

Mathematical Analysis of Drug-Drug Combinations under Non-Constant Ratio on the Cutaneous T-Cell Lymphoma Cell Line Hut-78

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Abstract

A set of designed experiments for evaluation of the combination effects between the alkylphosphocholine erufosine (ERF) and micellar curcumin with prolonged release (MCRM) combined in non-constant ratio were performed on the cutaneous T-cell lymphoma cell line HuT-78. Each non-constant ratio between ERF and MCRM doses consisted of five different concentrations of ERF (2, 4, 8, 16 and 32 μM) combined with one of three rationally chosen concentrations of MCRM (3.75, 7.5 or 15 μM). For the first time, a program with non-linear medium effect-dose model was coded in the MAPLE[®] software and used in all calculations. As a final calculation of the step by step methodology, the combination index (CI) was obtained to quantitatively determine the synergistic or additive effects of the designed combinations. The best synergism was achieved when using the lowest concentration of MCRM – 3.75 μM .

Keywords: modeling, non-constant ratio, combination index, erufosine, micellar curcumin, HuT-78 cells

Резюме

Върху клетъчната линия HuT-78 с произход от кожен Т-клетъчен лимфом бе проведен набор от планирани експерименти за оценка на комбинираните ефекти между алкилфосфохолина еруфозин (ERF) и мицелен куркумин с удължено освобождаване (MCRM), приложени в неконстантно съотношение. Всяко неконстантно съотношение между дозите на ERF и MCRM включваше комбинация на пет различни концентрации ERF (2, 4, 8, 16 и 32 μM) с една от три рационално избрани концентрации MCRM (3,75, 7,5 или 15 μM). За всички изчисления бе използвана за първи път програма с нелинеен модел за изчисление на корелацията доза-ефект, кодирана в софтуера MAPLE[®]. Като финален етап на алгоритъма за изчисление, бе получен комбинационния индекс (CI) за количествена оценка на синергичните или адитивните ефекти на планираните комбинации. Най-силен синергизъм бе постигнат след прилагане на най-ниската концентрация MCRM – 3,75 μM .

Introduction

The non-constant ratio for a combination of two or more drugs is a very popular method of studying their pharmacological potential for synergistic or additive effects. One of the most popular approaches for the calculation of drug combination effects in a non-constant ratio is based on the theory of Chou and Talalay (Chou and Talalay, 1984) which resulted in the CompoSyn Inc. software.

The latter is very widely used to calculate any possible drug-drug interactions. The basis of this software is a model of the medium effect of a single drug where IC_{50} is determined by the following equation:

$$D = D_m [F_a / (1 - F_a)]^{1/m}, \quad (1)$$

where D – stands for the dose; F_a and F_u are the fractions of the system – affected and unaffected by

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the dose D , respectively; D_m – represents the dose required to produce the median effect (in our case the IC_{50} value); “ m ” – is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve.

Since by definition, $F_a + F_u = 1$, this useful form of equation (1) is applied to describe many biological processes and proved its robustness because it is simple and does not require knowledge about the mechanism of inhibition. In the CompuSyn software, this non-linear equation is transformed into a linear form by representing it in a logarithmic form (Chou, 2006). Further, it is used in all calculations. In order to avoid all disadvantages of such an approach (linearization), we created a program in the MAPLE® software based on the non-linear identification procedure of model parameters and proved to be much better in many experiments with cell lines. Deep analysis and details can be found in our paper published elsewhere (Zaharieva *et al.*, 2018).

It must be noted that the algorithm for calculation of multiple drug effects (synergism, additive effect, and antagonism) requires the following steps to be made:

1. Dose-effect relationships for drug 1 (D_1), drug 2 (D_2), and their mixture $D_{1,2}$ (at a known ratio of drug 1 to drug 2) are required.

a. Measurements made with single doses of drug 1, drug 2, and their mixture can never alone determine synergism since the sigmoidicity of dose-effect curves and the exclusivity of drug effects cannot be determined from such measurements.

b. The dose-effect relationships should follow the basic mass-action principle relatively well (e.g. median-effect plots with correlation coefficients for the regression lines greater than 0.9).

c. Accurate determination of the model parameters “ IC_{50} ” and “ m ” sigmoidicity of dose-effect curves and the exclusivity of effects of multiple drugs are necessary. The slope of the median effect plot “ m ” gives a quantitative estimation of sigmoidicity.

Details about the quantification of these parameters with the different linear and non-linear procedures can be found in many works.

Attempts to interpret the effect of multiple drugs have a long history (Berenbaum, 1977). The pioneering work of Loewe about isobolograms (Loewe, 1928; 1953) and the fractional product concept of Webb in 1963 (Webb, 1963) opened new directions in the theoretical and practical aspects of the problem by studying the links between the

mechanisms of synergism and the methods of data analysis. In many works of Chou and co-workers (Chou and Talalay, 1983; Chou and Talalay, 1984; 1987; Chou and Martin, 2005) examples of applied formulas for evaluation of drug effects under the values of the CI index are presented. Quantitative evaluation of synergism, additive effect, and antagonism can be done on the base of a mathematical form of CI value. We will present the mathematical approach for calculating the combinational indices (CI) below. In the CompuSyn and MAPLE® programs, the CI values were determined on the basis of the already calculated IC_{50} of the single compounds D_1 and D_2 including their combination $D_{1,2}$ according to (Chou and Talalay, 1984) as follows:

1) For mutually exclusive drugs

$$CI_{(\alpha=0)} = D_1/Dx_1 + D_2/Dx_2 \quad (2)$$

2) For non-mutually exclusive drugs

$$CI_{(\alpha=1)} = D_1/Dx_1 + D_2/Dx_2 + \alpha \cdot ((D_1/Dx_1) \cdot (D_2/Dx_2)) \quad (3)$$

where $(Dx)_1$ = dose of a single drug 1 only to obtain 50% cell inhibition effect; $(D)_1$ = dose of drug 1 to obtain 50% cell inhibition effect in combination with $(D)_2$; $(Dx)_2$ = dose of single drug 2 only to obtain 50% cell inhibition effect; $(D)_2$ = dose of drug 2 to obtain 50% cell inhibition effect in combination with $(D)_1$; and the values of $\alpha = 0$ for mutually exclusive or $\alpha = 1$ for non-mutually exclusive actions of drugs. It should be noted that in equations (2) and (3) the values of D_x for single drugs are determined from equation (1).

According to the theory included in the software manuals, the CI values can be classified in details as follows: $CI > 1.3$ shows antagonism; $CI = 1.1$ to 1.3 moderate antagonism; $CI = 0.9$ to 1.1 additive effect; $CI = 0.8$ to 0.9 slight synergism; $CI = 0.6$ to 0.8 moderate synergism; $CI = 0.4$ to 0.6 synergism; and $CI = 0.2$ to 0.4 strong synergism.

In this paper, we are going to present experimental data and mathematical analysis of the non-constant combinations approach. For this aim, we investigated the combined effects of the alkylphosphocholine erufosine (ERF) (Zaharieva *et al.*, 2007; Zaharieva *et al.*, 2014) and micellar curcumin (MCRM) combined in a non-constant ratio experimental design. The evaluation was performed based on the theory of Chou and Talalay (Chou and Talalay, 1983) whereby we applied for the first time a non-linear model identification procedure coded in the MAPLE® software. ERF is a third-generation alkylphosphocholine with a promising antineoplastic activity on leukemia and lym-

phoma cell lines and antibacterial activity against the pathogenic bacteria *Staphylococcus aureus* in clinically relevant concentrations (Kaleagasioglu *et al.*, 2019; Zaharieva *et al.*, 2019). ERF has successfully passed the first phase of clinical trials in patients with chronic lymphocytic leukemia (Fiegl *et al.*, 2008). The preliminary results show that the plasma concentrations of 60 μM are well tolerated and can be achieved in humans without significant toxicity. Our previous publications revealed the potential of ERF for the treatment of CTCL (Yosifov *et al.*, 2014; Trochopoulos *et al.*, 2020) and therefore we chose for this study a cell line derived from a patient with Sézary syndrome, one of the most important subtypes of CTCL (Duvic, 2015). Curcumin (CRM) which is isolated from the plant *Curcuma long* (Zingiberaceae) is well known for its anti-inflammatory, antineoplastic and antibacterial effects (Aggarwal and Sung, 2009). CRM has also a gut activity against CTCL cell lines and because of its low toxicity is a suitable candidate for drug combinations (Trochopoulos *et al.*, 2020). As far as it is characterized by low solubility in water and fast metabolism, including it in micelles will increase its bioavailability and will provide opportunities for a water-soluble formulation. Our hypothesis for the synergistic potential of this combination is based on 1) the published knowledge about the antineoplastic mechanisms of action of both compounds on CTCL cells (Yosifov *et al.*, 2014; Trochopoulos *et al.*, 2020); 2) their antibacterial activity against Gram-positive bacterial pathogens as a single application and in combination (Zaharieva *et al.*, 2019) and 3) the fact that bacterial toxins, and especially the staphylococcal toxin can fuel the progression of CTCL. Considering the published data, we presumed that the combination between ERF and CRM will increase their antineoplastic effect. An additional benefit of this combination will be the antibacterial activity demonstrated in (Zaharieva *et al.*, 2019).

Materials and Methods

Mathematical models

The mathematical models used in this study are given in equations (1), (2), and (3). The dose of D_1 was kept constant while varying the D_2 doses. The CI values were calculated based on the of “ m ” and “ D_m ” values for every single drug and the combinations.

Drugs and chemicals

CRM was purchased from Sigma® Life Science (#C1386). ERF which was synthesized by

Prof. Eibl, MPI-Goettingen, Germany (Eibl *et al.*, 1992), was applied in all experiments after dilution of a stock solution (20 mM) prepared in 0.9% NaCl. The co-polymer methoxy poly(ethylene glycol)-block-poly(ϵ -caprolactone) (mPEG-PCL) purchased from Sigma® Life Science, Germany (#900649, mPEG-PCL) was used for the preparation of MCRM.

Curcumin loaded nano-delivery system

MCRM was prepared by dissolving mPEG-PCL and curcumin in dioxane at a ratio of 10:1 (wt/wt), gentle stirring (700 rpm, 30 min), and subsequent addition of purified water drop by drop. The physicochemical characteristics of the micelles (size, zeta-potential, and encapsulation efficiency) were determined in another study (data not shown) by photon correlation spectroscopy, electrophoretic laser doppler velocimetry (Zetamaster analyzer, Malvern Instruments, UK) and UV-spectrophotometry, respectively. The micelles were characterized by approx. 125 nm mean diameter, negative (-27 mV) zeta-potential, and approx. 80% encapsulation efficiency.

Cell lines and culture conditions

The T-cell lymphoma cell line HuT-78 (ATCC® TIB-161™) was selected as a cell model for evaluation of combination effects between ERF and MCRM applied in a non-constant ratio. The cell line originates from the American Type Culture Collection (ATCC). It was grown according to the recommendations of ATCC in a culture medium, supplemented with 4 mM L-Glutamine (#G7513, Sigma® Life Science, Germany), 20% fetal bovine serum (#FBS-HI-12A, Capricorn®, Germany), 25 mM HEPES buffer solution (#HEP-B, Capricorn®, Germany) and 4.5 g/l D-(+)-glucose (#G8769, Sigma® Life Science, Germany). The culture medium used for our experiments was RPMI-1640 without Phenol Red (#RPMI-XXA, Capricorn®, Germany) because our previous investigations showed that the presence of the dye Phenol Red interferes with the absorbance of curcumin and the MTT dye thus leading to false results (Trochopoulos *et al.*, 2020). Cells were maintained in concentrations between 5×10^4 and 8×10^5 viable cells/ml in a humidified atmosphere at 37°C and supply of 5% CO₂ (Panasonic CO₂ incubator, #MCO-18AC-PE, Japan).

Cell viability test

The combination effects between ERF and MCRM were measured using the MTT dye reduction assay (Mosmann, 1983) described in Annex C

of ISO 10993-5-2009 (ISO 10993-5:2009, 2017). Cells were seeded for the experiment in 96-well plates (3×10^5 cells/ml, 100 μ L/well) under sterile conditions (Laminar Air Flow Telstar Bio II Advance, Spain). The plates were incubated for 24 hours to allow the cells to enter the *log* phase of their growth. On the 24th hour, MCRM (D_1 in doses 3.75, 7.5, 15, 30, and 60 μ M) and ERF (D_2 in doses 2, 4, 8, 16, and 32 μ M) were applied as a single or combined treatment for an incubation period of 72 h. Every concentration was repeated fourfold. Three independent experiments were performed. Untreated cells served as a negative control. Cell viability was determined at the 72nd hour. Briefly, 10 μ L of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (#M5655-1G, Sigma® Life Science, Germany) dissolved in Phosphate Buffered Saline, pH 7.4 (#TS1101, HiMedia, India) to a concentration of 5 mg/ml and sterilized through filtration (0.22 μ m PES membrane filter) were added to each sample and the blanks. The blank samples contained only the culture medium and the compound or combination used for treatment. The plates were incubated for 3.5 h at 37°C. The formazan crystals formed by the dehydrogenases of the viable cells were dissolved with an appropriate organic solvent containing 5% HCOOH (Chimspektar OOD, Bulgaria) in isopropyl alcohol (#W292907-1KG-K, Sigma® Life Science, Germany). The absorption of the viable cells was measured on an Absorbance Microplate Reader EL-800 (Bio-Tek Instruments Inc., USA) at $\lambda=550$ nm against the blanks.

Statistics

GraphPad Prism software was used to calculate the mean absorbance (Am) and the effect (F_a) of each drug concentration, the untreated control, and the blank solution, as well as the standard deviations (SD). A Student *t*-test of the data was performed, as well whereby data were considered for statistically significant by $p \leq 0.05$. The Am values were presented as fractions from the untreated control (Am_f) and the F_a values were calculated according to the following equation: $F_a = 1 - Am_f$

Results and Discussion

In practice, the non-constant combinations approach has a meaning when the researcher needs to find out the range of changes of compounds D_1 and D_2 in order to maximize their combined effect of synergism in a simple way such as keeping D_1 constant while varying the D_2 doses. Therefore, obtaining the “*m*” and “ D_m ” values for every single drug is a base to calculate the CI value for each

combination data point under a non-constant ratio design. Additionally, the CI values for each data point on the F_a -CI graph will be available. In this section, we are going to show the results from the application of an algorithm based on the presented models which were described in section “Introduction”. Detailed analyses of synergism effects of applied by us experimental design of non-constant ratio of drugs doses will be demonstrated.

All experiments were performed with MCRM on HuT-78 cells for a 72 h incubation period (Table 1). The effects of erufosine as well as the combined effects of MCRM and ERF were studied for equal times of treatment (Table 2). MCRM in a single application diminished the cell viability by only 28% after treatment with the highest chosen dose 60 μ M.

Table 1. F_a of MCRM on HuT-78 cell line after 72 h of treatment

MCRM [μ M]	F_a^*	SD^{**}
0.00	0.000	0.006
3.75	0.068	0.011
7.5	0.051	0.016
15.00	0.095	0.006
30.00	0.108	0.007
60.00	0.283	0.006

* F_a stands for effect;

**SD stands for standard deviation.

All the data presented in Tables 1 and 2 were used to follow the strategy described in section “Introduction” to quantitatively evaluate the multiple drug effects – synergism, additive effect, and antagonism of the investigated combinations.

Equation 1 represents the simplest possible form of dose-effect relationship and it was used to fit the experimental data of D_1 and D_2 presented in Fig 1A, and B. Model parameters “ m ” and “ $D_m = IC_{50}$ ” were determined on the base of experimental data by using non-linear identification procedure coded in MAPLE software (Zaharieva *et al.*, 2018) unlike CompuSyn software that linearizes the model of equation (1) (Chou and Martin, 2005). Therefore, the dose and the effect are interchangeable since the dose (D) for any given degree of effect (F_a) in eq. 1 can be determined if the values for “ IC_{50} ” and “ m ” are known. The $IC_{50} = 18.85$ μ M value for erufosine (Fig.1A) is 10 times lower than that for MCRM ($IC_{50} = 191.55$ μ M). The correlation coefficients $R_1=0.988$ and $R_2=0.975$ for two drugs are much better than those determined by CompuSyn software. This is due to the advantages of using a non-linear

Table 2. Fa of erufosine and combinations between ERF and MCRM on HuT-78 cell line after 72 h of treatment

Concentration of ERF [μM]	ERF		ERF + 3.75 μM MCRM		ERF + 7.5 μM MCRM		ERF + 15 μM MCRM	
	F _a *	SD**	F _a *	SD**	F _a *	SD**	F _a *	SD**
0,00	0.000	0.006	-	-	-	-	-	-
2	0.096	0.016	0.072	0.013	0.051	0.016	0.105	0.019
4	0.192	0.009	0.227	0.005	0.274	0.018	0.292	0.037
8	0.292	0.024	0.541	0.033	0.537	0.007	0.487	0.012
16	0.525	0.013	0.670	0.023	0.594	0.003	0.594	0.003
32	0.701	0.020	0.804	0.021	0.705	0.022	0.705	0.022

*F_a stands for effect; **SD stands for standard deviation.

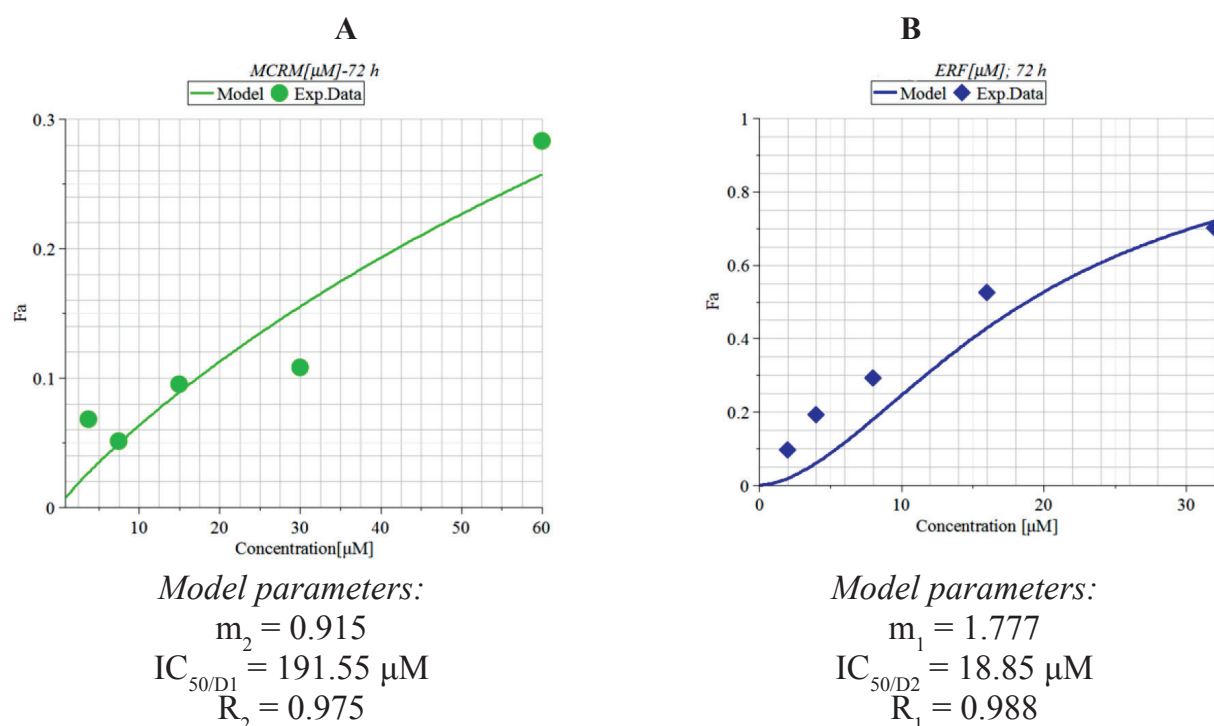


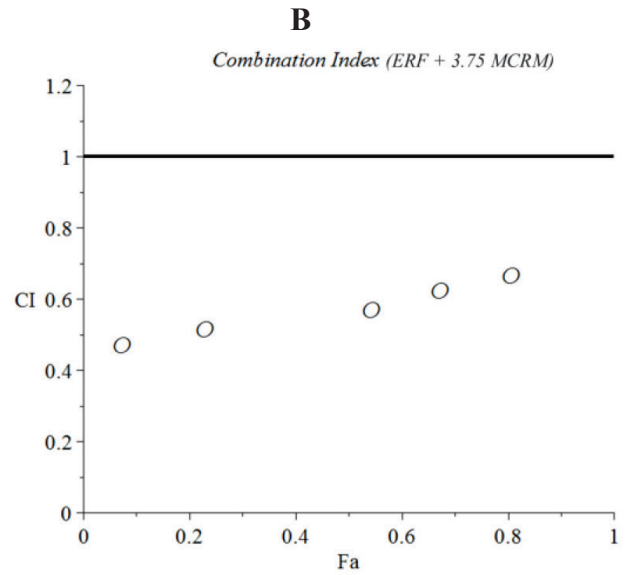
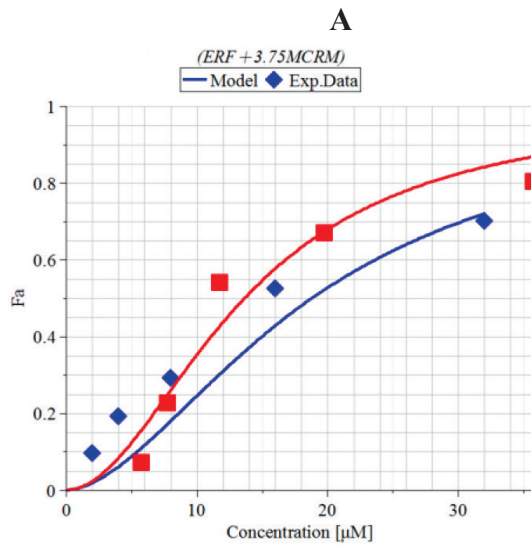
Fig. 1. Effects of ERF and MCRM as a single application on HuT-78 cells for 72 h.

identification procedure for the estimation of model coefficients. Further, experiments were performed with a combination of two drugs – ERF and 3.75 μM MCRM – under non-constant ratios between doses. Results are shown in Fig. 2A. It is visible that the dose of D_{1,2} significantly inhibited the cell line HuT-78 cells for 72 h. In Fig 2B the calculated CI values for each point are presented and all 5 points showed CI<1 for any given F_a which is indicative of a synergistic effect.

According to the theory, in the combination [ERF + 3.75 MCRM] we definitely succeeded to achieve a synergism (CI = 0.4 to 0.6 synergism) whereby the median inhibitory effect of erufosine in the combination ($\text{IC}_{50/\text{D1,2}} = 13.67 \mu\text{M}$) was lower than that of a single

When the combination [ERF + 7.5 MCRM] was applied a moderate synergism was obtained (CI = 0.6 to 0.8 moderate synergism, Fig 3B). The value of the median inhibitory effect of erufosine in the combination ($\text{IC}_{50, \text{D1,2}} = 18.85 \mu\text{M}$) was equal to that of erufosine as a single compound. Figure 3A demonstrates this relationship and the inhibition effect on the HuT-78 cell line after 72 h exposure to the combination.

In the case of the last combination [ERF + 15 MCRM], the increase of the MCRM concentration up to 15 μM MCRM was not in favor of a strong synergistic effect. In Fig. 4A we observed that the dose AB profile passed under the single drug 1 (ERF) profile. The median inhibitory effect of erufosine in the combination ($\text{IC}_{50, \text{D1,2}} = 26.1 \mu\text{M}$) was higher than that of



Model parameters of the combination
(red line, solid box):

$$m_{D1,2} = 1.96$$

$$IC_{50/D1,2} = 13.67 \mu M$$

$$R_{D1,2} = 0.984$$

CI values:

$$CI_{50alfa0}[1] = 0.467$$

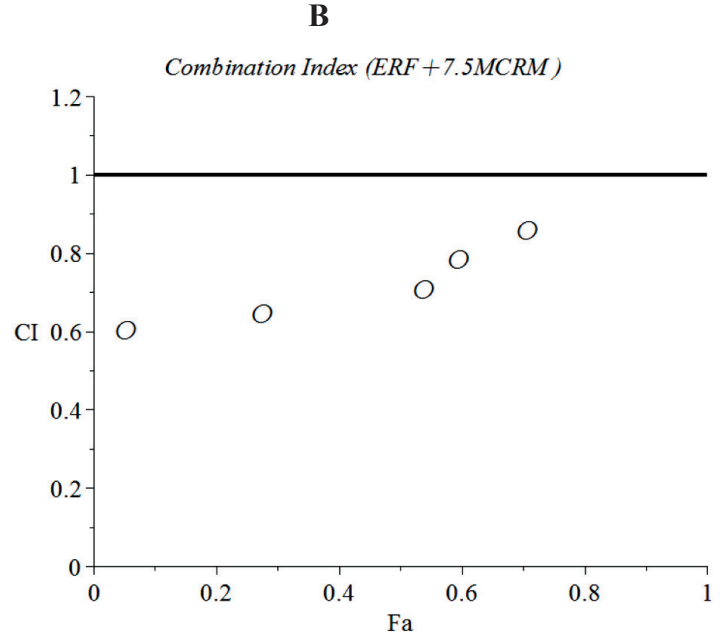
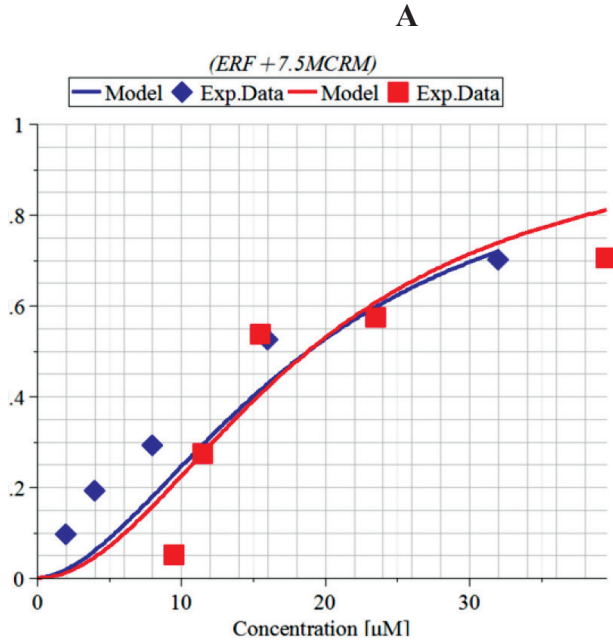
$$CI_{50alfa0}[2] = 0.512$$

$$CI_{50alfa0}[3] = 0.567$$

$$CI_{50alfa0}[4] = 0.621$$

$$CI_{50alfa0}[5] = 0.663$$

Fig. 2. Graphical comparison between the effects of ERF as a single application and the combination between ERF and 3.75 μM MCRM in the non-constant ratio on HuT-78 cells for 72 h



Model parameters of the combination
(red line, solid box):

$$m_{D1,2} = 1.96$$

$$IC_{50/D1,2} = 18.85$$

$$R_{D1,2} = 0.953$$

CI values:

$$CI_{50alfa0}[1] = 0.602$$

$$CI_{50alfa0}[2] = 0.644$$

$$CI_{50alfa0}[3] = 0.706$$

$$CI_{50alfa0}[4] = 0.782$$

$$CI_{50alfa0}[5] = 0.856$$

Fig. 3. Graphical comparison between the effects of ERF as a single application and the combination between ERF and 7.5 μM MCRM in the non-constant ratio on HuT-78 cell line for 72 h

erufosine as a single drug ERF ($IC_{50,D2} = 18.85 \mu M$) and this demonstrated a decrease of inhibition effect on HuT-78 cell line for 72 h (Fig. 4A). The value of the correlation coefficient $R_{D1,2} = 0.967$ (Figure 4A) is good enough and the analysis of the data cannot be misinterpreted. Figure 4B presents CI vs F_a where CI values were the highest from all designed experiments. The combination [ERF+ 15 MCRM] resulted in $CI = 0.8$ to 0.9 slight synergism and $CI = 0.9$ to 1.1 additive effect. It can be supposed that if the concentration of MCRM is increased above $15 \mu M$, most likely the combination of ERF and MCRM will represent antagonism. The third combination proved that increasing the concentration of MCRM was not in favor of the synergism between both compounds. Therefore, the concentration range of compounds 1 and 2 and their combinations must be carefully chosen in order to achieve the desired positive effect of synergism and additive effect without increasing the concentrations of the compounds above their median inhibitory concentration after a single application. Non-constant ratio in experimental design is a necessary step to localize the optimal range of drug concentrations. Further, having the knowledge database from a non-constant ratio, experiments with a constant ratio can be designed to search for the best effect of drug combinations.

Conclusion

Recently, a non-linear identification procedure was implemented in the MAPLE® software (Zaharieva *et al.*, 2018) in order to robustly evaluate the median dose-effect model (Chou and Talalay, 1984) widely used in medicine and pharmacy to calculate “ IC_{50} ” and “ m ” model parameters values of single drugs. The MAPLE® program was superior to the program used in CompuSyn software. Further, the combinations between drug doses for non-constant ratios were studied by applying CI as a criterion of synergism, additive effect, and antagonism. A set of designed experiments for the treatment of cancer cell line HuT-78 by erufosine and MCRM were performed. Three non-constant ratios between both compounds [ERF + $3.75 \mu M$ MCRM], [ERF + $7.5 \mu M$ MCRM], and [ERF + $15 \mu M$ MCRM] were applied and shown in Figures 1-4. In a step-by-step algorithm “ IC_{50} ” and “ m ” for single drugs and their combinations were determined. Nonlinear medium effect-dose model was used in all calculations. Combination index (CI) was calculated to qualitatively determine the synergistic and additive effects of the designed combinations. The best synergistic effect was achieved under the dose combination of [ERF + 3.75 MCRM] (Fig. 2 B). The lowest synergistic effect was observed when

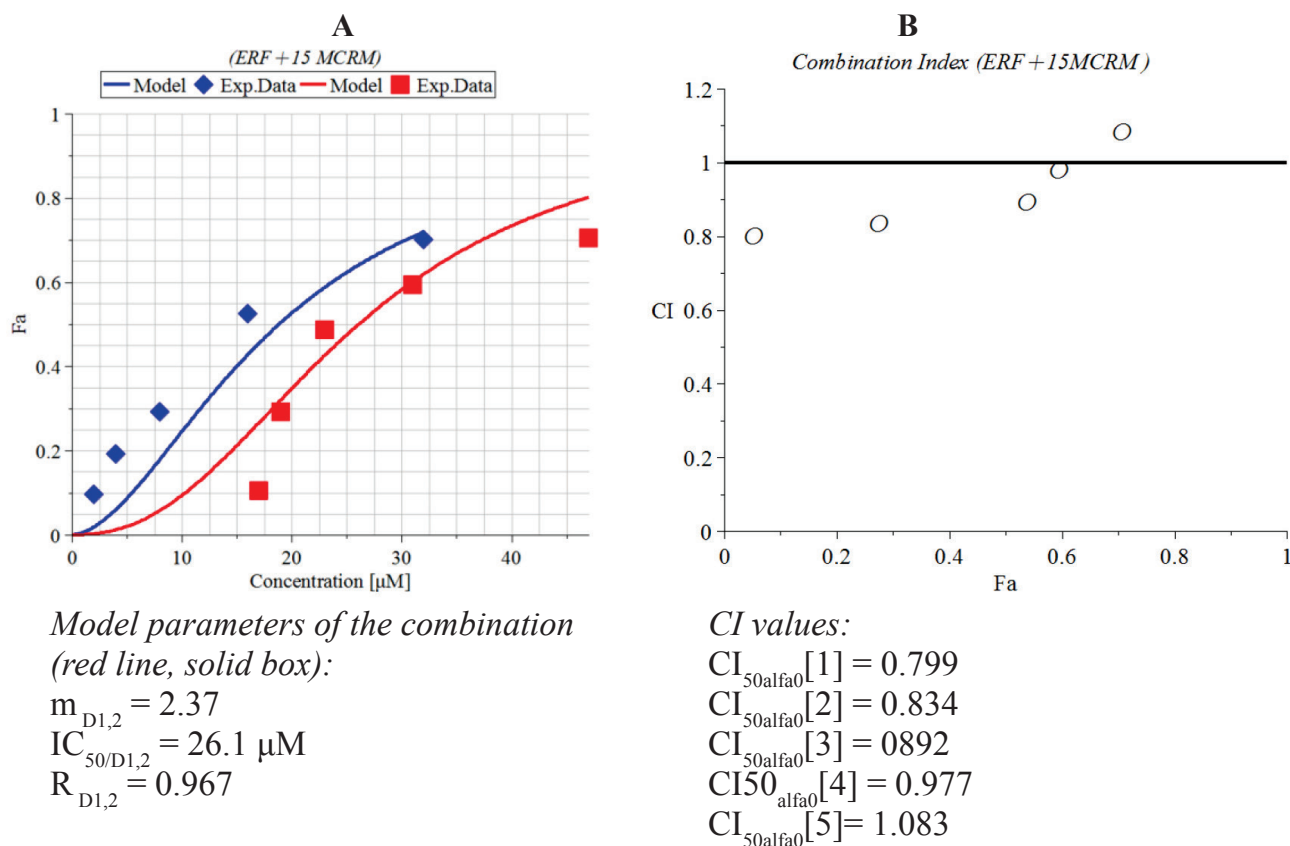


Fig. 4. Graphical comparison between the effects of ERF as a single application and the combination between ERF and $15 \mu M$ MCRM in the non-constant ratio on HuT-78 cells for 72 h

the concentration of MCRM increased up to 15 μM (Fig. 4 B). Hence, increasing the concentration of MCRM in this modality of non-constant dose ratio is not recommended because this diminishes the synergistic effect. From this study, the authors once again inspected the implemented software program in MAPLE[®] for modeling dose-effect experiments in cancer cell lines. The program robustly performed for different sets of experiments as well as when they were designed under the non-constant ratio of two chosen drugs. The described synergistic combination [ERF + 3.75 MCRM] is a perspective for further investigation of antineoplastic and antibacterial mechanisms of action.

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