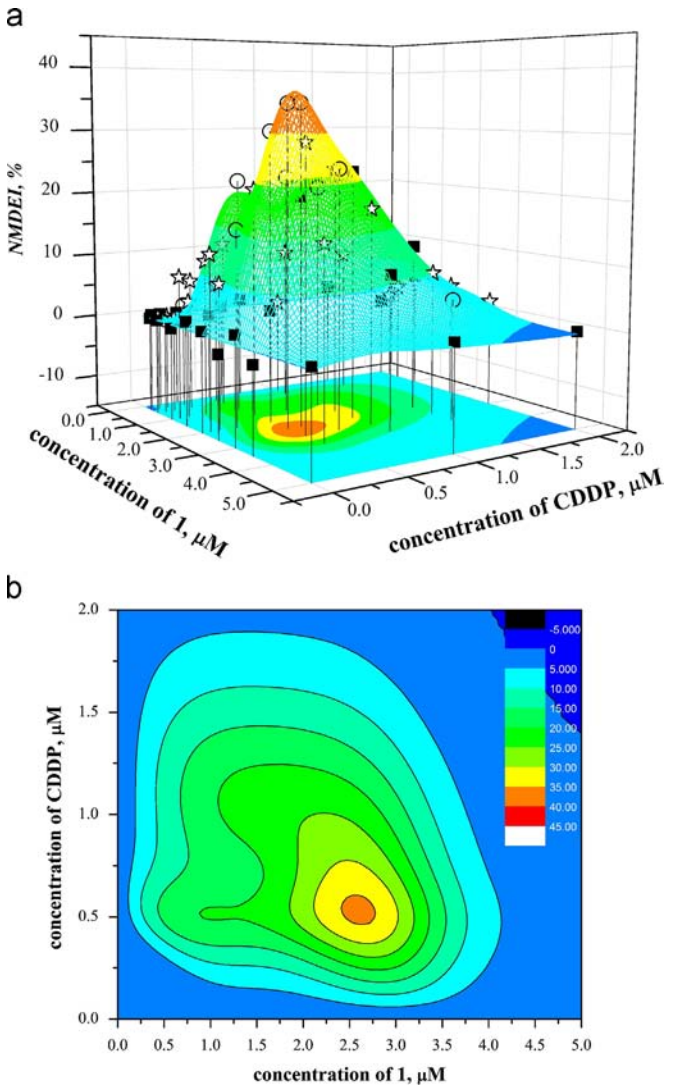

Fig. 4. *NMDEI* surface with experimental data (■ training set, ☆ validation set and ○ test set) (a) and contour plot of *NMDEI* iso-values (b) for the system **1-CDDP**.

points. For **1**, **2** and **C1**, a sigmoidal curve was obtained. The threshold doses (the minimum dose at which the drug presents an effect) for **1**, **2**, **C1** and **CDDP** are 1 μM, 0.5 μM, 0.25 μM and 0.375 μM, respectively. The calculated CC_{50} values are in the order: **CDDP** (0.922 μM)<**C1**(0.989 μM)<**2**(1.26 μM)⩽**1** (3.01 μM).

4.5. Calculation of the NAAE and the NMDEI surface

The *NAAE* was calculated according to Eq. (1) and subtracted from the cytotoxicity values for all the points of the used grid to determine the *NMDEI* (Eq. (5)). The plot of the *NMDEI* as a function of the drug concentrations and the related contour plot is shown in Fig. 4 for **1-CDDP** system. The results for **2-CDDP** and **C1-CDDP** systems are shown in Figs. S5 and S6. Only positive values of *NMDEI* are present for the **1-CDDP** system (Fig. 4a), indicating a synergistic effect between the two drugs. The maximum *NMDEI* is present at a concentration of **1** equal to 2.63 μM and a concentration of **CDDP** equal to 0.55 μM (Fig. 4b). As can be seen from Fig. 2b, this combination induces 73.6% mortality, while **1** and **CDDP** alone show at the same concentrations as the combination mortality values of 29.3 and 11.8%, respectively. The same considerations can be done for the systems **2-CDDP** and **C1-CDDP**. In fact, the maximum *NMDEI* indicating a synergistic effect is revealed (Fig. S5a, S5b) at **2** equal to 1.05 μM and **CDDP** equal to 0.85 μM. This combination induces 81.6% cytotoxicity (Fig. S3), while **2** and **CDDP** alone show at the same concentrations as the combination cytotoxicity values of 19.7% and 18.5%, respectively. For the **C1-CDDP** system (Figs. S6a and S6b), a maximum is revealed at **C1** equal to 0.87 μM and **CDDP** equal to 0.45 μM. This combination induces 82.8% cytotoxicity (Fig. S4), while **C1** and **CDDP** alone show at the same concentrations as the combination cytotoxicity values of 32.9% and 27.5%, respectively.

No negative values of *NMDEI* are present in any case, indicating that **CDDP** and the studied copper(II) complexes do not act antagonistically in the studied range of concentrations.

4.6. Test of the obtained result

In order to confirm the predicted maxima of *NMDEI* and to check the overall predictive ability of the network, new sets of 11 combinations of **1-CDDP**, 10 combinations of **2-CDDP** and six combinations of **C1-CDDP** were prepared (test sets) and the corresponding cytotoxic activities were measured. These test data values were corrected for the *NAAE* and compared with the calculated values. It was found that all the data predicted by the network were experimentally confirmed. The test points are superimposed to the surfaces in Figs. 1, 2, 4 and S3–S6. The quality of the prediction can also be seen from Fig. 1b, d and f ("o" symbol).

4.7. Mass spectrometry

Since the highest values of *NMDEI* occurs for almost integer ratios of copper complexes and **CDDP** concentrations (5:1, 1:1 and 1:2 Cu(II)/**CDDP** for **1**, **2** and **C1**, respectively), we verified the possible formation of mixed complexes between either **1**, **2** or **C1** with **CDDP** by mass spectrometry. Both the LDI and MALDI mass spectra of the mixtures of Cu(II) complexes and **CDDP** at 1:5 and 5:1 molar ratios were recorded. No mixed copper-platinum complexes were detected (mass spectra are not shown).

4.8. Comparison with IB and CI methods

4.8.1. Brief description of the methods

The IB method [5] consists of the construction of a graph in which equally effective doses of two drugs are reported for a single effect level (isoboles). Once a specific level is chosen, such as 50% of the maximum, the doses of the two drugs A and B that give the same degree of effect are plotted as axial points in a Cartesian plot. The straight line connecting A and B defines the combinations that produce a pure additive effect. The experimental points which fall below or above that line represent drug combinations that have synergistic or antagonistic effect, respectively.

The CI method [3,4,6,7] tries to measure the extent of drug interactions. It considers the existence of a relationship between the fraction affected (f_a) and unaffected ($1-f_a$) by a drug and the ratio $(D/D_m)^m$, according to Eq. (10), where D is the dose of the drug and D_m is the median effect dose, *i.e.* the concentration at which the drug inhibits the system by 50% (the fraction affected (f) coincides with the cytotoxic activity divided by 100).

$$\frac{f_a}{(1-f_a)} = \left(\frac{D}{D_m}\right)^m \tag{10}$$

The values of m and D_m are determined by applying a logarithm to Eq. (10), which gives $\log(f_a/(1-f_a)) = m \log(D) - m \log(D_m)$ and plotting the function $y = \log(f_a/(1-f_a))$ *vs.* $x = \log(D)$. By linear fitting, the variables m and D_m can be obtained as the slope and intercept, respectively.

Once m and D_m are defined, as the second step of the method, the value of D_x for each drug (Eq. (11)) and finally the *CI* (Eq. (12)) have to be calculated (the numbers 1 and 2 indicate the two

drugs).

$$D_x = D_m \left(\frac{f_a}{1-f_a} \right)^{1/m} \quad (11)$$

$$CI = \frac{D^1}{D_x^1} + \frac{D^2}{D_x^2} \quad (12)$$

A *CI* lower than, equal to and higher than 1 indicates synergism, additivity and antagonism, respectively.

4.8.2. Selected examples

Both CI and IB methods require that the concentrations of the individual drugs and that of their combinations providing the same cytotoxic effect have to be compared. Let us consider the solutions reported in Table 1. In the solutions *a* and *b*, **CDDP** and **1** have a concentration of 1.0 and 3.0 μM, respectively; at these concentrations, they both show a cytotoxicity value of 50%. Following the IB and CI methods, these two solutions should be compared with all the combinations which show the same cytotoxic activity, for example solution *c*. On the basis of the results of the isobologram (Fig. S7a, triangle symbol and dotted line) and of *CI* calculus (*CI*=1.28), this combination presents antagonistic effect. Instead, in our view, the final effect of a combination should be compared with the effects that the individual drugs provide at the same concentration. In fact, solution *c* should be compared with *d* and *e*, *i.e.* where **1** and **CDDP** have the same concentration of their combination. The drugs **1** and **CDDP** alone present a cytotoxicity value of 9% and 18%, respectively. Comparing these two values with the cytotoxicity of their combination (50%), a clear synergistic effect is evident, as also pointed out by the *NMDEI* calculated by our approach, being positive and higher than zero (*NMDEI*=25).

To stress the concept, we can consider the solutions *f*, *g* and *h*. If we compare the cytotoxic activity of **1** at the concentration of 2.63 μM, which is equal to 29%, and that of **CDDP** at a concentration of 0.55 μM, which is equal to 12%, it appears evident that the cytotoxic activity of their combination (74%) is even greater than a simple algebraic sum of the effects, and, without any kind of calculation, a synergistic effect is evident. The *CI* (*CI*=1.23) instead suggests an antagonistic effect. Note that solution *f* has the maximum value of *NMDEI* (*NMDEI*=36, Fig. 4).

The same considerations may be presented for the **2-CDDP** (solutions *i*–*n*) and **C1-CDDP** systems (solutions *o*-*u*). In particular, the solutions *l* and *s* that give rise to the maximum value of *NMDEI* for the **2-CDDP** and **C1-CDDP** systems, respectively, present a *CI* indicating an additive effect (*CI*=0.98) or a weak synergistic effect (*CI*=0.88).

Let us now analyze some other results reported in the literature for the system **CDDP**-paclitaxel [6] on 833 K teratocarcinoma cells. Considering the solutions *aa*, *ab* and *ac*, we find that the cytotoxic activity of paclitaxel at 0.01 μM is 88%, that of **CDDP** at 1.0 μM is 82%, while their combination has a cytotoxic activity of 97%. According to the *CI*, this combination has a synergistic effect (*CI*=0.602), but, applying our method, we find that the *NMDEI* for the two drugs is equal to −1%; therefore, a slight antagonistic rather than synergistic effect should be considered. Let us now analyze the solutions *ad*, *ae* and *af*; the compounds paclitaxel at 0.002 μM and **CDDP** at 0.2 μM present a cytotoxicity value of 43% and 30%, respectively, while their combination has a cytotoxicity value of 70%. According to the *CI*, this combination has a moderate synergistic effect (*CI*=0.815), lower than that shown by the solution *ac*, as it is closer to 1. According to our calculation, the *NMDEI* of this combination is 10%, indicating a synergistic effect, unlike the previous case.

Table 1
Results for selected experimental data analyzed using the method proposed in this work and the combination index (*CI*).

System	Solution	Conc. of drug$_1$ (μM)	Conc. of CDDP (μM)	Experimental cytotoxicity (%)	*NMDEI (%)*[a]	*CI.I.*[b]
CDDP	***a***		1.0	50		
1-CDDP	***b***	3.00	–	50	–	–
	c	1.88	0.60	50	25	1.28
	d	1.88	–	9	–	–
	e	–	0.60	18	–	–
	f	2.63	0.55	74	36	1.23
	g	2.63		29	–	–
	h		0.55	12	–	–
2-CDDP	***i***	1.25		50	–	–
	j	0.75	0.80	50	29	1.50
	k	0.75		7	–	–
	l	1.05	0.85	82	44	0.98
	m	1.05		20	–	–
	n		0.85	19	–	–
C1-CDDP	***o***	0.98	–	50	–	–
	p	0.50	0.45	50	16	1.40
	q	–	0.45	28	–	–
	r	0.50	–	8	–	–
	s	0.87	0.45	83	33	0.88
	t	0.87		32	–	–
	u		0.45	28	–	–
PAC-CDDP[c]	***aa***	0.01	–	88	–	–
	ab		1.0	82	–	–
	ac	0.01	1.0	97	−1	0.602
	ad	0.002	–	43	–	–
	ae	–	0.2	30	–	–
	af	0.002	0.2	70	10	0.815

[a] Calculated by Eq. (5).
[b] Calculated as proposed by Chou [6] (a number greater, lower or equal to 1, indicates antagonism, synergism and additive effect, respectively).
[c] PAC is paclitaxel [6].

4.8.3. Our considerations

In our opinion, comparing the concentrations of individual drugs and that of their combination providing the same cytotoxic effect, as the CI and IB methods suggest, might lead to misleading results. A better comprehension of the studied system arises from a comparison between the effect of a given combination and the effect that the individual drugs provide at the same concentration.

The CI method tries to explain the cytotoxic effect of drug mixtures considering the inhibition of enzymatic activity, a mechanism that follows an exponential trend. In our opinion, this is actually a limitation. If the mechanism of drug action is not due only to the inhibition of enzymatic kinetics, then linearizing a pattern which is not *a priori* exponential leads to approximate results. In fact, the application of the logarithm mathematical operator to linearize a function is justified only when the trend of the function is exponential over its entire interval of existence. The common choice to consider only the points that lie on a portion of a straight line, *i.e.* those that lie around the inflection point on the dose–response curve, is not mathematically rigorous and shows even more the inability of the method to generalize. The limits of the method are clearly understandable from Fig. S7b–d, where the data of **1** and **CDDP** and paclitaxel alone are reported treated following Eq. (11). As can be seen, the linear fitting of the trend does not give acceptable results, in particular for **1** and **CDDP**.

4.8.4. The choice of the combinations

According to Chou [6,7], the most efficient and economical way to study the combinations of the drugs in order to save data points, costs and time is to keep the ratio of the concentrations constant. In our opinion, this procedure is hazardous because some drug combinations that may exhibit synergism could be lost. In fact, synergism (as well as antagonism) may be due to chemical or bio-chemical reactions (formation of adducts, complexes, redox reactions, ligand/metal exchange, *etc.*) that depend on the experimental conditions, for example the molar ratio. Therefore, by working at a constant molar ratio, the optimal combinations of the drugs might go unnoticed. On the contrary, our method based on the combined approach of ED and ANN, allows the evaluation of all the possible combinations on the entire space defined by the selected concentration intervals.

4.9. Multiple linear regression

As stated in the introduction, the response surface is usually modeled using MLR methods. These were applied to model the cytotoxicity response surface and in this section the results are given. For MLR treatment, the same data used to train the network (Section 4.1) were used.

Polynomials with the general expression shown in Eq. (13) were used.

$$y = \alpha_0 + \sum_{i=1}^{n} \alpha_i x_1^i + \sum_{i=1}^{n} \beta_i x_2^i + \sum_{i=1}^{n}\sum_{j=1}^{n}\sum_{k=1}^{n\times n} \gamma_k x_1^i x_2^j \quad (13)$$

At first, the concentrations of the two drugs could be considered uncorrelated to each other. However, because the possible synergistic or antagonistic effect may be due to a chemical or biochemical reaction between the drugs, the concentrations of the drugs are actually related. In fact, models without the interaction terms ($x_1^i x_2^j$) provided non-acceptable regression parameters (confidence lower than 80%).

A third degree polynomial was used for the system **2-CDDP** and a fourth degree polynomial for the systems **1-CDDP** and **C1-CDDP** (confidence ≥ 95%). Polynomials with a degree higher than four or lower than two gave unreliable results (confidence ≤ 60%). The models obtained for the studied systems are given below (***y*** is the percentage of mortality, $\boldsymbol{x_1}$ and $\boldsymbol{x_2}$ are the concentrations of the two drugs and *RSS* is the residual sum of squares):

- ***System* 1-CDDP**:

$$y = -0.9x_1^4 + 2.9x_1^3x_2 + 6.1x_1^2x_2^2 - 2.4x_1x_2^3 + 31.8x_2^4 + 5.6x_1^3 - 33.4x_1^2x_2 - 22.0x_1x_2^2 - 168.4x_2^3 - 0.659x_1^2 + 78.3x_1x_2 + 276.5x_1^2 - 10.8x_1 - 89.1x_2 + 5.1;\ RSS = 141.$$

- ***System* 2-CDDP**:

$$y = 10.4x_1^3 - 43.2x_1^2x_2 - 9.4x_1x_2^2 - 16.3x_2^3 + 0.5x_1^2 + 83.0x_1x_2 + 61.7x_2^2 + 4.7x_1 - 12.6x_2 + 2.9;\ RSS = 189.$$

- ***System* C1-CDDP**:

$$y = -16.6x_1^4 + 20.9x_1^3x_2 + 36.0x_1^2x_2^2 + 39.0x_1x_2^3 + 28.1x_2^4 + 19.0x_1^3 - 134.4x_1^2x_2 - 151.6x_1x_2^2 - 129.4x_2^3 + 58.1x_1^2 + 164.4x_1x_2 + 164.9x_2^2 - 9.5x_1 + 11.3x_2 + 0.9;\ RSS = 45.$$

The response surface was calculated according to a grid with dimensions of 40 × 40. The calculated and experimental data for the training sets are in agreement (Fig. S8 a, c and e; *R* ranges from 0.9972 to 0.9993). On the contrary, no satisfactory agreement is achieved for both the validation and test sets (*R* ranges from 0.8870 to 0.9869). The shapes of the obtained response surfaces are quite irregular, indicating that overfitting had occurred (Fig. S8 b, d and f).

Moreover, the polynomial model does not allow for properly fitting the experimental dose-response curves (Fig. S9) as done by the neural network (Fig. 3). This result is instead crucial for the application of the method proposed in this work, as described in Section 4.5. Finally, the resulting *NMDEI* surfaces do not agree with the experimental values (Fig. S10).

5. Conclusions

A generalized and rigorous mathematical form for the definition of additive effect for an arbitrary number of drugs has been proposed. An advanced *model-free* approach of data exploration and analysis, based on the ANN and ED was set up to predict and quantify the synergism of drugs.

From the analysis of the calculated cytotoxicity surface, the drug combinations with the desired cytotoxicity and the related lowest dose of the drugs can be found. From the analysis of the calculated *NMDEI* surface, the drug combination with the highest synergistic effect can be chosen. Unlike traditional methods, the use of ANN allows to evaluate the cytotoxicity of all the possible combinations on the entire space defined by the chosen concentration intervals.

The copper(II) compounds Cu(1,10-orthophenanthroline)$(H_2O)_2(ClO_4)_2$, [Cu(1,10-orthophenanthroline)$_2(H_2O)$]$(ClO_4)_2$ or [Cu(1,10-orthophenanthroline)$_2$(imidazolidine-2-thione)]$(ClO_4)_2$ act synergistically with **CDDP**. Against *in vitro* tested cells, the optimal compositions of the combinations present an anti-proliferative effect 3 to 6 times greater than that of **CDDP** alone. The combinations with the maximum synergistic effect present high cytotoxic activity (from 74% to 83%), with required doses significantly lower than those needed for the individual drugs. The predicted combinations that presented the highest synergistic effect were actually prepared and experimentally tested. In all cases, the data predicted by the network were experimentally confirmed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.031.

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