New Insights in Routine Procedure for Mathematical Evaluation of *in vitro* Cytotoxicity Data from Cancer Cell Lines

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Abstract: *In oncopharmacology, the common procedure to evaluate median-effect concentrations (IC_{50}) on experimental data is based on the use of well-established kinetic models representing inhibition effects of drugs on human cancer cell lines. Several widespread software programs, such as GraphPad Prism and CompuSyn offer possibilities for calculation of IC_{50} through the model of Chou. In recent study, we analyzed the results from those two software programs and compared them with the non-linear programming procedure written by us in the MAPLE symbolic software. The last evaluated IC_{50} more precisely and the correlation coefficient R value was better in all trails. We demonstrated the efficiency of non-linear programming procedures in examples of two cancer cell lines treated with three different drugs. The response surface analysis showed the potential of the applied kinetic model. As a result, we were able to define better the IC_{50} values and to use them in planning further experiments in human cancer cell lines related to single drug influence and drug-drug interference.*

Keywords: *Non-linear programming, Kinetics models, Drugs, in vitro cytotoxicity, Human cancer cell lines.*

Introduction

The development of algorithms and optimal treatment approaches in modern medicine is based on theoretical achievements in the cell biology and the involvement of disease related signal transduction pathways in a complex hierarchic system. The deep and enhanced understanding of the biology of the disease is crucial, as it is a complex system with metabolic pathways, which are interconnected. Nevertheless, the simultaneous action of several drugs can tremendously increase the robustness and reliability of combination therapies [27]. This will reflect on overall efficiency, as toxicity will be substantially decreased and last but not leas the adaptation and development of drug resistance will be reduced. Therefore, the establishment of long winning therapeutic strategies in the medical practice often relays on synergistic drug combinations. If we use the neologism "-omics" (which refers to fields of study in biology known as genomics, proteomics or metabolomics), we should highlight that the recent advances in Omics and cell biology have a great impact on the increasing use of drug combinations in the modern medicine [16]. Much evidence of the superiority of combinations of drugs as compared to single agent use has been published already [5, 6, 14]. Some methodologies are discussed in details elsewhere [12, 25]. In the present study, we focus on median-dose effect methodology and its practical advantages and drawbacks during particular calculations by using the most popular software from CompuSyn Inc., which is based on the work of Chou and Martin [7].

The concepts of synergy/antagonism have been clearly defined as follows: they represent greater or lesser efficacy of the drugs in combination in comparison with the simple additive effect expected from each drug separately. However, their implementation into a robust and working methodology is still not an easy task to solve and requires much more than simplified solutions [5, 12]. It is useful to highlight the methodological basis of combination effect studies in order to understand effect-based approaches and dose-effect based approaches, in order to evaluate their practical advantages and limitations. Methods following an effect-based strategy compare the effect resulting from the combination of two drugs (E_{AB}) directly to the effects of its respective components (E_A and E_B). The exact decision process that allows a conclusion of positive, negative, or null combination effect can vary among four main strategies which are:

(1) Combination Subthresholding,
(2) Highest Single Agent,
(3) Response Additivity, and
(4) Bliss Independence model as previously described and explained in detail [11].

Dose-effect based strategy

The effect-based methods compare the influence of different agents having nonlinear dose-effect curves and search the amount or concentration of each component, which produces the same quantitative effect. The expected (additive) effect of a combination depends on the individual dose-effect curves and enables the formulation of unequivocal definitions of synergism, additivity, and antagonism. In particular dose-effect based approaches rely on the mathematical framework known as Loewe additivity, first defined by Loewe [20-23].

Mathematical base of dose-effect framework

Loewe additivity relies on both the dose equivalence principle (that for a given effect, dose a of drug A is equivalent to dose b_a of drug B, and reciprocally) and *the sham combination principle* (that b_a can be added to any other dose b of drug B to give the additive effect of the

combination). The additive effect of drugs *A* and *B* depends on the individual dose-effect curves and can be expressed as:

$$Effect(a+b) = E_A(a+a_b) = E_b(b_a+b) = E_{AB},$$

where E_A is measured on the dose-effect curve of drug *A*, ($a + a_b$) corresponds to the dose *A* giving the effect E_{AB} and for drug *B*, respectively. It can be assumed that the drugs have a constant potency ratio ($R = A/B$). In practice, dose-effect curves with constant potency ratio have a constant ratio of doses at each effect-level and hence are parallel on a log-dose scale, and have equal individual drug maximum effects [28]. From there, we define the relation between all pairs of doses (*a*, *b*) producing the combination effect E_{AB} and the single doses *A* and *B* necessary to reach this effect:

$$a + a_b = A \leftrightarrow a + bR = A \leftrightarrow a + bA/B = A,$$

which leads to the most influential mathematical relation of the Loewe additivity as basis of most dose-effect based approaches developed subsequently:

$$a/A + b/B = 1.$$

Practical limitations

It is most important to basically understand and identify main practical limitations and restrictions of each model of the combination analysis based on Loewe additivity.

One important issue is the accuracy of estimation of dose-effect curves in order to support the calculation of the effective doses (*A* and *B*) for a defined effect (E_{AB}). In most cases, the dose-effect relationship follows the Hill equation (also called sigmoid or logistic function) defined by:

$$F_a = F_a(\max)\, Dose^m / (IC_{50}^m + Dose^m), \quad (1)$$

where F_a stands for the effect reached at value of particular *Dose*; F_a(max) stands for the maximum effect; the median-effect concentration IC_{50} is the half maximum effective *Dose* and corresponds to the inflection point of the curve, and *m* stands for the shape parameter linked to the slope of the curve.

The estimation of dose-effect curves for the drug-drug interactions requires a certain amount of data (experimental data must be much larger than the number of model parameters, for example if Eq. (1) is used with 2 parameters. Therefore it is better to use at least 5-6 data points in order to obtain robust estimation of the parameter values. Some authors [18] pointed out that in some cases the identification procedure can rapidly become expensive as well as experimentally and computationally highly demanding, and makes the analysis of drug combination prohibitive. Hence, the Loewe additivity model becomes unusable when a dose-effect curve is not available or difficult to model [33]. This is the most common restriction for every unknown system. The ideal situation in medical and pharmaceutical practice is when the additive isoboles are representing straight lines. The researchers often do not use constant potency ratio (*R*), a situation that would apply when the individual log-dose-effect curves are not parallel and/or when the individual drug maximum effects differ and lead to curvilinear

additive isoboles [13]. The mentioned publication shows how in this case calculation of the Combination Index and the isobologram analyses can be performed.

Finally, a number of other algebraic and graphical approaches have been built based on Loewe's equations. Most are reviewed and discussed in Greco et al. [14]. It is at least worth mentioning the median-effect approach of Chou and Talalay, where combination effects are analyzed on the basis of the principle of mass action, and this has been the subject of numerous publications [3, 5, 6, 8]. On the base of their theoretical and practical achievements Chou and Talalay developed a strategy for evaluating drug-drug interactions. This strategy was coded in the CompuSyn software published and accessible in Internet. The CompuSyn software program is free of charge and provides detailed analysis of experimental data using mathematical models discussed in the user's manual of the software.

Before continuing, we have to address the problem of evaluating the single effect step studies. The *discovery step* is often dedicated to the *in vitro* screening of combinations including a set of candidate drugs administered at various doses in order to identify one or several combinations of interest [11]. For each drug, screening experiments should explore drug doses that span the anticipated region of activity upon and below the IC_{50} depending on the current state of knowledge. Furthermore, *in vitro* studies have to determine more precisely the applicability of combination effects selected from the *discovery step*. At this stage, the crucial point is to obtain knowledge about robust and precisely determined values of IC_{50} and m from single dose-effect curves. Further, these values (IC_{50} and m) determine or jeopardize the dose-effect approach based on *Loewe additivity* with *Combination index*, *Isobologram analysis* etc.

Therefore, the aim of this work is to systematize the knowledge about the *discovery step* by comparing the approaches applied in three different software programs (GraphPadPrism, CompuSyn and MAPLE) in order to find the best-evaluated values of IC_{50} and m. In our recent study the effects of the natural product curcumin and the synthetic drugs from the group of anticancer alkylphosphocholines (APC) on cell lines from cutaneous T-cell lymphoma (CTCL) were of particular interest. CTCL is a rare extranodal T-cell lympho-proliferative disorder (non-Hodgkin's lymphoma), which primarily affects the skin by clonal accumulation of neoplastic T-lymphocytes [1, 2, 26]. Most of the patients have poor prognosis and overall survival up to 5 years or less [29]. Treatment is often empiric and stage based because of the limited insight into the genetic basis of CTCL [9] and single drug therapy is usually not applicable. Therefore, the development of successful therapeutic strategies based on new drug combinations represents an effective algorithm to meet medical needs for cure of this orphan disease.

For the specific aim of this study, we obtained experimental data for calculation of the median-dose effects of curcumin and the APC miltefosine and erufosine by the treatment of two CTCL cell lines, HuT78 and MJ, representative for Sèzary syndrome and Mycosis fungoides, respectively. These cell lines were reported in previous studies of our and other research groups as sensitive to these compounds [17, 30-32] and therefore were chosen as suitable for providing robust experimental data for median-dose effects calculations. In addition, we used all the available theoretical information [8], which is a base for the extremely popular free of charge CompuSyn software [7]. We created our own program coded in MAPLE environment and compared the results of the three above mentioned software programs. The key difference between the programs was that our program used non-linear identification procedure to find the values of model parameters which will be explained in detail in the next paragraph.

Materials and methods

Mathematical models

Extremely popular in medicine and pharmacokinetic studies is the median-effect principle (MEP) first formulated by Chou [4]. The equation based on the mass-action law can be written as follows:

$$F_a / F_u = (Dose / D_m)^m , \quad (2)$$

where F_a stands for affected fraction; F_u stands for unaffected fraction $(1 - F_a) = F_u$; *Dose* stands for a dose of drug; D_m stands for a dose giving median-effect i.e. $D_m = IC_{50}$ (in our case); m is a slope of median-effect plot which means the shape of the dose-effect curve. For $m = 1$ the curve is hyperbolic; for $m > 1$ sigmoidal; for $m < 1$ negative (flat) sigmoidal.

Having in mind the above mentioned Eq. (2), we may write its logarithmic form in order to evaluate the two unknown model parameters D_m and m on the base of enough experimental data:

$$\log(F_a /(1 - F_a)) = m \log(Dose) - m \log(D_m), \quad (3)$$

where y is $\log(F_a /(1 - F_a))$, x is log(*Dose*), a is a slope m and b is (y-intercept) = $-m\log(IC_{50})$.

If we accurately determine the values of $D_m = IC_{50}$ and m by using linear regression procedure, we will be able to determine the value of *Dose* for every given F_a and vice versa. Chou used the principle of analogy from enzyme kinetics (Michaelis-Menten equation and its graphical analysis by Lineweaver-Burk's plot, 1934 [19]); similar approach is used in microbiology when working with Monod's equation) and after rearrangement developed an equation and linearization method for determination of its parameters. It is well known that such linearization approach and its use are very limited because of the dependence on experimental data errors and values of *Dose* as an independent variable. The median-effect plot of Chou in a form of the straight line $y = ax + b$ in CompuSyn software is as follows:

$$\log(F_a / F_u) = m \log(Dose) - m \log(IC_{50}) . \quad (4)$$

From here, at the median-effect dose $F_a = F_u = 0.5$, $\log(F_a/F_u) = 0$ and $\log(Dose) = \log(IC_{50})$. The x-intercept of the plot stands for $\log(IC_{50})$, and IC_{50} can be calculated from antilog of the x-intercept $IC_{50} = 10^{-(y-intercept/m)}$. Further, the values of the statistical criterion (R) show the goodness of fitting the experimental data to this equation and its validity. $R = 1$ shows perfect conformity of the data to the applied equation and its assumption. $R < 1$ indicates the decreasing of the correlation between mathematical model and real experimental data.

Hence, the *discovery step* is completed by determination of the evaluated values of IC_{50} and m. The use of IC_{50} value of a single drug is critically important for planning the experiments in order to find the effects of drug-drug interactions in particular cell lines.

In order to avoid any drawbacks mentioned above and about linear regression application applied to Chou median-effect model (Eq. (2)), we developed the non-linear regression procedure coded in MAPLE® software of symbolic mathematics based on weighted least squares statistical criterion as an objective function of the search. Further, numerical

optimization algorithm was used in order to minimize the sum of weighted squares and to find the estimates of best-fitting parameter values. We applied the median-dose model (Eq. (2)), obtained the evaluated values of IC_{50}, m and R, and compared them with those calculated by the commercially available GraphPad Prism software and the CompuSyn software of Chao and Martin using the same sets of experimental data on tumor cell lines. Moreover, we used the Response Surface Analysis (RSA) methodology in order to show the predictive power of the model (Eq. (2)) as a function of the parameters' values IC_{50} and m. The range of their values changes in the RSA 3D plot were determined on the base of standard deviation of IC_{50} and m obtained during the statistical evaluation of the experimental data by GraphPad Prism software (inhibition dose-response model: *log* inhibitor versus normalized response). Our approach showed the usefulness of the applied model for the robust prediction of the experimental data for a particular tumor cell line.

Drugs and chemicals

Curcumin was purchased from Sigma® Life Science (#C1386); the working solution was prepared prior usage in absolute ethanol (#46139, Sigma® Life Science) at a concentration of 10 mM. Miltefosine (#M5571, Sigma® Life Science) was disolved in ethanol/PBS (1:1, v/v) to stock concentrations of 10 mM and stored at 4 °C. The compound erufosine, synthesized by Prof. Eibl, MPI-Goettingen, Germany [10], was applied in all experiments after dilution of a stock solution (20 mM) prepared in 0.9% NaCl.

Cell lines and culturing procedure

The experimental data were obtained after treating the T-cell lymphoma cell lines HuT78 (ATCC® TIB-161™) and MJ (ATCC® CRL-8294™) with the selected compounds. The cell lines were purchased from the American Type Culture Collection and grown in RPMI-1640 without Phenol Red (#RPMI-XRXA, Capricorn®, Germany), supplemented with 4 mM L-Glutamin (#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). Cells were incubated in a humidified atmosphere by 37 °C and 5% CO_2 (Panasonic CO_2 incubator, #MCO-18AC-PE, Japan) and maintained in concentrations between $5 \cdot 10^4$ and $8 \cdot 10^5$ viable cells/ml.

Cell viability assay

The effects of the three test compounds were estimated using the MTT reduction assay [24] with some modifications based on ISO 10993-5-2009 [15]. Briefly, prior treatment cells were seeded in 96-well plates ($3 \cdot 10^5$ cells/ml) under sterile conditions (Laminar Air Flow Telstar Bio II Advance, Spain), incubated for 24 hours until entering the *log*-phase of the growth curve, treated thereafter with curcumin (0-100 μM), erufosine (0-25 μM) or miltefosine (0-25 μM) and incubated for 72 h. The dose density for each compound was five doses (plus an untreated control) in serial twofold dilutions.

All experiments were performed in triplicate, wherein every sample was repeated four times. Cell viability was determined after adding of 10 μl 5 mg/ml solution of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (#M5655-1G, Sigma® Life Science, Germany) in Phosphate Buffered Saline, pH 7.4 (#TS1101, HiMedia, India) and incubating of the plate for 3.5 h at 37 °C. Formazan crystals were dissolved by an equivalent volume of 5% HCOOH (Chimspektar OOD, Bulgaria) inisopropyl alcohol (#W292907-1KG-K, Sigma® Life Science, Germany). Absorption was measured on an Absorbance Microplate

Reader ELx-800 (Bio-Tek Instruments Inc., USA) at λ = 550 nm against a blank solution (culture medium, MTT and solvent).

Statistics

The mean absorbance (*Am*) and the effect (F_a) of each concentration, the untreated control and the blank solution, as well as the standard deviations (*SD*), were calculated with the GraphPad Prism software. A Student *t*-test of the data was performed with the same software. The *Am* values were presented as fractions from the untreated control (Am_f) and the F_a values were calculated as follows:

$$F_a = 1 - Am_f \,. \tag{5}$$

Results and discussion

In this study, we presented a set of analysis on median-effect dose methodology using a non-linear programming procedure coded by us in the MAPLE symbolic software. In all simulations and fitting of experimental data the median-dose effect model of Chou was used (Eq. (2)). The dose-range of the drugs and compounds used was chosen based on the sensitivity of the cells, so that the experimental points were below and above the median-effect doses, where this was possible. Serial fivefold and tenfold dilutions were avoided according to the recommendations of the CompuSyn software [7].

The presented non-liner approach for calculation differs from the methods used in CompuSyn by the possibility to analyze a wide range of data and because it gives the advantage of superiority when the particular experimental points were considered as the key ones and the model has to pass through them. The objective function (OF) (weighted least squares) can be modified in the way to be more sensitive to the small changes of the parameters, which accelerate the search of its minimum and gives the best fitting of experimental data to the model. The NLPSolve command in MAPLE solves a non-linear program (NLP), which is computing the minimum (or maximum) of a real-valued objective function (OF) subject to constraints. In our case, the OF was built on weighted least-squares. Generally, a local minimum is returned unless the problem is convex. In some cases, global search is available as described in the MAPLE manual.

Most of the algorithms used by the NLPSolve command are assuming that the objective function and the constraints are twice continuously differentiable. It is important to notice, that NLPSolve command will succeed sometimes and even if these conditions are not met. In our case (nonlinear evaluation of two parameters values of the median-dose effect model), modified Newton's method was used as the most suitable for all studies. The obtained results by using this method were compared with the linearization method of CompuSyn and GraphPad Prism reports and always showed superiority during the fitting procedure by minimizing the OF value, respectively maximizing the value of correlation coefficient *R*. The simulation results by fitting the experimental data will be compared and discussed in details below.

Effect of curcumin on HuT78 cells

The IC_{50} value for curcumin (CRM) on the cell line HuT78 was calculated after treatment of the cells with 6 concentrations of the drug ranging from 0.0 to 100 μM. The results showed dose-dependent increase of F_a of CRM which was in line with data published in previous studies [31]. F_a 0.9 or higher was achieved after application of concentrations ≥ 50 μM

(Table 1). The IC_{50} was between 25 and 50 μM. The data (Table 1) were analyzed with the GraphPad Prism, CompuSyn and MAPLE software and the parameters IC_{50}, m and R were compared (Table 2).

Table 1. F_a of CRM on HuT78 cell line after 72 h of treatment

CRM concentration, [μM]	F_a*	SD**
0.0	0.000	0.025
6.25	0.001	0.022
12.5	0.001	0.014
25.0	0.128	0.128
50.0	0.935	0.014
100.0	0.906	0.039
*F_a stands for effect, **SD – for standard deviation		

Table 2. Determination of m and IC_{50} by non-linear (MAPLE Report) and linearization methods (CompuSyn Report, GraphPad Prism Report)-HuT78 cells after 72 h treatment with CRM

Parameters	Software reports		
	MAPLE	CompuSyn	GraphPad Prism
m	6.58	4.028	6.56 (4.682-8.448)*
IC_{50}, [μM]	33.62	42.74	33.48 (30.53-36.72)* [μM]
R	0.9998	0.9411	0.9806
*95% confidence interval was taken from the GraphPad Prism statistics evaluation.			

Analyzing the experimental data (Table 1) obtained for CRM in HuT78 cells, it is obvious that the values of m and IC_{50}, determined with the MAPLE software, corresponded to the highest R = 0.9998 value and were also in the range of the 95% confidence interval given by the GraphPad Prism software (Table 2). This, practically, can be considered as a perfect match between the model and the data (Fig. 1). The result obtained for the IC_{50} value by CompuSyn report was outside of the 95% confidence interval range and this reflected on the lowest value of R = 0.9411. The GraphPad Prism software showed comparable results with those of MAPLE with slightly lower value of R = 0.9806. Therefore, the computational results from MAPLE and GraphPad Prism software were very robust and reliable compared to those in CompuSyn report. In Fig. 1 the model behavior for the optimal value of parameters is shown (Table 2) when using the data from Table 1.

RSA studies were designed in 3D form in order to show the power of the model and its sensitivity for the changes of m and IC_{50} values. Noteworthy, the ranges of these constants were chosen to be the same as those in the 95% confidence interval range calculated by the GraphPad Prism program (see Table 3). This is especially important when the researcher deals with higher deviation of his experimental data. Not all cell lines followed the perfect inhibitory curve because of the specific differences in the human cell lines biology, reflecting the different biochemical pattern of growth inhibition under the influence of different drug's concentrations. In all RSA studies, points stand for experimental data.

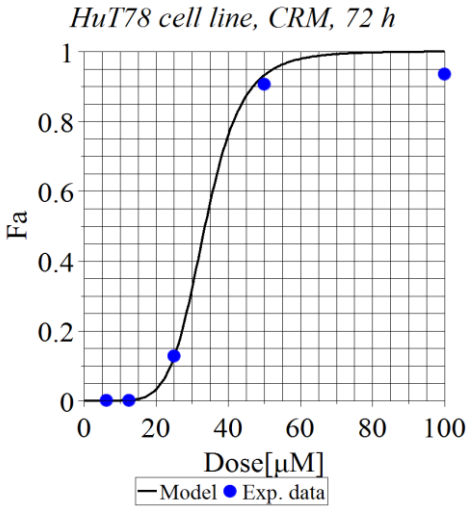
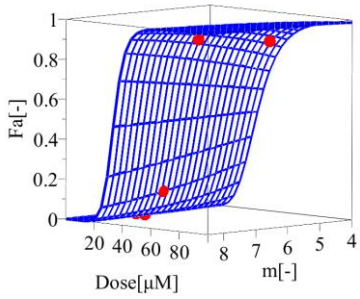
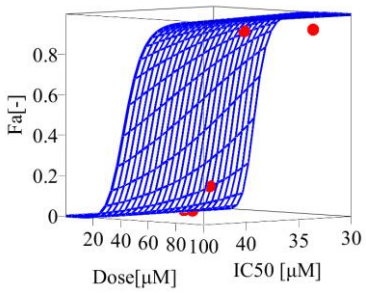


Fig. 1 Non-linear identification of parameters based on experimental data obtained from the cell line HuT78 after single drug treatment with CRM for 72 h

Table 3. RSA by using the median-dose effect model of Chou – HuT78 cells treated with CRM for 72 h

MAPLE RSA	
Dose = 0.1-100 [μM]	
m = 4.0-8.5	*m* = 6.58
IC_{50} = 33.62 [μM]	IC_{50} = 30-44 [μM]
RSA-HuT78-CRM,[μM] 	RSA-HuT78, CRM,[μM] 

Effect of ERF on HuT78 cells

The IC_{50} of ERF for the cell line HuT78 was calculated after treatment of the cells with 6 concentrations of the drug ranging from 0.0 to 25 μM. The dose-dependent increase of F_a (ERF) confirmed the tendency from previously published data of our research group [31]. The increase in the IC_{50} value as compared to previous studies was due to the use of higher content of FBS in the culture media. Concentration of 25 μM led to F_a = 0.7 (Table 4). The IC_{50} value or the median dose effect was between 12.5 and 25 μM. The experimental data analyzed with the GraphPad Prism, CompuSyn and MAPLE software programs and the parameters IC_{50}, *m* and *R* are presented in Table 5.

Table 4. F_a of ERF on HuT78 cell line after 72 h of treatment

ERF concentration, [μM]	F_a*	SD**
0.0	0.000	0.019
1.563	0.101	0.03
3.125	0.131	0.031
6.25	0.247	0.026
12.5	0.409	0.022
25.0	0.704	0.015
*F_a stands for effect, **SD – for standard deviation		

Table 5. Determination of *m* and IC_{50} by non-linear (MAPLE Report) and linearization methods (CompuSyn Report, GraphPad Prism Report) – HuT78 cells after 72 h treatment with ERF

Parameters	Software reports		
	MAPLE	CompuSyn	GraphPadPrism
m	1.379	1.1	1.286 (1.116-1.455)*
IC_{50}, [μM]	14.12	14.668	14.55(13.24-15.98)* [μM]
R	0.9962	0.9784	0.9774
*95% confidence interval was taken from the GraphPadPrism statistics evaluation.			

The ERF experimental data (Table 4) on HuT78 cells, were well described by the three applied programs and the evaluated values of *m* and IC_{50} were inside the range of the 95% confident interval. Once again, the highest *R* value (R = 0.9962, Table 5) was obtained by the MAPLE program. Noteworthy, the results presented by CompuSyn report were very reliable and comparable with the two other programs. Also, the values of *m* and IC_{50} were inside the range of the 95% confidence interval. Moreover, the R = 0.9784 value was slightly higher than the one obtained by GraphPad Prism – R = 0.9774. Therefore, the chosen range of ERF concentrations on HuT78 cells and performance of the experimental study were excellently executed. Fig. 2, shows the model and the experimental data (Table 4) according to the MAPLE report. It must be noticed, that (if it is possible) the experimental verification of the IC_{50} value may differ from the computational one, but obviously will fall within the range of confidence interval given by statistical evaluation of GraphPad Prism.

We observed similar behavior of RSA simulations on ERF and HuT78 cell line (Table 6). The deviation of the model from the experimental data (see points) for the chosen 95% confident interval was not very high. Therefore, all values of the constants can be considered reliable and robust.

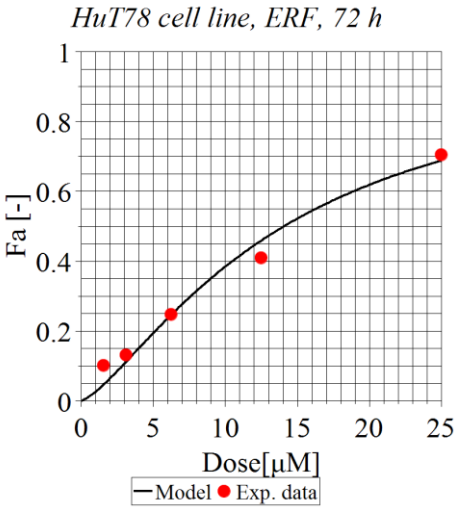
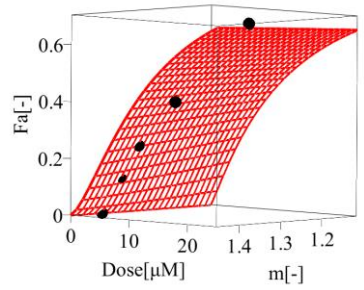
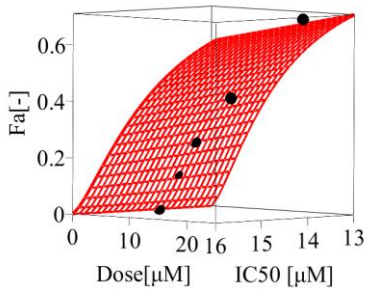


Fig. 2 Non-linear identification of parameters on the base of experimental data from HuT78 cell line and single drug ERF, 72 h

Table 6. Response Surface Analysis by using the median-effect model of Chou – HuT78 cells treated with ERF for 72 h

MAPLE RSA	
Dose = 0.1-25 [μM]	
m = 1.116-1.5	m = 1.379
IC_{50} = 14.12 [μM]	IC_{50} = 13-16 [μM]
RSA-HuT78-ERF,[μM] 	RSA-HuT78, ERF,[μM] 

Effect of CRM on MJ cells

The IC_{50} of CRM for the cell line MJ was calculated after treatment of the cells with 6 concentrations of the drug ranging from 0.0 to 80 μM. The effect F_a of CRM increased on a dose-dependent manner confirming our previous results [31]. Concentrations higher than 40 μM led to $F_a \geq 0.8$ (Table 7). The IC_{50} value was between 12.5 and 25 μM. The data were analyzed by applying the GraphPad Prism, CompuSyn and MAPLE software and the parameters IC_{50}, m and R were compared in Table 8.

Table 7. F_a of CRM on MJ cell line after 72 h of treatment

CRM concentration, [μM]	F_a*	SD**
0.0	0.000	0.033
5.0	0.000	0.021
10.0	0.102	0.003
20.0	0.566	0.051
40.0	0.857	0.014
80.0	0.918	0.011
*F_a stands for effect, **SD – for standard deviation		

Table 8. Determination of m and IC_{50} by non-linear (MAPLE Report) and linearization methods (CompuSyn Report, GraphPad Prism Report) – MJ cells after 72 htreatment with CRM

Parameters	Software reports		
	MAPLE	CompuSyn	GraphPadPrism
m	2.9	3.26	2.886 (2.1-3.67)*
IC_{50}, [μM]	19.37	26.53	19.27 (17.38-21.36)* [μM]
R	0.9985	0.9446	0.9822
*95% confidence interval was taken from the GraphPadPrism statistics evaluation.			

By analyzing the experimental data for MJ cells (Table 7), one may figure out that MAPLE values of m and IC_{50} not only fall within the 95% confident interval defined by the GraphPad Prism program, but also determine the highest value for R (R = 0.9985, Table 8), i.e. they are closer to the perfect match between the model and the data as compared to the other two programs (Fig. 3). The CompuSyn results for the same parameters showed that the IC_{50} value was outside the range of 95% confidence interval. Accordingly, this reflected on the lowest value of R = 0.9446.

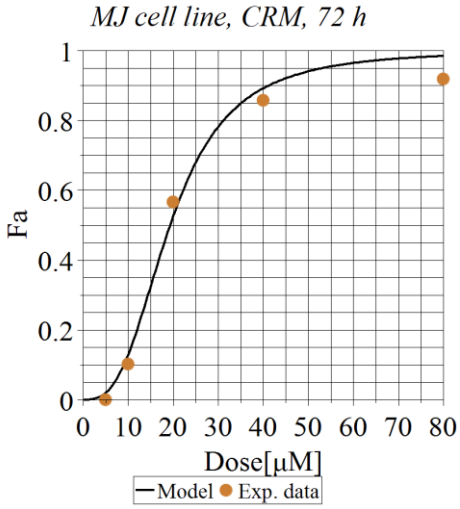
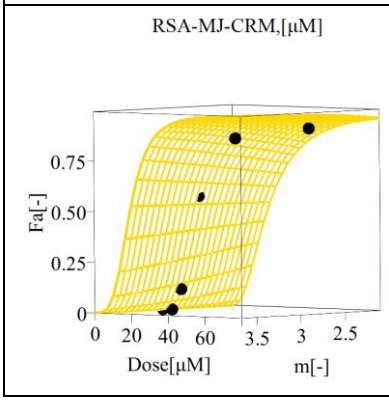
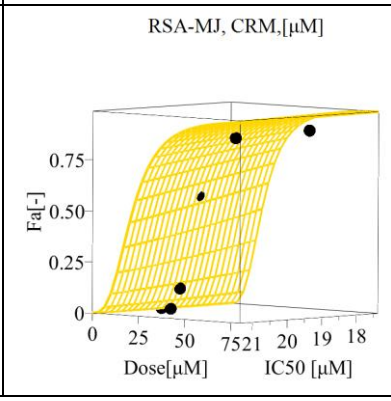


Fig. 3 Non-linear identification of parameters on the base of experimental data from MJ cell line and single drug CRM, 72 h.
The median-dose effect model of Chou was coded in MAPLE software.

The behavior of RSA simulations on CRM and MJ cells is shown in Table 9. The deviation of the model from experimental data (see points) for the chosen 95% confident interval was not very high, which means that the values of all constants in the chosen ranges of *m* and IC_{50} can be considered as reliable and robust.

Table 9. Response Surface Analysis by using the median-effect model of Chou – MJ cell, treated with CRM for 72 h

MAPLE RSA	
Dose = 0.1-80 [μM]	
m = 2.1-3.67	*m* = 2.9
IC_{50}=19.37 [μM]	IC_{50} = 17.38-21.36 [μM]
	

Effect of MLT on MJ cells

The median-effect dose of MLT on the MJ cell line was calculated after treatment of the cells with 6 concentrations of the drug ranging from 0.0 to 25 μM. The dose-dependent increase of F_a of MLT and the IC_{50} value did not differ from previously published studies of our research group [31]. Concentration of 25 μM led to F_a = 0.46 (Table 10). The *m* and IC_{50} values obtained by GraphPad Prism, CompuSyn and MAPLE (Table 11) fell within the 95% confidence interval. The MAPLE evaluated values of constants and correlation coefficient showed perfect match between the model and the experimental data (Fig. 4). In this particular case, the CompuSyn' report showed better analysis than that generated from the GraphPad Prism software.

Table 10. F_a of MLT on MJ cell line after 72 h of treatment

MLT concentration, [μM]	F_a*	**SD****
0.0	0.0	0.013
1.563	0.109	0.037
3.125	0.140	0.022
6.25	0.221	0.028
12.5	0.351	0.033
25.0	0.458	0.028
*F_a stands for effect, **SD – for standard deviation		

Table 11. Determination of *m* and IC_{50} by non-linear (MAPLE Report) and linearization methods (CompuSyn Report, GraphPad Prism Report) – MJ cells after 72 h treatment with MLT

Parameters	**Software reports**		
	MAPLE	**CompuSyn**	**GraphPadPrism**
m	0.650	0.73085	0.757 (0.658-0.8574)*
IC_{50}, [μM]	30.574	31.9235	30.5 (25.78-36.07)* [μM]
R	0.9989	0.9934	0.9737
*95% confidence interval was taken from the GraphPadPrism statistics evaluation.			

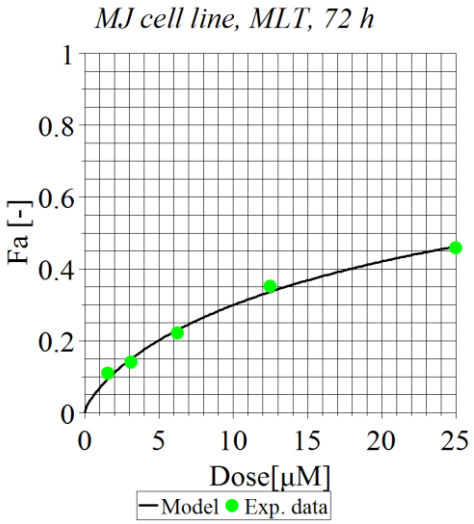
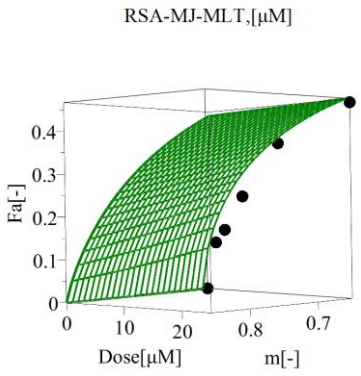
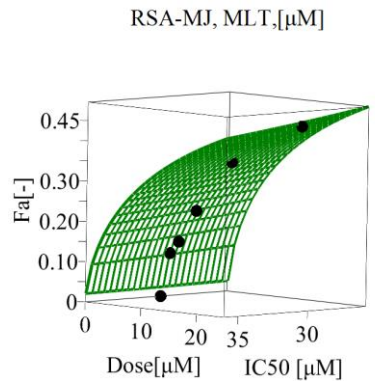


Fig. 4 Non-linear identification of parameters on the base of experimental data from MJ cell line and single drug MLT, 72 h

The RSA simulations on MLT and MJ cell line are shown in Table 12. A slight deviation of the model from experimental data (see points) for the chosen 95% confidential interval was observed, which means, that for the chosen experimental concentrations, the model was more sensitive to the changes of *m* and IC_{50} values as compared to the previously analyzed RSA data.

To perform all RSA studies, we used the 95% confidence interval of parameter values obtained from the GraphPad Prism statistics evaluation. By using this approach, we were able to preserve all the knowledge of statistics and modeling and to find the flexibility and reliability of the model parameter values *m* and IC_{50} describing experimental data. As it can be seen from the RSA simulations, the changes of the *m* and IC_{50} for the given interval of confidence did not show drastic deviations from the trend of real experimental data. It is noteworthy, that in all our previously studied cell lines, this was not the common model behavior (data not shown) and this finding can be explained by the nature of the processes and mechanisms involved.

Table 12. Response Surface Analysis by using the median-effect model of Chou – MJ cells, treated with MLT for 72 h

MAPLE RSA	
Dose = 0.1-25 [μM]	
m = 0.658-0.8574	m = 0.658
IC_{50} = 30.574 [μM]	IC_{50} = 25.78-36.07 [μM]
	

Conclusion

In pharmacodynamics, common procedure is to evaluate IC_{50} from experimental data using well-established kinetic models. They should reflect inhibition effects of drugs on human cancer cell lines and based on a widespread linearization method coded in software programs such as GraphPad Prism and CompuSyn. We analyzed the obtained results from those two software programs and compared them with the non-linear programming procedure coded by us in the MAPLE symbolic software. Furthermore, we evaluated m and IC_{50} values very precisely and the correlation coefficient R in all trails reached values above 0.99. It was evidenced, that the performed experiments with the studied cancer cell lines were extremely well designed and executed as well. We demonstrated on several examples reliability of this approach by including RSA simulations in order to highlight the potential of the applied kinetic model. Based on the simulation results we were able to interpret the m and IC_{50} values better and to use them in planning active experiments in both single drug influence and drug-drug interference on human cancer cell lines. RSA studies showed the sensitivity of the model to the changes of m and IC_{50} values inside the statistically determined 95% confidence interval estimated by GraphPad Prism program. The developed methodology can be successfully applied when using more sophisticated non-linear kinetic models, reflecting specific cell signal transduction changes in terms of sub-cellular metabolic mechanisms or enzymatic inhibitions.

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