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Mathematical models of cytotoxic effects in endpoint tumor cell line assays: critical assessment of the application of a single parametric value as a standard criterion to quantify the dose–response effects and new unexplored proposal formats†

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The development of convenient tools for describing and quantifying the effects of standard and novel therapeutic agents is essential for the research community, to perform more precise evaluations. Although mathematical models and quantification criteria have been exchanged in the last decade between different fields of study, there are relevant methodologies that lack proper mathematical descriptions and standard criteria to quantify their responses. Therefore, part of the relevant information that can be drawn from the experimental results obtained and the quantification of its statistical reliability are lost. Despite its relevance, there is not a standard form for the *in vitro* endpoint tumor cell lines' assays (TCLA) that enables the evaluation of the cytotoxic dose–response effects of anti-tumor drugs. The analysis of all the specific problems associated with the diverse nature of the available TCLA used is unfeasible. However, since most TCLA share the main objectives and similar operative requirements, we have chosen the sulforhodamine B (SRB) colorimetric assay for cytotoxicity screening of tumor cell lines as an experimental case study. In this work, the common biological and practical non-linear dose–response mathematical models are tested against experimental data and, following several statistical analyses, the model based on the Weibull distribution was confirmed as the convenient approximation to test the cytotoxic effectiveness of anti-tumor compounds. Then, the advantages and disadvantages of all the different parametric criteria derived from the model, which enable the quantification of the dose–response drug-effects, are extensively discussed. Therefore, model and standard criteria for easily performing the comparisons between different compounds are established. The advantages include a simple application, provision of parametric estimations that characterize the response as standard criteria, economization of experimental effort and enabling rigorous comparisons among the effects of different compounds and experimental approaches. In all experimental data fitted, the calculated parameters were always statistically significant, the equations proved to be consistent and the correlation coefficient of determination was, in most of the cases, higher than 0.98.

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1. Introduction

A wide range of *in vitro* TCLA endpoint assays have been developed for the discovery of therapeutic molecules,[1] investigation of their mechanisms of action,[2–4] and study of signal transduction, cell division, and other biological processes using chemical biology approaches.[5] In general, these assays use established tumor cell lines from human patients or animals and differ in the mechanism of displaying the tumor cell response and/or target molecules, and in the end products measured. The methods described include tetrazolium reduction (MTT, MTS, XTT, and WST-1), resazurin reduction, sulforhodamine B (SRB), protease markers, and ATP detection.[6–8] Each assay has its own set of advantages and disadvantages and it is difficult to point to the most convenient assay that conducts the best evaluation. These

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techniques still left many open questions, only ranking the cytotoxicity in their reaction system.

Originally, most TCLA were performed in a "single-test" response format, and the cell counts were determined after several steps, preventing researchers from testing a large number of samples simultaneously. At that time, the lack of proper application of mathematical models turned out simple formalisms, which were more practical.[9,10] Since the development of equipment able to perform multiple analyses (such as microplate readers), new protocols have been developed to reconfigure the methods extending the applicability of the methodologies, enabling higher numbers of samples, reducing the quantity of reagents and increasing the precision and reproducibility.[11]

Although consistent response results are obtained with TCLA, the quantification of the results has been left in the oldest format with simple calculation formalisms to abbreviate the testing procedure. The quantification relies on the determination of the dose that would decrease the tumor cell population down to 50%, commonly known as the 50% lethal dose (LD_{50}). Although cytotoxic results are expressed fundamentally by the LD_{50} values, other typical metric values are also used such as the fraction of viable cells at the highest agent concentration (E_{max}) or the area under the dose–response curve (AUC).[5] Their calculation can be obtained by non-mathematical means (graphical analysis, linear interpolations, *etc.*) or by mathematical expressions (typically by sigmoidal curves). The application of non-mathematical means has caused much controversy because: (1) the LD_{50} values are less reproducible and highly depend on the slope of the response;[12] (2) it prevents the quantification of its statistical reliability;[9] (3) increases the accumulation of procedural restrictions that over-standardize the protocol;[13] and (4) loses a part of the relevant information that can be drawn from the experimental results.[14] When mathematical expressions are applied, comprehensive metric information of the dose–response is provided. However, a variety of mathematical models (from 2 to 5 parameter curves), the range and number of doses, among other experimental factors, has recently been determined for large-scale drug-response, in which data responses vary unacceptably between different studies.[5,15–18]

The analysis of all the problems of TCLA with the diverse quantification criteria used for each method is unfeasible. However, since most of the methods share the main objectives and operative requirements,[6,19] the SRB colorimetric assay for cytotoxicity screening of tumor cell lines has been chosen as a case study. The SRB assay is a well-accepted model for testing cytotoxicity and is a highly reproducible procedure, currently performed using microplate readers, providing an appropriate tool which ensures that samples and controls of the reaction can be simultaneously assessed, producing abundant data with lower experimental error.[8] It is, therefore, a robust and meaningful example that can be used as a case study for developing criteria to evaluate and compare the cytotoxicity process of TCLA.

In mathematical terms, there are several approaches that can be transferred from microbiology, enzymology, pharmacology, among other dose–response fields, to describe the complex mortality process of tumor cell lines.[11,20–24,26] In fact, from the dose–response theory, the two parameter (2P) equations (such as the Bertalanffy and Michaelis–Menten equations) and the three parameter (3P) sigmoidal group of functions (such as the logistic, Weibull, Hill, Gompertz, Richards–Chapman, among others) would be, in general, acceptable solutions to fit individually the dose–response profiles corresponding to the cytotoxic responses of tumor cells after being treated with individual or a mixture of compounds. In general, these models would improve, aid and guide researchers to compute and, occasionally, to precisely predict the results obtained in TCLA. These models would be able to produce key parameters to summarize the responses, such as the asymptote, LD_{50}, maximum and average rate processes or the lag-phase period values, among others.

In this work, the most biologically relevant and practical non-linear dose–response mathematical models were tested against the experimental data obtained. Their fitting results were evaluated with different statistical criteria to rank the suitability of the mathematical equations for describing TCLA responses. The most satisfactory solution was determined and when properly applied, it would certainly improve the efficiency and precision of the description, characterization, quantification and prediction of TCLA. The advantages are a simple application, provision of parametric estimations that characterize the response, simplification of the protocol, economization of experimental effort and facilitation of rigorous comparisons among the effects of different compounds and experimental approaches.

2. Materials and methods

2.1. Reagents

Cell lines HeLa, NCI-H460 and MCF-7 were purchased from Deutshe Sammlung von Mikroorganismen and Zellkulturen Gmbit (DSMZ). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U mL^{-1} and 100 mg mL^{-1}, respectively) were purchased from Gibco Invitrogen Life Technologies (Carlsbad, CA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). The anti-tumor agents ellipticine (EL), cisplatin (CDDP) and etoposide (VP-16) were purchased at Adooq Bioscience (Irvine, CA).

2.2. Anti-tumor agents

2.2.1. Commercial agents. Stock solutions of 10 mM were prepared for each commercial agent. The solvent used for EL and VP-16 was DMSO, whereas phosphate buffered solution (PBS, 1 M) was used for CDDP.

2.2.2. Natural agents. In this study, two of the classical solvents with different degrees of polarity were used to extract the anti-tumor agents previously identified in *Achillea millefolium* L.[25] Briefly:

- Methanol extraction (ME): One gram was extracted twice by stirring with 30 mL of methanol (25 °C at 150 rpm) for 1 h and afterwards the solvent was evaporated at 40 °C to dryness.

- Water extraction (WE): One gram was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained extracts were frozen and lyophilized.

2.3. Evaluation of the cytotoxic properties by the sulforhodamine B colorimetric assay

Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7, NCI-H460 HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (1.0×10^4 cells per well in 200 μL of final volume) in 96-well plates and allowed to attach for 24 h. The cells were then incubated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilized with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm.

Natural agents were dissolved in water, meanwhile commercial agents were dissolved in DMSO. Initial experiments were carried out to determine the dose range that will describe the profile from none to maximum cytotoxic effects.

Each compound was tested with 11 different concentrations plus the control (absence of agent) using all the row-wells in the 96-well plate and replicated 8 times, for each column of the 96-well plate. The maximum dose-ranges of the 11 subsequent serial dilutions of 4/3-fold dilutions for each compound and the cell lines were described as follows:

- For NCI-H460: ME (200 μg mL^{-1}), WE (200 μg mL^{-1}), EL (20 μM), VP-16 (200 μM) and CDDP (100 μM).

- For HeLa: ME (200 μg mL^{-1}), WE (90 μg mL^{-1}), EL (10 μM), VP-16 (150 μM) and CDDP (50 μM).

- For MFC7: ME (200 μg mL^{-1}), WE (150 μg mL^{-1}), EL (8 μM), VP-16 (160 μM) and CDDP (60 μM).

- For HepG2: ME (200 μg mL^{-1}), WE (150 μg mL^{-1}), EL (10 μM), VP-16 (200 μM) and CDDP (30 μM).

2.4. Data normalization

In cellular growth media, the maximum response achieved by the application of the anti-tumor agent at sufficiently long times would be the complete death of all cells, producing asymptotic responses that would correspond to the total percentage of the tumor cell population mixed in the solution. Consequently, the spectrophotometric inhibition responses (I) should be carefully standardized. Therefore, before the data are analyzed, the response (R) must be normalized as a function of the maximum inhibition value (I_{max}) that can be achieved, as follows:

$$R = (I/I_{max}) \times 100 \quad (1)$$

This simple normalization will transform the R as a function of the inhibited population cells in percentage. The I_{max} corresponds to the spectrophotometric values produced by the control response (absence of any agent) that should be included in each analytical test using as many replicates as possible and between different tests of the same cellular line it can be used as an indicator of the reproducibility of the results generated. The I value corresponds to the spectrophotometric inhibition responses achieved at different doses of the agent tested.

The normalization of raw data adjusts the resulting sample variation (human error, excessive or defective dilution, number of red blood cells present, *etc.*). Fitting the normalized data would result in a significant increase in the reproducibility and accuracy of the results obtained, but not in a significant change in the parameters produced.

2.5. Formal mathematical expressions from the dose–response theory and related fields of study

All the models are formulated as a function of the dose (D) and they are reparametrized to explicitly show the asymptotic value K and the dose corresponding to the half-maximum ($K/2$) inhibition response known as the LD_{50} value. These models with three parameters would present an additional shape parameter a.

2.5.1. First-order functions without intercept

M1: Michaelis–Menten equation. Michaelis and Menten[27] published their now classic paper in which they showed that the rate of an enzyme reaction is proportional to the concentration of the enzyme/substrate complex predicted by the Michaelis–Menten equation with rigor and precision.[28] Since then, the equation has been used successfully in many other fields of study to describe symmetrical hyperbolic non-linear responses. The expression can be written as follows:

$$R(D) = K[D/(LD_{50} + D)]. \quad (2)$$

M2: Bertalanffy equation. A simplification of a more complex four parameter equation,[21] the Bertalanffy equation has been applied to describe potential and first order responses. The expression can be written as follows:

$$R(D) = K(1 - 2^{-D/LD_{50}}). \quad (3)$$

2.5.2. Sigmoid functions with intercept

M3: Verhulst growth equation. The so-called Verhulst model, first proposed as a model of population growth[29,30] or logistic growth curve, is one of the simplest of the S-shaped growth curves. Authors have used the Verhulst equation in different fields of study including the dose–response one[31–34] and can be written in the following functional form:

$$R(D) = K/\{1 + \exp[a(\mathrm{LD}_{50} - D)]\}. \quad (4)$$

M4: Gompertz growth equation. The Gompertz equation, Gompertz (1825),[35] has been altered into growth curve analysis (initial value $R_0 \neq 0$) for the description of tumors and microorganisms, among others.[36] The equation, known as a Gompertz curve, is now used in many areas to analyze those time series where growth decreases at the start and termination periods. The equation can be described as follows:

$$R(D) = K\{1 - 2^{-\exp[-a(D-\mathrm{LD}_{50})]}\}. \quad (5)$$

2.5.3. Sigmoid functions without intercept

M5: Weibull equation. What was first developed by Fréchet (1927)[37] and first applied by Rosin & Rammler (1933)[38] to describe a particle size distribution is today the well-known Weibull distribution, named in honor of the author who described its capabilities in detail[39] and then applied it to analyze the mechanical failure of machines.[40] The distribution equation to describe dose–response profiles can be written as follows:

$$R(D) = K\{1 - \exp[-\ln 2(D/\mathrm{LD}_{50})^a]\}. \quad (6)$$

M6: Hill equation. Hosselfed IV (1822)[41] suggested a way to describe the tree growth, an equation that was lately reinterpreted to measure the rate of mortality. The equation was popularized after the application by A.V. Hill[42] to describe the equilibrium relationship between oxygen tension and the saturation of hemoglobin. In pharmacology, the Hill equation has been extensively used to analyze quantitatively the drug–receptor relationships[43] by describing the non-linear dose–response relationships:

$$R(D) = K/[1 + (\mathrm{LD}_{50}/D)^a]. \quad (7)$$

M7: Modified Verhulst equation. The Verhulst equation[29,30] can be transferred from its habitual formulation (as a model for describing autocatalytic kinetics, or a biological growth) to the context of the dose–response relationships eliminating the intercept (to make $R_0 = 0$),[44] whereby its form would be:

$$R(D) = K/\{1 + \exp[a(\mathrm{LD}_{50} - D)]\} - K/\{1 + \exp(a\mathrm{LD}_{50})\}. \quad (8)$$

M8: Modified Gompertz equation. Gompertz (1825)[35] suggested that a "law of geometric progression pervades" in mortality after a certain age and with the rate of mortality. This equation was designed to describe the age distribution of the human population. It has been successfully applied to many biological processes demonstrating sufficient flexibility. It has also been called the exponential decay function.[45] The equation can be presented as follows:

$$R(D) = K\{1 - 2^{-[(\exp(aD)-1)/(\exp(a\mathrm{LD}_{50})-1)]}\}. \quad (9)$$

M9: Richards–Chapman equation. The trademark of the Richards–Chapman equation is its flexibility. Although it was first reported by Mitscherlich (1919),[46] it became known after the publication of Richards (1959).[47] The Richard–Chapman function is a modification of the Bertalanffy function. It is yet another three-parameter model that behaves similarly to the Weibull distribution, which has an upper asymptote and goes through the origin. Researchers have used this model for different purposes and different fields including the dose–response one, finding a successful performance.[48] The equation can be written as follows:

$$R(D) = K[1 - (1 - 2^{-1/a})^{D/\mathrm{LD}_{50}}]^a. \quad (10)$$

2.6. Fitting procedure to the mathematical models

All fitting procedures, coefficient estimates and statistical calculations were performed on a Microsoft Excel spreadsheet. Fitting and statistical analysis of the experimental results to the proposed equations are as follows:

2.6.1. Coefficient determination. Parametric estimates were performed by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro Solver in Microsoft Excel 2003,[49] which allows a quick testing of a hypothesis and its consequences.[50]

2.6.2. Coefficient significance and model consistency. The parametric confidence intervals were determined by incorporating the 'SolverAid' macro (Prieto *et al.*, 2012)[12] for estimating the confidence intervals. The model was simplified by dropping those terms, which did not show a statistically significant p-value of >0.05. The Fisher F test (α = 0.05) was used to determine whether the constructed models were adequate to describe the observed data.[51] Finally, the correlation coefficient R^2 is interpreted as the proportion of the variability of the dependent variable explained by the model.

2.7. Criteria used to assess the selection of the best model

2.7.1. Criteria based on model selection criteria (MSC). In the present work, the AICc, BIC, RIC, Cp, R^2_{adj}, FPE and MSIC criteria (Table A1†) were obtained directly by applying an Excel spreadsheet using the Excel add-in 'Solverstat' macro.[52] This selected group is a combination of different criteria that can discriminate the models based on their goodness of fit, complexity, overfitting and generalizability.

2.7.2. Additional intuitive criteria. Additional statistical criteria to evaluate the mathematical models are based on the following common features:[53] (a) the residual distribution; and (b) the number of non-significant parameters (α = 0.05).

3. Results

3.1. Analysis of the most common dose–response mathematical models for the description of cytotoxic responses from TCLA by selective statistical criteria

Currently, the cytotoxicity reaction mechanism of tumor cells *in vitro* is becoming increasingly better understood.[3,4,54] However, the development of a mechanistic model to evaluate the cytotoxic effect of agents is very sophisticated and often difficult to accomplish due to the quantity of intermediate components that need to be tested. In addition, the detailed

mechanistic description of cytotoxic TCLA responses is even more complex due to the heterogeneity of the cell lines used that varies from one to the other assay, making it difficult to characterize in mechanistic terms the molecular pathways and perhaps unfeasible to propose a single mechanistic system for all the TCLA techniques.[5,9] Therefore, researchers tend to search for empirical general models, able to quantify and evaluate the complex responses and summarize the results in simple parameters.[21,41,55,56] In this regard, previous reports from related fields of study have reviewed different empirical non-linear mathematical expressions useful in biological systems (such as microbiology, toxicology, pharmacology, immunology, population dynamics, *etc*.) for wide diverse circumstances (analysis such as growth, kinetic, dose–response, *etc*.).[20–22,24,48,57,58] These studies provide the key concepts to guide the development of a standard solution for the TCLA.

Currently, it is unclear which model or models are consistent or inconsistent with experimental data. A key problem is that a direct comparison between the models has not been carried out, in part because they have been formulated under different frameworks.[59] To make sense from the diversity of TCLA quantification criteria, a variety of verbal and mathematical ones are assessed. Next, the two parameter equations (Bertalanffy and Michaelis–Menten equations) and the three-parameter sigmoidal group of functions with intercept (Verhulst and Gompertz equations) and without intercept (Weibull, Hill, modified Gompertz, Richards–Chapman and modified Verhulst equations) are tested with rigorous and selective statistical criteria against experimental data specifically obtained to determine the most efficient model solution.

3.1.1. Selection by statistical model selection criteria (MSC). The usefulness of MSC to choose the best solution and model is well-documented (Rivers & Vuong, 2002).[74] A model should be complex enough to extract the regularities in data, but simple enough to not overfit it and thereby, reduce productiveness. MSC adjust the goodness of fit in order to penalize model complexity, overfitting and lack of generalizability. Currently, there are a variety of MSC available (Forster, 2000; Myung & Pitt, 2004),[75,76] but there is no one criterion that can lead to a perfect choice (Roland T. Rust, Simester, Brodie, & Nilikant, 1995).[77] A summary of the MSC used to evaluate the results obtained for the nine models is given in Table A1† (AIC, AICc, BIC, RIC, Cp, R^2_{adj}, FPE, MSIC, and MA^2 criteria). Experimental data used to test the models were the dose–responses of 3 commercial agents (EL, VP-16 and CCDP, as a single compound) and 2 natural extracts (ME and WE as a mixture of compounds) in four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) by one of the common TCLA techniques, the SRB colorimetric assay (125 data points per agent and tumor cell line).

Table 1 reports the estimated numerical values and confidence intervals at a level of 95% for each of the parameters K, LD_{50} and a of eqn (2)–(10), after fitting the dose–response values of four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) by the SRB colorimetric assay for screening the effects of 3 commercial agents (EL, VP-16 and CCDP, as a single compound) and 2 natural extracts (ME and WE as a mixture of compounds). These parametric values were used to test the nine models selected from the bibliography (eqn (2)–(10)). The statistical value of the correlation coefficient R^2 is presented as proof of the fitting results obtained. The italicised parametric values are non-significant and therefore, the fitting result should be considered as non-valid analysis.

Table 2 shows the model rank (Rk) obtained for each MSC and the global ranking based on the ranking sum of each MSC ($\sum$Rk) for the five agents used for one tumor cell line (NCI-H460) as a representative example of the work performed for all other tumor cell lines. Model selection criteria help to differentiate the most "true solution". In the ESI, Tables A2, A3 and A4† show the results for HeLa, MCF-7 and HepG2 tumor cell lines, respectively. In general, all statistical MSC analyses have different features of the models as described in Table A1† providing efficient solutions from different statistical angles.

Finally, Table A5† reports the global ranking ($\sum$Rk) for all the four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) and for all the agents experimentally tested using the SRB assay (EL, VP-16, CCDP, ME and WE). These ranking values were used to order the nine models selected from the bibliography (eqn (2)–(10)) from the most to the least efficient one for each tumor cell line and finally for all of them. The global ranking ($\sum$Rk) denoting the efficiency of the fitting analysis for each of the tumor cell lines assessed, is as follows in a decreasing order:

- For NCI-H460: Weibull > Hill > Chapman > VerhulstM > GompertzM > Bertalanffy > Verhulst > Michaelis > Gompertz.
- For HeLa: Hill > Weibull > Chapman > VerhulstM > Bertalanffy > GompertzM > Michaelis > Verhulst > Gompertz.
- For MFC7: Weibull > VerhulstM > Chapman > Hill > GompertzM > Bertalanffy > Verhulst > Michaelis > Gompertz.
- For HepG2: VerhulstM > Weibull > Chapman > Hill > GompertzM > Bertalanffy > Michaelis > Verhulst > Gompertz.

Therefore, in global terms the models most likely to be correct, presenting a final global ranking, are as follows in a decreasing order: Weibull > Hill > VerhulstM > Chapman > GompertzM > Bertalanffy > Verhulst > Michaelis > Gompertz.

3.1.2. Additional intuitive criteria. The residuals should be randomly scattered around zero to avoid autocorrelation.[49] These residuals should not be grouped and should not increase or decrease as a function of the independent variable. In addition, a large group of runs in certain zones of the residual plot indicate a cluster distribution suggesting a lack of estimation or overestimations. In general terms, all the models used showed a relatively good distribution of the residuals. Only the Bertalanffy and Michaelis models showed autocorrelations when the responses presented a sigmoid or potential profile. All other models showed a relatively good distribution of the residuals and autocorrelation was not observed (data not shown).

Another important aspect is the confidence in intervals at a level of 95% for each parameter (reported in Table 1). The parametric estimates in many cases led to large confidence intervals and therefore, these parameters were considered not

Table 1 Estimated numerical values and confidence intervals of the parameters K, LD_{50} and a of eqn (2)–(10), after fitting the dose–response values of four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) by the SRB colorimetric assay for screening the effects of three commercial agents (EL, VP-16 and CCDP, as a single compound) and two natural extracts (ME and WE as a mixture of compounds). These parametric values were used to test the nine models selected from the bibliography (eqn (2)–(10)). The statistical value of the correlation coefficient R^2 is presented as proof of the fitting results obtained. The italic parametric values are non-significant and therefore, the fitting result should be considered as non-valid analysis

		NCI-H460				HeLa				MFC7				HeG2			
		K	LD_{50}	a	R^2	K	LD_{50}	a	R^2	K	LD_{50}	a	R^2	K	LD_{50}	a	R^2
Methanol extract (ME)	Weibull	100.0 ± 9.3	58.83 ± 6.42	1.98 ± 0.39	0.9785	100.0 ± 21.5	50.16 ± 14.78	1.27 ± 0.50	0.9359	100.0 ± 12.2	57.89 ± 8.79	1.53 ± 0.34	0.9772	100.0 ± 60.4	96.02 ± 71.64	1.28 ± 0.44	0.9639
	Hill	100.0 ± 11.6	56.59 ± 7.46	2.88 ± 0.76	0.9776	100.0 ± 20.7	48.39 ± 13.19	2.01 ± 0.94	0.9377	100.0 ± 13.7	55.48 ± 9.37	2.31 ± 0.67	0.9752	100.0 ± 43.8	92.96 ± 55.09	1.68 ± 0.67	0.9593
	Chapman	100.0 ± 10.0	57.34 ± 7.06	3.77 ± 1.83	0.9781	100.0 ± 18.8	49.81 ± 13.40	1.62 ± 1.19	0.9370	100.0 ± 11.9	56.83 ± 8.85	2.27 ± 1.07	0.9767	100.0 ± 48.2	95.31 ± 58.89	1.49 ± 0.79	0.9634
	VerhulstM	100.0 ± 8.7	59.45 ± 6.22	0.05 ± 0.01	0.9788	100.0 ± 20.5	50.46 ± 14.20	*0.02 ± 0.02*	0.9350	100.0 ± 10.7	58.57 ± 8.00	0.03 ± 0.01	0.9771	100.0 ± 62.8	97.37 ± 73.11	*0.01 ± 0.02*	0.9641
	GompertzM	100.0 ± 9.0	60.92 ± 6.50	0.03 ± 0.01	0.9752	100.0 ± 27.7	50.49 ± 18.24	*0.01 ± 0.02*	0.9327	100.0 ± 10.4	59.44 ± 7.88	*0.01 ± 0.01*	0.9760	100.0 ± 108.2	*98.57 ± 122.51*	*0.01 ± 0.02*	0.9644
	Verhulst	100.0 ± 8.5	60.11 ± 6.16	26.3 ± 14.4	0.9761	100.0 ± 12.8	53.49 ± 10.84	8.21 ± 5.1	0.9104	100.0 ± 8.6	60.86 ± 6.66	19.8 ± 12.8	0.9640	100.0 ± 34.8	101.68 ± 38.13	9.92 ± 3.52	0.9342
	Gompertz	100.0 ± 9.2	62.26 ± 6.48	0.04 ± 0.01	0.9679	100.0 ± 12.4	56.28 ± 11.35	0.03 ± 0.01	0.8831	100.0 ± 8.4	63.69 ± 6.42	0.04 ± 0.01	0.9502	100.0 ± 58.0	106.78 ± 60.16	0.02 ± 0.01	0.9124
	Michaelis	100.0 ± 42.3	*20.00 ± 25.82*	—	0.7003	100.0 ± 29.8	41.32 ± 33.83	—	0.8721	100.0 ± 28.7	48.58 ± 35.55	—	0.9173	100.0 ± 45.7	105.33 ± 89.01	—	0.9108
	Bertalanffy	100.0 ± 34.6	55.38 ± 34.56	—	0.9333	100.0 ± 18.6	47.28 ± 17.85	—	0.9272	100.0 ± 18.6	53.21 ± 18.97	—	0.9588	100.0 ± 40.0	98.40 ± 58.18	—	0.9521
Water extract (WE)	Weibull	100.0 ± 8.1	46.96 ± 5.23	1.73 ± 0.31	0.9347	100.0 ± 4.5	21.16 ± 1.43	1.32 ± 0.19	0.9696	100.0 ± 12.9	44.32 ± 7.11	1.35 ± 0.15	0.9683	100.0 ± 17.6	38.22 ± 9.00	1.25 ± 0.22	0.9772
	Hill	100.0 ± 19.2	49.31 ± 12.99	2.20 ± 0.83	0.9325	100.0 ± 7.9	22.00 ± 2.11	2.50 ± 0.69	0.9749	100.0 ± 20.3	39.04 ± 9.71	2.15 ± 0.66	0.9705	100.0 ± 25.4	36.60 ± 12.93	1.78 ± 0.65	0.9642
	Chapman	100.0 ± 15.6	50.41 ± 11.31	2.32 ± 1.33	0.9324	100.0 ± 6.5	22.28 ± 2.10	2.52 ± 1.33	0.9745	100.0 ± 20.0	40.16 ± 10.03	2.11 ± 1.04	0.9704	100.0 ± 21.7	37.83 ± 11.55	1.46 ± 0.64	0.9750
	VerhulstM	100.0 ± 13.9	52.24 ± 9.93	0.04 ± 0.02	0.9304	100.0 ± 6.2	22.58 ± 2.18	0.08 ± 0.03	0.9737	100.0 ± 21.3	41.47 ± 10.55	0.04 ± 0.03	0.9682	100.0 ± 21.5	38.81 ± 10.89	0.03 ± 0.02	0.9792
	GompertzM	100.0 ± 13.2	53.49 ± 9.61	0.02 ± 0.01	0.9254	100.0 ± 6.1	22.59 ± 2.41	0.03 ± 0.02	0.9709	100.0 ± 32.1	42.24 ± 15.51	*0.02 ± 0.02*	0.9657	100.0 ± 28.6	39.36 ± 13.93	*0.01 ± 0.02*	0.9813
	Verhulst	100.0 ± 12.4	53.39 ± 9.17	17.0 ± 11.5	0.9196	100.0 ± 5.9	23.34 ± 2.27	12.1 ± 7.15	0.9659	100.0 ± 13.4	42.85 ± 6.53	18.6 ± 11.7	0.9470	100.0 ± 14.2	41.42 ± 8.06	7.99 ± 2.59	0.9587
	Gompertz	100.0 ± 12.6	55.97 ± 9.10	0.04 ± 0.01	0.9012	100.0 ± 6.4	24.17 ± 2.62	0.07 ± 0.02	0.9518	100.0 ± 12.3	45.12 ± 5.90	0.05 ± 0.01	0.9237	100.0 ± 15.3	43.84 ± 8.83	0.03 ± 0.01	0.9387
	Michaelis	100.0 ± 43.4	55.70 ± 52.68	—	0.8641	100.0 ± 23.7	15.52 ± 12.49	—	0.8842	100.0 ± 36.0	39.42 ± 30.76	—	0.9065	100.0 ± 32.5	35.67 ± 26.22	—	0.9101
	Bertalanffy	100.0 ± 29.9	52.42 ± 28.83	—	0.9038	100.0 ± 11.3	19.68 ± 5.30	—	0.9511	100.0 ± 28.3	39.59 ± 18.89	—	0.9477	100.0 ± 19.7	37.09 ± 12.67	—	0.9673
Ellipticine (EL)	Weibull	100.0 ± 12.3	0.78 ± 0.34	0.65 ± 0.16	0.9546	100.0 ± 26.7	1.21 ± 0.51	0.94 ± 0.46	0.9474	100.0 ± 21.0	1.42 ± 0.43	1.12 ± 0.42	0.9685	100.0 ± 82.2	1.16 ± 0.89	0.72 ± 0.51	0.8775
	Hill	100.0 ± 14.7	0.72 ± 0.35	0.98 ± 0.33	0.9550	100.0 ± 23.2	1.20 ± 0.40	1.56 ± 0.90	0.9479	100.0 ± 20.8	1.38 ± 0.40	1.77 ± 0.85	0.9661	100.0 ± 61.6	1.17 ± 0.26	1.16 ± 0.50	0.8774
	Chapman	100.0 ± 10.8	0.82 ± 0.33	0.52 ± 0.16	0.9519	100.0 ± 22.5	1.21 ± 0.45	0.90 ± 0.67	0.9472	100.0 ± 18.6	1.42 ± 0.40	1.22 ± 0.80	0.9682	100.0 ± 73.5	1.17 ± 0.79	0.57 ± 0.16	0.8779
	VerhulstM	100.0 ± 111.3	*0.74 ± 1.62*	*0.04 ± 3.38*	0.9554	100.0 ± 33.4	1.20 ± 0.60	*0.47 ± 1.10*	0.9475	100.0 ± 21.0	1.43 ± 0.43	*0.65 ± 0.73*	0.9684	100.0 ± 260.7	*1.17 ± 5.53*	*0.15 ± 5.41*	0.8784
	GompertzM	100.0 ± 12.7	0.96 ± 0.32	*0.00 ± 0.65*	0.9406	100.0 ± 37.6	1.24 ± 0.68	*0.01 ± 0.66*	0.9470	100.0 ± 29.5	1.43 ± 0.57	*0.11 ± 0.53*	0.9684	100.0 ± 72.0	*1.34 ± 1.46*	*0.01 ± 1.07*	0.8638
	Verhulst	100.0 ± 13.1	1.11 ± 0.45	5.65 ± 3.21	0.8791	100.0 ± 12.6	1.39 ± 0.34	5.45 ± 3.3	0.8888	100.0 ± 10.6	1.61 ± 0.24	14.3 ± 12.3	0.9365	100.0 ± 24.0	1.49 ± 0.73	3.90 ± 2.54	0.7615
	Gompertz	100.0 ± 14.9	1.20 ± 0.53	1.11 ± 0.56	0.8461	100.0 ± 12.3	1.46 ± 0.36	0.81 ± 0.33	0.8533	100.0 ± 10.5	1.70 ± 0.25	1.26 ± 0.47	0.9154	100.0 ± 22.5	*1.58 ± 1.77*	0.57 ± 0.32	0.7107
	Michaelis	100.0 ± 9.6	0.73 ± 0.26	—	0.9551	100.0 ± 17.4	1.00 ± 0.55	—	0.9238	100.0 ± 19.4	1.14 ± 0.65	—	0.9462	100.0 ± 21.1	*1.10 ± 1.69*	—	0.8754
	Bertalanffy	100.0 ± 10.5	0.96 ± 0.32	—	0.9406	100.0 ± 11.4	1.24 ± 0.31	—	0.9470	100.0 ± 12.7	1.37 ± 0.37	—	0.9674	100.0 ± 19.7	*1.34 ± 1.56*	—	0.8638
Etoposide (VP-16)	Weibull	100.0 ± 6.4	18.84 ± 2.60	1.25 ± 0.26	0.9667	100.0 ± 26.8	15.43 ± 7.37	0.66 ± 0.36	0.9751	100.0 ± 10.2	25.02 ± 3.64	1.18 ± 0.29	0.9847	100.0 ± 38.7	43.40 ± 28.53	0.92 ± 0.27	0.9831
	Hill	100.0 ± 7.2	18.23 ± 2.52	1.99 ± 0.47	0.9688	100.0 ± 22.6	15.43 ± 5.01	1.08 ± 0.72	0.9795	100.0 ± 10.6	24.67 ± 3.36	1.97 ± 0.61	0.9850	100.0 ± 28.0	41.75 ± 20.34	1.30 ± 0.44	0.9816
	Chapman	100.0 ± 6.1	18.75 ± 2.53	1.57 ± 0.59	0.9678	100.0 ± 21.8	15.42 ± 6.42	0.50 ± 0.33	0.9737	100.0 ± 9.1	25.02 ± 3.40	1.43 ± 0.68	0.9848	100.0 ± 33.1	43.55 ± 24.86	0.88 ± 0.33	0.9830
	VerhulstM	100.0 ± 6.4	18.95 ± 2.67	0.06 ± 0.03	0.9657	100.0 ± 136.0	*15.42 ± 38.75*	*0.01 ± 0.20*	0.9790	100.0 ± 9.8	25.07 ± 3.56	0.04 ± 0.03	0.9845	100.0 ± 71.5	*43.13 ± 49.98*	*0.01 ± 0.04*	0.9831
	GompertzM	100.0 ± 6.8	18.78 ± 2.84	*0.01 ± 0.02*	0.9635	100.0 ± 22.8	18.78 ± 5.58	*0.01 ± 0.04*	0.9536	100.0 ± 13.2	24.80 ± 4.39	*0.01 ± 0.02*	0.9839	100.0 ± 93.4	*43.76 ± 60.41*	*0.01 ± 0.03*	0.9816
	Verhulst	100.0 ± 6.7	20.46 ± 3.03	9.11 ± 5.01	0.9498	100.0 ± 11.8	20.46 ± 6.17	3.91 ± 2.61	0.8436	100.0 ± 7.4	27.75 ± 3.04	17.2 ± 14.3	0.9651	100.0 ± 30.9	48.89 ± 22.76	5.44 ± 1.91	0.8990
	Gompertz	100.0 ± 7.6	21.45 ± 3.50	0.07 ± 0.02	0.9293	100.0 ± 12.0	21.74 ± 7.27	0.04 ± 0.02	0.7900	100.0 ± 7.8	28.98 ± 3.18	0.08 ± 0.03	0.9497	100.0 ± 49.9	52.34 ± 37.46	0.02 ± 0.01	0.8561
	Michaelis	100.0 ± 14.4	13.86 ± 6.94	—	0.9195	100.0 ± 7.0	14.92 ± 3.83	—	0.9787	100.0 ± 15.6	18.70 ± 9.48	—	0.9616	100.0 ± 16.3	41.75 ± 15.01	—	0.9688
	Bertalanffy	100.0 ± 7.1	17.52 ± 3.39	—	0.9594	100.0 ± 8.6	18.42 ± 4.14	—	0.9557	100.0 ± 8.1	23.54 ± 4.34	—	0.9827	100.0 ± 12.7	43.75 ± 9.38	—	0.9817
Cisplatin (CCDP)	Weibull	100.0 ± 7.1	6.49 ± 1.30	0.93 ± 0.17	0.9817	100.0 ± 12.4	6.80 ± 1.24	1.08 ± 0.34	0.9690	100.0 ± 14.7	12.17 ± 2.17	1.50 ± 0.31	0.9810	100.0 ± 17.3	1.45 ± 0.94	0.50 ± 0.41	0.9851
	Hill	100.0 ± 9.5	6.28 ± 1.50	1.47 ± 0.40	0.9787	100.0 ± 11.8	6.82 ± 1.03	1.87 ± 0.69	0.9723	100.0 ± 16.1	11.63 ± 2.32	2.18 ± 0.62	0.9765	100.0 ± 17.6	1.84 ± 1.29	0.98 ± 0.81	0.9870
	Chapman	100.0 ± 6.7	6.51 ± 1.30	0.88 ± 0.23	0.9818	100.0 ± 10.7	6.84 ± 1.13	1.21 ± 0.69	0.9695	100.0 ± 14.0	11.93 ± 2.17	2.09 ± 0.89	0.9797	100.0 ± 8.3	2.26 ± 1.40	0.50 ± 0.49	0.9777
	VerhulstM	100.0 ± 7.6	6.49 ± 1.26	0.09 ± 0.08	0.9817	100.0 ± 12.7	6.78 ± 1.26	0.12 ± 0.11	0.9687	100.0 ± 12.4	12.34 ± 1.89	0.14 ± 0.06	0.9813	100.0 ± 334.6	*1.91 ± 12.12*	*0.01 ± 3.66*	0.9870
	GompertzM	100.0 ± 8.0	6.67 ± 1.24	*0.01 ± 0.05*	0.9818	100.0 ± 20.8	6.69 ± 1.85	*0.01 ± 0.09*	0.9679	100.0 ± 13.5	12.57 ± 1.99	0.06 ± 0.04	0.9813	100.0 ± 8.2	3.26 ± 0.79	*0.01 ± 0.15*	0.9720
	Verhulst	100.0 ± 9.5	7.77 ± 1.89	8.22 ± 3.91	0.9481	100.0 ± 8.4	7.55 ± 1.19	7.08 ± 4.26	0.9359	100.0 ± 9.8	12.92 ± 1.54	18.3 ± 10.3	0.9639	100.0 ± 6.1	4.23 ± 0.92	*10.7 ± 13.9*	0.9522
	Gompertz	100.0 ± 11.2	8.28 ± 2.00	0.20 ± 0.06	0.9261	100.0 ± 9.0	7.87 ± 1.38	0.18 ± 0.06	0.9080	100.0 ± 9.7	13.63 ± 1.51	0.17 ± 0.04	0.9474	100.0 ± 6.5	4.46 ± 0.93	0.44 ± 0.22	0.9413
	Michaelis	100.0 ± 10.9	5.34 ± 2.04	—	0.9660	100.0 ± 16.8	5.14 ± 2.95	—	0.9287	100.0 ± 30.8	10.81 ± 7.85	—	0.9181	100.0 ± 4.5	1.88 ± 0.55	—	0.9871
	Bertalanffy	100.0 ± 6.2	6.67 ± 1.23	—	0.9818	100.0 ± 8.1	6.59 ± 1.26	—	0.9677	100.0 ± 20.9	11.39 ± 4.28	—	0.9614	100.0 ± 5.2	3.26 ± 0.64	—	0.9720

Table 2 Main fitting statistics and model ranking (Rk) obtained for each MSC for the effects of five agents (EL, VP-16, CCDP, ME and WE) for the NCI-H460 tumor cell lines as an illustrative example case. The results obtained for each MSC are presented along with individual rankings (Rk) which are presented in brackets. Finally, the global ranking based on the ranking sum of each MSC (∑Rk) for each agent is presented

		Main statistics						Model selection criteria																	
								AIC		AICc		BIC		FPE		R^2_{adj}		RIC		Cp		MSC		MA^2		Global ranking (∑Rk)
Compound & model		k	P	RSS	R^2	ESS	S^2	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	
Methanol extract (ME)	Weibull	3	3	3026.7	0.9785	151 214.5	1219.5	404.4	(1)	398.4	(1)	1016.4	(1)	39 6941	(1)	0.9780	(2)	989.4	(1)	191.2	(1)	3.86	(1)	52.0	(1)	(1)
	Hill	3	3	3449.8	0.9776	169 859.2	1369.8	420.7	(4)	414.8	(4)	1032.7	(4)	452 430	(4)	0.9771	(4)	1005.3	(4)	195.8	(2)	3.85	(2)	60.4	(4)	(4)
	Chapman	3	3	3342.2	0.9781	145 824.6	1176.0	416.8	(3)	410.8	(3)	1028.8	(3)	438 316	(3)	0.9776	(3)	1001.5	(3)	236.2	(4)	3.73	(4)	60.3	(3)	(3)
	VerhulstM	3	3	3299.5	0.9788	144 547.6	1165.7	415.2	(2)	409.2	(2)	1027.2	(2)	432 721	(2)	0.9783	(1)	999.9	(2)	234.8	(3)	3.73	(3)	57.5	(2)	(2)
	GompertzM	3	3	4112.0	0.9752	143 854.8	1160.1	442.7	(6)	436.7	(6)	1054.7	(6)	539 278	(6)	0.9746	(6)	1026.8	(6)	324.1	(6)	3.51	(6)	72.6	(6)	(6)
	Verhulst	3	3	3924.2	0.9761	143 821.1	1159.8	436.8	(5)	430.9	(5)	1048.8	(5)	514 646	(5)	0.9755	(5)	1021.1	(5)	303.9	(5)	3.55	(5)	71.0	(5)	(5)
	Gompertz	3	3	5742.9	0.9679	143 199.0	1154.8	484.4	(7)	478.5	(7)	1096.4	(7)	753 161	(7)	0.9671	(7)	1067.5	(7)	502.6	(7)	3.17	(7)	99.0	(7)	(7)
	Michaelis	2	1	86 579.6	0.7003	172 081.2	1387.8	821.6	(9)	817.6	(9)	1430.8	(9)	11 174 399	(9)	0.6954	(9)	1406.1	(9)	7677.6	(9)	0.65	(9)	44 104	(9)	(9)
	Bertalanffy	2	2	16 380.2	0.9333	144 316.7	1163.8	613.4	(8)	609.5	(8)	1222.6	(8)	2 114 109	(8)	0.9322	(8)	1201.3	(8)	1638.3	(8)	2.14	(8)	196.2	(8)	(8)
Water extract (WE)	Weibull	3	3	3023.0	0.9347	110 994.2	895.1	404.2	(1)	398.3	(1)	1016.2	(1)	396 461	(1)	0.9330	(1)	989.2	(1)	303.2	(1)	3.56	(1)	51.8	(1)	(1)
	Hill	3	3	7431.9	0.9325	110 000.1	887.1	516.7	(2)	510.7	(2)	1128.7	(2)	974 671	(2)	0.9308	(2)	1099.0	(2)	928.2	(2)	2.65	(2)	136.5	(2)	(2)
	Chapman	3	3	7445.0	0.9324	109 469.1	882.8	516.9	(3)	510.9	(3)	1128.9	(3)	976 389	(3)	0.9308	(3)	1099.2	(3)	935.2	(3)	2.64	(3)	139.1	(3)	(3)
	VerhulstM	3	3	7752.5	0.9304	109 776.1	885.3	521.9	(4)	516.0	(4)	1134.0	(4)	1 016 715	(4)	0.9287	(4)	1104.1	(4)	975.6	(4)	2.60	(4)	142.7	(4)	(4)
	GompertzM	3	3	8396.1	0.9254	109 995.8	887.1	531.9	(5)	526.0	(5)	1143.9	(5)	1 101 130	(5)	0.9236	(5)	1113.9	(5)	1064.1	(5)	2.52	(5)	157.6	(5)	(5)
	Verhulst	3	3	9268.9	0.9196	110 346.7	889.9	544.3	(6)	538.3	(6)	1156.3	(6)	1 215 599	(6)	0.9176	(6)	1125.9	(6)	1183.0	(6)	2.43	(6)	173.8	(7)	(6)
	Gompertz	3	3	11 755.1	0.9012	110 940.4	894.7	574.0	(7)	568.0	(7)	1186.0	(7)	1 541 648	(7)	0.8987	(8)	1154.9	(7)	1523.4	(7)	2.20	(7)	208.2	(8)	(7)
	Michaelis	2	2	22 734.5	0.8641	109 415.6	882.4	654.4	(9)	650.5	(9)	1263.6	(9)	2 934 233	(9)	0.8619	(9)	1241.6	(9)	3099.6	(9)	1.54	(9)	309.9	(9)	(9)
	Bertalanffy	2	2	14 057.0	0.9038	109 868.7	886.0	594.3	(8)	590.4	(8)	1203.5	(8)	1 814 269	(8)	0.9022	(7)	1182.4	(8)	1862.1	(8)	2.02	(8)	161.5	(6)	(8)
Ellipticine (EL)	Weibull	3	3	5834.6	0.9546	132 833.5	1071.2	486.4	(1)	480.5	(1)	1098.4	(2)	765 189	(1)	0.9535	(4)	1069.4	(1)	561.8	(2)	3.08	(2)	106.8	(3)	(1)
	Hill	3	3	6013.2	0.9550	159 292.9	1284.6	490.2	(4)	484.2	(3)	1102.2	(4)	788 620	(4)	0.9539	(3)	1073.1	(3)	466.1	(1)	3.23	(1)	112.7	(4)	(3)
	Chapman	3	3	6435.1	0.9519	121 286.9	978.1	498.7	(5)	492.7	(5)	1110.7	(5)	843 953	(5)	0.9507	(5)	1081.4	(5)	703.4	(5)	2.89	(5)	117.9	(5)	(5)
	VerhulstM	3	0	6001.9	0.9554	122 683.1	989.4	489.9	(3)	484.0	(2)	1102.0	(3)	787 139	(3)	0.9543	(2)	1072.9	(2)	639.3	(4)	2.97	(4)	4932.6	(8)	(4)
	GompertzM	3	2	10 370.3	0.9406	121 790.2	982.2	558.3	(7)	552.4	(7)	1170.3	(7)	1 360 044	(7)	0.9391	(7)	1139.6	(6)	1200.8	(7)	2.42	(7)	185 517	(9)	(7)
	Verhulst	3	3	19 380.2	0.8791	117 360.4	946.5	636.5	(8)	630.5	(8)	1248.5	(8)	2 541 667	(8)	0.8761	(8)	1215.9	(8)	2440.6	(8)	1.75	(8)	370.2	(6)	(8)
	Gompertz	3	3	24 906.9	0.8461	117 480.8	947.4	667.8	(9)	661.9	(9)	1279.8	(9)	3 266 479	(9)	0.8423	(9)	1246.5	(9)	3167.1	(9)	1.50	(9)	475.9	(7)	(9)
	Michaelis	2	2	6020.9	0.9551	122 799.8	990.3	488.3	(2)	484.4	(4)	1097.5	(1)	777 081	(2)	0.9544	(1)	1078.2	(4)	639.0	(3)	2.98	(3)	59.7	(1)	(2)
	Bertalanffy	2	2	10 369.6	0.9406	121 790.5	982.2	556.3	(6)	552.3	(6)	1165.5	(6)	1 338 352	(6)	0.9396	(6)	1145.0	(7)	1198.7	(6)	2.43	(6)	102.0	(2)	(6)
Etoposide (VP-16)	Weibull	3	3	3874.1	0.9667	129 575.9	1045.0	435.2	(1)	429.3	(1)	1047.2	(1)	508 078	(1)	0.9658	(3)	1019.5	(1)	344.4	(1)	3.46	(1)	66.7	(2)	(1)
	Hill	3	3	4049.0	0.9688	134 177.8	1082.1	440.7	(2)	434.8	(2)	1052.8	(2)	531 010	(2)	0.9680	(1)	1024.9	(2)	348.7	(2)	3.45	(2)	70.2	(3)	(2)
	Chapman	3	3	4297.8	0.9678	125 255.8	1010.1	448.2	(3)	442.3	(3)	1060.2	(3)	563 643	(3)	0.9670	(2)	1032.2	(3)	412.8	(3)	3.32	(3)	75.9	(4)	(3)
	VerhulstM	3	3	4601.6	0.9657	125 309.0	1010.6	456.7	(4)	450.8	(4)	1068.8	(4)	603 494	(4)	0.9649	(4)	1040.5	(4)	450.2	(4)	3.26	(4)	82.9	(5)	(4)
	GompertzM	3	2	4910.3	0.9635	125 387.1	1011.2	464.8	(5)	458.9	(5)	1076.9	(5)	643 979	(5)	0.9626	(5)	1048.4	(5)	488.0	(5)	3.19	(5)	2515.6	(9)	(5)
	Verhulst	3	3	7558.6	0.9498	124 573.0	1004.6	518.8	(7)	512.8	(7)	1130.8	(7)	991 287	(7)	0.9486	(7)	1101.0	(7)	821.5	(7)	2.75	(7)	137.5	(6)	(7)
	Gompertz	3	3	10 932.8	0.9293	124 691.4	1005.6	564.9	(8)	559.0	(8)	1176.9	(8)	1 433 810	(8)	0.9275	(8)	1146.1	(8)	1240.0	(8)	2.39	(8)	192.6	(8)	(8)
	Michaelis	2	2	14 312.1	0.9195	128 022.9	1032.4	596.6	(9)	592.6	(9)	1205.8	(9)	1 847 194	(9)	0.9182	(9)	1184.7	(9)	1611.8	(9)	2.16	(9)	152.6	(7)	(9)
	Bertalanffy	2	2	5510.2	0.9594	125 984.6	1016.0	477.3	(6)	473.3	(6)	1086.5	(6)	711 172	(6)	0.9588	(6)	1067.3	(6)	556.9	(6)	3.10	(6)	50.3	(1)	(5)
Cisplatin (CCDP)	Weibull	3	3	2427.8	0.9817	147 973.2	1193.3	376.8	(1)	370.9	(1)	988.8	(1)	318 396	(1)	0.9813	(4)	962.5	(1)	135.3	(1)	4.06	(1)	42.1	(2)	(1)
	Hill	3	3	3321.8	0.9787	186 457.7	1503.7	416.0	(6)	410.1	(6)	1028.0	(6)	435 651	(6)	0.9781	(6)	1000.7	(6)	157.1	(2)	3.98	(2)	59.0	(6)	(5)
	Chapman	3	3	2718.1	0.9818	132 974.4	1072.4	390.9	(2)	385.0	(2)	1002.9	(2)	356 477	(2)	0.9813	(3)	976.3	(2)	197.8	(3)	3.84	(3)	47.7	(3)	(2)
	VerhulstM	3	3	2756.2	0.9817	133 239.0	1074.5	392.7	(3)	386.7	(3)	1004.7	(4)	361 462	(3)	0.9812	(5)	978.0	(3)	201.6	(4)	3.83	(4)	52.7	(4)	(4)
	GompertzM	3	2	2830.9	0.9818	133 155.9	1073.8	396.0	(5)	390.1	(5)	1008.0	(5)	371 266	(5)	0.9813	(2)	981.2	(4)	210.5	(6)	3.80	(6)	5386.3	(9)	(6)
	Verhulst	3	3	9153.9	0.9481	128 873.8	1039.3	542.7	(8)	536.8	(8)	1154.7	(8)	1 200 517	(8)	0.9468	(8)	1124.4	(8)	982.0	(8)	2.60	(8)	167.7	(7)	(8)
	Gompertz	3	3	13 188.9	0.9261	128 580.6	1036.9	588.4	(9)	582.4	(9)	1200.4	(9)	1 729 693	(9)	0.9243	(9)	1168.9	(9)	1470.9	(9)	2.23	(9)	236.5	(8)	(9)
	Michaelis	2	2	5862.2	0.9660	134 697.6	1086.3	485.0	(7)	481.0	(7)	1094.2	(7)	756 605	(7)	0.9655	(7)	1074.9	(7)	553.6	(7)	3.10	(7)	58.9	(5)	(7)
	Bertalanffy	2	2	2830.2	0.9818	133 156.9	1073.8	394.0	(4)	390.0	(4)	1003.2	(3)	365 281	(4)	0.9815	(1)	985.3	(5)	208.4	(5)	3.82	(5)	25.6	(1)	(3)

Other notations: k, number of fitted parameters; P, number of significant fitted parameters; RSS, residual sum of squares; ESS, explained sum of squares; S^2, standard deviation.

significant (italicised in Table 1). For example, the VerhulstM and GompertzM showed many cases in which one or two parameters were found to be non-significant. Only the models of Weibull, Hill and Chapman showed in all cases that all the parameters were statistically significant ($\alpha = 0.05$).

3.1.3. Advantages/disadvantages of each model solution and final model selection. The non-linear model of the Michaelis–Menten and Bertalanffy equations, which is a two parameter solution, that only describes a very particular symmetrical hyperbolic case can adjust only first-order kinetic profiles, but fails to accomplish complex model solutions with different order levels such as potential and sigmoidal ones, which in contrast are very common responses in the TCLA.

The other tested alternatives, the sigmoid group without intercept (Weibull, Hill, Chapman, VerhulstM and GompertzM) and the sigmoid group with intercept (Verhulst and Gompertz), are models that cover the maximum possible responses in a 2D frame (from potential to sigmoid profiles including the first-order ones). However, they show some relevant differences that may end-up being key characteristics that may make the difference when analyzing TCLA. In order to properly understand and to illustrate the different capabilities of applying one or another sigmoid group of functions, Fig. 1 shows the application of these seven models in four specific cases typical in dose–response analysis: case 1, a potential profile; case 2, a first-order profile; case 3, a slightly sigmoid profile; and case 4, a strong sigmoid profile. The models are simulated under the same parametric conditions for each case in order to be able to compare the differences in the pattern profiles: the asymptotic K value is established for all cases at a value of 100% of inhibition; the LD_{50} is equal for all the models and placed in specific locations for each case in order to magnify as much as possible the profile differences (for cases 1 & 2 and cases 3 & 4, the LD_{50} is located at the hypothetical value of 10% and 50% of the total dose units measured, respectively); and the shape parameter a is adjusted by means of least squares to minimize the differences between each model. Since we are fitting each specific profile case to an identical parametric value of K and LD_{50} the main differences are only present in the initial and final stages. These differences are in many cases difficult to visualize and therefore, to illustrate them in each case, an augment section at the early and final stages is included. Additionally, each line is identified in a decreasing order in each of the stages augmented.

As it was already underlined by other authors,[13] proved by the MSC results and visually illustrated in Fig. 1, the equations with intercept (Verhulst and Gompertz) cause some problems, and in general, it is preferable to consider the null response as zero. The major issues arise when these models (with intercept) describe the initial and final parts of the reaction. If used in a real analysis of TCLA dose–responses, the fitting procedure would cause that the residual distribution and the correlations between the observed and predicted data will show deviations as a function of the dependent variable, which would result in increasing the interval of confidence of the parameters, reducing the correlation coefficients (such as the R^2) and causing difficulty in the production of robust results. Such deviations disappear when the fitting is performed with model approaches without intercept. In general, the sigmoid function without intercept is the best solution to fit individually the profiles corresponding to a series of increasing levels of an agent. However, the models without intercept of VerhulstM and GompertzM show other issues related to high parametric confidence intervals (many of them are non-significant) and poor ranking values by the MSC (see Table A5†), in comparison with the other ones (Weibull, Hill and Chapman). Therefore, Weibull, Hill and Chapman sigmoid models without interception are found to be the most efficient ones with global ranking results better than the other alternatives. Although the differences are narrow, the Weibull model shows overall better results and therefore, without any doubt, can be considered as the most appropriate solution for TCLA.

3.2. Extensive description of the capabilities of the mathematical model selected for the dose–response quantification of the inhibited population growth of tumor cell lines

The Weibull model was described in detail by Weibull & Sweden (1951).[40] The model was adapted to analyze the mechanical failure of machines after an event was repeatedly induced. If we consider the distribution of the number of times that this event needs to be repeated to cause the machine failure as doses to damage cells which may lead to death, we have an analogy with mortality or inhibition of cells. Therefore, it seems not a casual coincidence that this model was the most appropriate solution.

The model transcribes the following power reaction affinity of agents to cells as a function of the dose (D):

$$A(D) = bD^{(c-1)} \tag{11}$$

in which A is the affinity of agents to cells and b and c are parametric values. If the affinity of agents of a population R changes as a function of D (dR/dD) can be initially defined by the following expression:

$$dR/dD = -AR. \tag{12}$$

Therefore, by inserting eqn (11) into (12) and integrating the resulting expression, the basis of the Weibull model can be obtained. By multiplying the resulting function by an asymptotic K parameter and rearranged[60] to explicitly show the LD_{50} parameter, the following increasing inhibition or mortality can be found as described initially in eqn (6):

$$R(D) = K\{1 - \exp[-\ln 2(D/LD_{50})^a]\}. \tag{6}$$

In addition, the Weibull model is a versatile and flexible equation with an intuitive shape parameter to define the possible type of profile that can adjust: (1) when $a < 1$ describes factionary-order kinetics; (2) when $a = 1$ describes a first-order kinetics; and (3) when $a > 1$ produces a variety of sigmoidal profiles that are the common solution for the system.

Although the use of all of them is not frequent, from this equation, other essential parametric values for characterizing

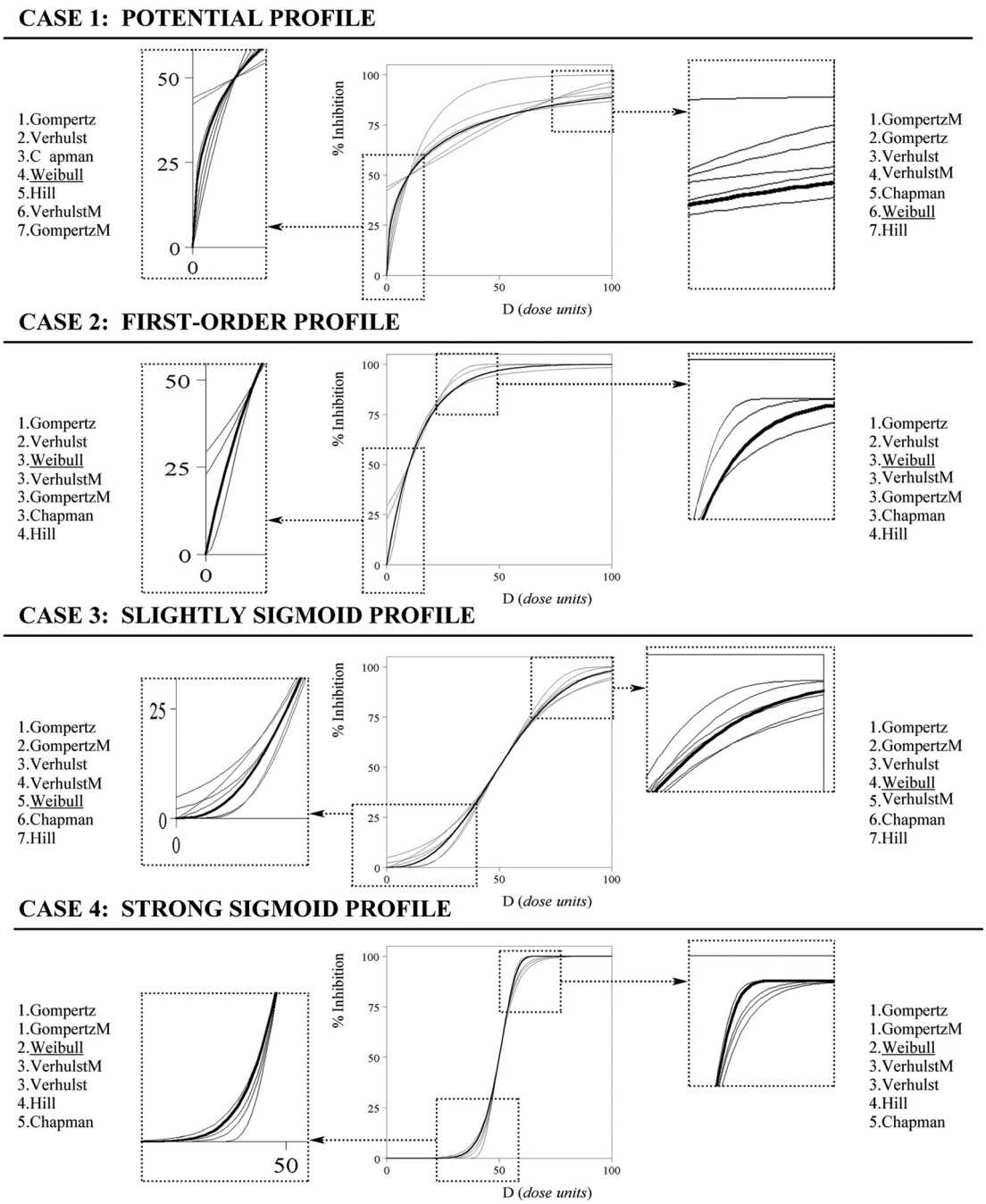

Fig. 1 Differences between the seven-sigmoid group of models to fit specific cases of the TCLA dose–responses. Four different cases are presented: case 1, a potential profile; case 2, a first-order profile; case 3, a slightly sigmoid profile; and case 4, a strong sigmoid profile.

the inhibition process of tumor cells from TCLA can be computed. The analysis results can be directly obtained from the parameters of eqn (6) or with some algebraic modifications of eqn (6) reparametrized to make such values explicit in an independent equation and therefore, their confidence intervals can be computed. Next the most useful values for cytotoxic analysis of the response inhibition in TCLA are:

(1) The dose that shows the maximum affinity of the reaction process (D_A) that translates the % inhibition achieved by increase of each dose unit, which can be computed directly or explicitly as follows:

$$D_A = \frac{K \ln 2}{2LD_{50}};\ \text{therefore } R(D) = K \exp\left[-(\ln 2)^{1-a}\left(\frac{2D_A}{K} \times D\right)^a\right] \quad (13)$$

(2) The lag-phase parameter (LD_λ, dose units), that accounts for the dose value in which the rate of the process can be con-

sidered negligible and corresponds to the intersection point obtained at the dependent variable axis (dose units) by a linear extrapolation tangent to the curve at the inflection point:

$$\mathrm{LD}_\lambda = \frac{\mathrm{LD}_{50}}{\sqrt[a]{\ln 2}}\left[G^{1/a} + \frac{\mathrm{e}^{-G}-1}{aG^G\mathrm{e}^{-G}}\right];\ \text{where } G = \frac{a-1}{a}$$
$$\text{therefore } R(D) = K\ \exp\left[-\left(\left(G^{1/a} + \frac{\mathrm{e}^{-G}-1}{aG^G\mathrm{e}^{-G}}\right)\frac{D}{\mathrm{LD}_\lambda}\right)^a\right] \quad (14)$$

(3) Any dose corresponding to any response R representing any lower fraction of K (or LD_n) can be computed using the following expression:

$$\mathrm{LD}_n = \mathrm{LD}_{50}\{\ln[1-(R_n/K)]/-\ln 2\}^{1/a} \quad (15)$$

by providing the n values of eqn (15) between 0 and 100% any desired dose level of the response would be achieved (LD_{10}, LD_{25}, LD_{75}, LD_{95}, *etc.*). As in the previous parametric values, they can be reparametrized into an explicit equation to compute the confidence intervals. In general, for the computation of LD_n corresponding to a response equivalent to n% of the maximum one (nK/100), the reparametrized equation is:

$$R(D) = K\{1-\exp[\ln(1-0.01n)(D/\mathrm{LD}_n)^a]\} \quad (16)$$

(4) An additional quantitative feature is the dose that elapses 100% of the tumor cell inhibition or in other words, the dose at which the response reaches the asymptotic parametric value of K (LD_K). This value can be obtained by arranging eqn (16) with a tricky simplification (considering 100% of the response as ~99.995%) as follows:

$$R(D) = K\{1-\exp[-10(D/\mathrm{LD}_K)^a]\} \quad (17)$$

Therefore, the general characterization of the inhibition process of tumor cells from TCLA can be described rigorously with the combinatory information provided by the parameter criteria of LD_{50}, D_A, LD_λ, LD_K or any desired LD_n values. Although the explicit eqn (13), (14), (16) and (17) have the same total number of parameters as eqn (6), the algebraic manipulation is less operative and less practical than the standard eqn (6) with the LD_{50} parameter.[61]

3.3. Problems with each of the possible parameters from the mathematical analysis that could be used as a single criterion to analyze the dose–response results of TCLA

When describing the cytotoxicity of a new agent, most of the research studies focus only on the production of the LD_{50}, but this parametric value on its own is much less reliable than generally anticipated. In fact, on their own any of the criteria values pointed out previously (LD_{50}, D_A, LD_λ, LD_K or any desired LD_n values) cannot produce reliable results in comparative terms with other agents even if the results are very reproducible.

To illustrate this fact, Fig. 2 shows a representation of the problems associated with the application of the possible parametric values to describe the dose–response inhibition of tumor cells. In the first part of Fig. 2 (part A), a graphical identification of the possible characterizing parameters for the analysis of TCLA is presented (including LD_{50}, D_A, LD_λ and LD_K). In the second part of Fig. 2 (part B) the lack of reliability of all the parametric criteria values is illustrated by means of simulation with eqn (6), (13), (14) and (17). By simulating and comparing the inhibition activity of five hypothetical agents (X_1 to X_5) for each of the characterizing parameters four different case scenarios (cases B1 to B4) illustrate the associated problems and are depicted in detail as follows:

- For LD_{50} parametric criteria (case B1): the graphical illustration shows how five well differentiated profile responses of agents (X_1 to X_5) would present identical LD_{50} values, but different D_A, LD_λ and LD_K parametric values.

- For D_A parametric criteria (case B2): the illustration presents five well differentiated profile responses (X_1 to X_5) that would lead to identical D_A values, but different LD_{50}, LD_λ and LD_K parametric values.

- For LD_λ parametric criteria (case B3): the illustration presents five differentiated profile responses (X_1 to X_5) that would lead to identical LD_λ values, but different LD_{50}, D_A and LD_K parametric values.

- For LD_K or any desired LD_n parametric criteria (case B4): the illustration presents five differentiated profile responses (X_1 to X_5) that would end up with identical LD_K values, but different LD_{50}, D_A and LD_λ parametric values. This trend can be applied to any desired LD_n values that produces a specific percentage of the mortality response.

These problems have been studied in other fields, notably in theoretical biology, in which the estimations are proposed to be based on the combinatory analysis of many parametric values for comparative purposes. Probably, because an unreliable solution has been found, as those illustrated in Fig. 2 (part B), when considering the response of only one parametric value. To add more complexity to the analytical characterization of the responses, different hypotheses could provide more complex scenarios with similar estimates. The imprecision of the estimates is generally larger than the differences between the approaches used. Moreover, all parametric results are highly dependent on the quality of the dataset. Thus, it becomes clear that when, any of these criteria used on their own as the single general response value of the action of an agent, they are very dependent on the shape of the curve and could produce easily untrustworthy comparative conclusions. Therefore, all parametric analyses need to be presented in conjunction to be able to compare the inhibitory activity of agents rigorously, and the more criteria are used the less would be the chances of being tricked by the effects accounted for.

3.4. Model application to experimental results specially designed to validate the model

Once that it has been performed, a detailed analysis (in statistical terms) of the most effective model solution for TCLA dose–responses, an extensive description of the parametric values that can be derived from that model solution and a discussion of the advantages and disadvantages of using one or another parametric value to characterize the TCLA dose–responses, in this section the selected model solution of eqn (6)

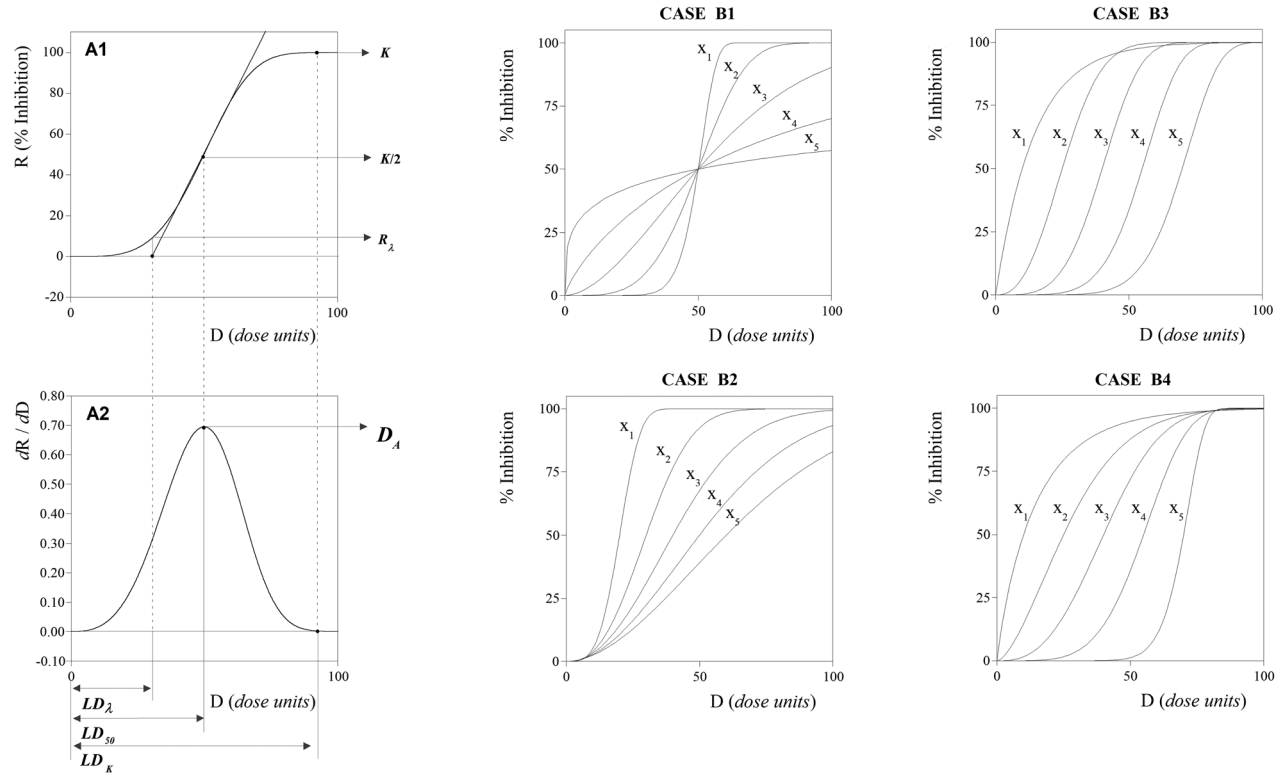

Fig. 2 Part A: Identification of the possible parameters for the analysis of TCLA responses by a conventional graphical representation. In the top part, the percentage of inhibition of tumor cells is plotted against the dose–response of a simulated agent. In the bottom part, the rate of the inhibition is presented (the corresponding numerical derivative). Part B: Illustration of the problems associated with the application of the possible parametric values to describe the dose–response inhibition of tumor cells: case B1, all profiles present the same LD_{50} values; case B2, all profiles present the same D_A values; case B3, all profiles present the same LD_λ values; and case B4, all profiles present the same LD_K values.

would be finally applied to describe, characterize, quantify and predict rigorously the dose response effects of agents from a range of TCLA.

Fig. 3 shows the fittings of the dose–response model of eqn (6) to the data obtained by the SRB colorimetric assay in four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) for screening the effects of the three commercial agents (EL, VP-16 and CCDP) and two natural extracts (ME and WE as a mixture of compounds). The experimental dose–responses of 125 data points per agent and tumor cell line are represented by the circles (○), meanwhile the lines (—) represent the fittings achieved by the mathematical model selected. Table 3 shows the parametric estimations of eqn (6) and the additional model values (eqn (13), (14) and (17) in order to obtain LD_{50}, D_A, LD_λ and LD_K parametric values, respectively), confidence intervals (α = 0.05) and statistical information of the dose–response results of the inhibition.

The fitting of results was always robust and consistent (p-values < 0.001 from Fisher's F test), the residuals were randomly distributed and autocorrelations were not observed by the Durbin–Watson test (data not shown). The statistical analysis, parameter assessment tools and model prediction uncertainties are in agreement accordingly. Furthermore, the correlation coefficients R^2 between the predicted and observed values were always greater than 0.93, with a wide majority of the fittings superior at 0.97.

3.5. Some examples of the analytical capabilities of the parametric values produced

Focusing on the standard commercial compounds and knowing that in each well 1.0×10^4 cells were placed (in 200 μL of final volume), we can extend the analysis in terms of molecules needed to inhibit the whole cell population. For example, for the cell line NCI-H460, for the EL case and using the parametric value of LD_K (31.6 ± 6.1 μM of EL, Table 3), 3.80×10^{11} molecules of EL were needed to inhibit each tumor cell in global terms (average inhibitory rate of molecules needed to inhibit the entire cell population). Because the process was non-linear, meaning that this global value varies depending on the dose, this average value cannot be computed by the LD_{50} as it depends on the profile of the curve.

Another interesting fact of the non-linear process here described is the maximum killing rate achieved by the compound, which it can be computed by using the parametric value of D_A (33.96 ± 1.1% Inh per μM for EL for NCI-H460 as an example, Table 3), which in this case was 3.55×10^{10} mole-

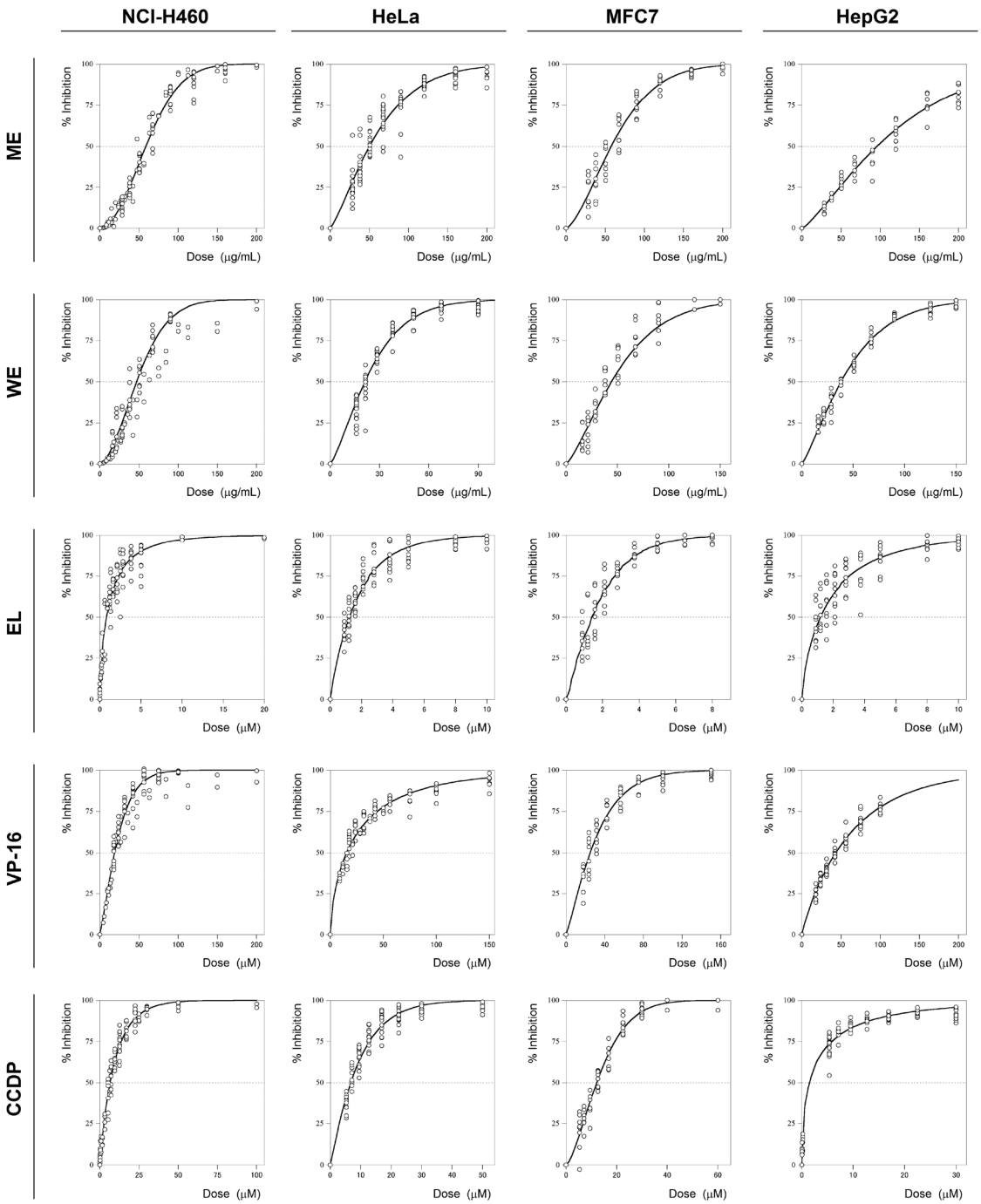

Fig. 3 Experimental dose–response data (O) to four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) by the SRB colorimetric assay for screening the effects of three commercial agents (EL, VP-16 and CCDP, as a single compound) and two natural extracts (ME and WE as a mixture of compounds). These data (125 data points per agent and tumor cell line) were used to test the nine models selected from the bibliography. Lines (—) represent the fittings achieved by the mathematical model selected. The obtained parametric fitting values are presented in Table 3.

cules of EL that were needed to inhibit each tumor cell. At the maximum rate value of the process, the cells were inactivated with ~11 times less molecules than at the average value computed above with the parametric value of LD_K.

For all other compounds in each cell line the following values (molecules per cell) were identified:

- For NCI-H460: EL (as described, 3.80×10^{11} with the maximum rate value of 3.55×10^{10} using ~11 times less molecules), VP-16 (1.55×10^{12}; 4.42×10^{11}; ~4) and CDDP (1.55×10^{12}; 2.06×10^{11}; ~5).

- For HeLa: EL (1.87×10^{11}; 3.72×10^{10}; ~5), VP-16 (7.06×10^{12}; 6.90×10^{11}; ~10) and CDDP (7.47×10^{11}; 1.76×10^{11}; ~4).

Table 3 Parametric estimations, confidence intervals (α = 0.05) and statistical information of the dose–response results of the inhibition process of four tumor cell lines (NCI-H460, HeLa, MCF-7 and HepG2) by the SRB colorimetric assay for screening the effects of three commercial agents (EL, VP-16 and CCDP, as a single compound) and two natural extracts (ME and WE as a mixture of compounds). The samples were fitted to the model selected of eqn (6) and the additional model rearrangements (eqn (13), (14), (16) and (17)) in order to obtain LD_{50}, D_A, LD_λ and LD_K parametric values. The dose units (D) ME and WE are in μg mL^{-1}, meanwhile the units for EL, VP-16 and CCDP are in μM. Table A6 shows the results in a comparative mode in μg mL^{-1} for all compounds

Cellular line & compound		Main fitting parameters			Other valuable parameters			Statistics
		K (% Inh)	LD_{50} (D)	a (—)	D_A (% Inh per D)	LD_K (D)	LD_λ (D)	R^2
NCI-H460	ME	100.0 ± 9.3	58.8 ± 6.4	1.98 ± 0.29	1.27 ± 0.2	196.9 ± 12.4	11.91 ± 3.00	0.9785
	WE	100.0 ± 8.1	47.0 ± 5.2	1.73 ± 0.11	1.12 ± 0.2	187.2 ± 13.5	6.50 ± 1.88	0.9347
	EL	100.0 ± 12.3	0.8 ± 0.3	0.65 ± 0.06	33.96 ± 1.1	31.6 ± 6.1	0	0.9546
	VP-16	100.0 ± 6.4	18.8 ± 2.6	1.25 ± 0.16	2.73 ± 0.5	128.5 ± 12.9	0.43 ± 0.17	0.9667
	CCDP	100.0 ± 7.1	6.5 ± 1.3	0.93 ± 0.17	5.85 ± 1.6	84.7 ± 11.4	0	0.9817
HeLa	ME	100.0 ± 21.5	50.2 ± 14.8	1.27 ± 0.10	1.04 ± 0.2	329.6 ± 32.4	1.38 ± 0.54	0.9359
	WE	100.0 ± 4.5	21.2 ± 1.4	1.32 ± 0.19	2.85 ± 0.5	129.6 ± 12.3	0.79 ± 0.30	0.9696
	EL	100.0 ± 26.7	1.2 ± 0.5	0.94 ± 0.16	32.42 ± 3.7	15.5 ± 2.1	0	0.9474
	VP-16	100.0 ± 26.8	15.4 ± 7.4	0.66 ± 0.06	1.75 ± 0.7	586.4 ± 11.3	0	0.9751
	CCDP	100.0 ± 12.4	6.8 ± 1.2	1.08 ± 0.04	6.85 ± 1.6	62.0 ± 7.2	0.02 ± 0.01	0.9690
MFC7	ME	100.0 ± 12.2	57.9 ± 8.8	1.53 ± 0.34	1.04 ± 0.2	275.5 ± 22.4	5.06 ± 1.65	0.9772
	WE	100.0 ± 12.9	44.3 ± 7.1	1.35 ± 0.15	1.38 ± 0.3	262.2 ± 24.3	1.89 ± 0.70	0.9683
	EL	100.0 ± 21.0	1.4 ± 0.4	1.12 ± 0.42	31.92 ± 7.2	12.2 ± 1.4	0.01 ± 0.00	0.9685
	VP-16	100.0 ± 10.2	25.0 ± 3.6	1.18 ± 0.29	2.00 ± 0.4	189.0 ± 20.0	0.33 ± 0.14	0.9847
	CCDP	100.0 ± 14.7	12.2 ± 2.2	1.50 ± 0.31	4.69 ± 0.8	59.9 ± 5.0	0.97 ± 0.32	0.9810
HeG2	ME	100.0 ± 60.4	96.0 ± 7.6	1.28 ± 0.44	0.45 ± 0.1	620.2 ± 60.4	2.86 ± 1.11	0.9639
	WE	100.0 ± 17.6	38.2 ± 9.0	1.25 ± 0.22	1.21 ± 0.2	258.6 ± 25.8	0.92 ± 0.37	0.9772
	EL	100.0 ± 82.2	1.2 ± 1.9	0.72 ± 0.01	24.93 ± 2.7	32.6 ± 5.7	0	0.8775
	VP-16	100.0 ± 38.7	43.4 ± 2.5	0.92 ± 0.07	0.78 ± 0.2	583.0 ± 79.1	0	0.9831
	CCDP	100.0 ± 17.3	1.5 ± 0.9	0.50 ± 0.01	13.39 ± 6.7	174.7 ± 43.7	0	0.9851

- For MFC-7: EL (1.47×10^{11}; 3.77×10^{10}; ~4), VP-16 (2.28×10^{12}; 6.02×10^{11}; ~4) and CDDP (7.21×10^{11}; 2.57×10^{11}; ~3).

- For HepG2: EL (3.93×10^{11}; 4.83×10^{10}; ~8), VP-16 (7.02×10^{12}; 1.55×10^{12}; ~5) and CDDP (2.10×10^{12}; 9.01×10^{10}; ~23).

Therefore, the application of these parametric values of the dose–response curve offers new formats of comprehending the dose effects of the compounds assessed, which can open to a greater extent the analytical capabilities of researchers and therefore, its conclusions. These new analytical capabilities can be used in complex scenarios. As an example, when combining compounds in pharmacology for synergistic purposes, it is likely that by applying the doses of these compounds at their respective maximum rates of inhibition (or maximum effective doses), the synergistic effects, if any, will show their maximum synergistic interaction. The maximum effective doses at which the D_A occurs are easily computable by eqn (13).

This analysis can be transferred to the mixture of compounds of the extracts (in this case ME and WE). Because the mixture is so complex, it will be nearly impossible to establish relationships between molecules and their effects on the cells, but its essence is preserved.

3.6. Potential equivalent cytotoxic activity of methanol and water extracts

Beyond quantitative differences, WE and ME extracts promote the cytotoxic activity in the four tumor cell lines. Note that the dose units (D) for ME and WE are in μg mL^{-1}, meanwhile for EL, VP-16 and CCDP the units are in a standard form of μM (μmol L^{-1}). Although the results between pure compounds and a mixture of compounds are not comparable, the total computable effects of the mixture of compounds (synergistic and antagonistic) present in the extracts can be compared in a concentration format to the commercial standards. In order to compare the mixture of compounds with standard molecules, the results of Table 3 of EL, VP-16 and CCDP need to be expressed in μg mL^{-1}. Such a conversion is presented in Table A6† and a visual comparison of the transformed model parametric values (LD_{50}, D_A, LD_λ and LD_K) is presented in Fig. 4. As the parameter LD_λ was not meaningful for all responses it will not be used for discussion. Using the equivalences of the relevant parametric values (LD_{50}, D_A, and LD_K) between each extract mixture of compounds (ME and WE) against each pure commercial compound (EL, VP-16 and CCDP) for each tumor cell line some extra relevant information will illustrate the problems highlighted in this document regarding the problem of using only one value as standard criterion. As an example, for the ME in the NCI-H460 cell line the equivalent values of EL show for the LD_{50} ~308 less equivalent effective values, for the D_A ~109 and for LD_K ~25.

For the ME all the equivalent values for the commercial compounds were the following:

- For NCI-H460: EL (as described, LD_{50} ~308; D_A ~109; LD_K ~25), VP-16 (LD_{50} ~5; D_A ~4; LD_K ~3) and CDDP (LD_{50} ~30; D_A ~15; LD_K ~8).

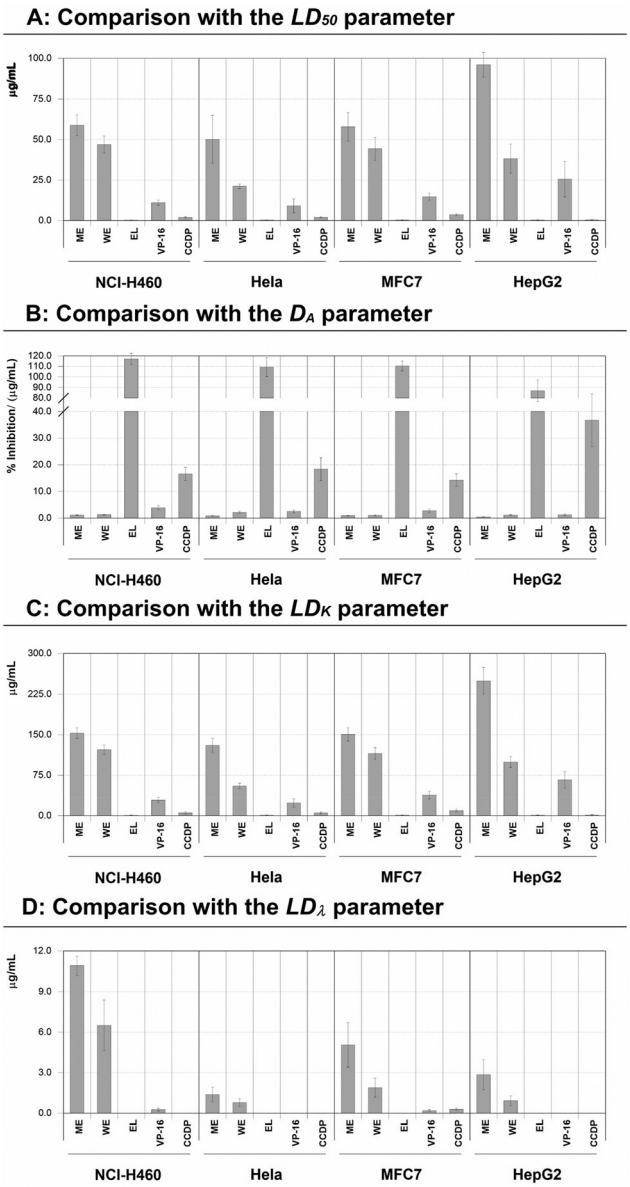

Fig. 4 Graphical comparison of the main parametric values to assess the experimental responses. Parametric values were obtained by eqn (6) for LD_{50}, eqn (13) for D_A, eqn (14) for LD_λ and eqn (17) for LD_K. Error bars display the confidence intervals (α = 0.05) of the parametric values.

- For HeLa: EL (LD_{50} ~169; D_A ~127; LD_K ~86), VP-16 (LD_{50} ~6; D_A ~3; LD_K ~1) and CDDP (LD_{50} ~25; D_A ~22; LD_K ~18).

- For MFC-7: EL (LD_{50} ~165; D_A ~125; LD_K ~92), VP-16 (LD_{50} ~4; D_A ~3; LD_K ~2) and CDDP (LD_{50} ~16; D_A ~15; LD_K ~15).

- For HepG2: EL (LD_{50} ~335; D_A ~224; LD_K ~77), VP-16 (LD_{50} ~4; D_A ~3; LD_K ~2) and CDDP (LD_{50} ~220; D_A ~99; LD_K ~12).

For the WE all the equivalent values for the commercial compounds were the following:

- For NCI-H460: EL (LD_{50} ~246; D_A ~123; LD_K ~24), VP-16 (LD_{50} ~4; D_A ~4; LD_K ~2) and CDDP (LD_{50} ~24; D_A ~17; LD_K ~7).

- For HeLa: EL (LD_{50} ~71; D_A ~46; LD_K ~34), VP-16 (LD_{50} ~2; D_A ~1; LD_K ~0.4) and CDDP (LD_{50} ~10; D_A ~8; LD_K ~7).

- For MFC-7: EL (LD_{50} ~127; D_A ~94; LD_K ~87), VP-16 (LD_{50} ~3; D_A ~2; LD_K ~2) and CDDP (LD_{50} ~12; D_A ~11; LD_K ~15).

- For HepG2: EL (LD_{50} ~133; D_A ~83; LD_K ~32), VP-16 (LD_{50} ~1; D_A ~1; LD_K ~0.75) and CDDP (LD_{50} ~88; D_A ~37; LD_K ~5).

Therefore, as it can be illustrated using values LD_{50} will underestimate the equivalent results of WE and ME in an average term about ~2 times than if we use D_A and up to ~5 times if we use LD_K. Presenting some extreme cases, as an example for the CDDP in the HeG2 cell line in which by using the LD_{50} or LD_K will show equivalent differences underestimating the activity of WE ~18 times. In another illustrative case, for the VP-16 in HeLa cell line the LD_{50} shows that the WE needs ~2 times the dose values to achieve the same response, but when the analysis is performed based on the LD_K parametric value the effect is inverted showing ~2 times higher cytotoxic effects of the WE than the VP-16.

In consequence, these results corroborate the conclusions previously discussed, that all parametric analyses need to be presented in conjunction to be able to compare the inhibitory activity of agents rigorously, and the more criteria are used the less would be the chances of being tricked by the effects accounted for.

4. Conclusions

Despite the existence of very rigorous results, the advances in the manipulation of cell lines, the technological sophistication of equipment to determine at a molecular level the cellular changes and the mechanization of the TCLA response, the quantification of the dose-effect results has been left aside as they were described between two and three decades ago, when the access to computerized systems was limited. Except for lineally dependent responses, in almost all the other cases, which are the most frequent ones in biology, non-linear expressions must be used to properly describe the effects of variables such as the dose ones. Avoiding the explicit application of mathematical models for the analysis of TCLA in endpoint assay responses could end in lower results reproducibility, which often would lead to an excessive quantity of restrictions in the protocol application. Similar to other authors in the cancer research area,[62–69] we suggest alternative forms to the simplistic ways of characterization of cytotoxic responses of TCLA and attempt to address this issue by bringing across theoretical, mathematical concepts from related areas to overcome the existing quantification difficulties and propose a unified criterion.

The range of mechanistic or empirical non-linear expressions available is large[70] and the preferable options are always those models that have a lower number of parameters and models with parameters that provide direct meaning of the processes under analysis.[53,58] In this sense, for the dose–response description of the mortality (inhibition) or survival (viability) rates of a tumor cell, they can be assessed by a group

of alternatives that already have been well described in the bibliography covering a wide spectrum of profile responses, from potential to sigmoid ones, with and without intercepts.[22,48,57,71] Independent of the intrinsic mechanistic rate of processes that those models translate, the empirical description of the cytotoxic effect of those processes show a wide range of 2D responses (hyperbolic, potential, first-order and sigmoidal). In general, most of the above models tested can be transferred to study the cytotoxic effects in TCLA and help to compute, in a proper form, the inhibitory features produced by different agents. Those models would be able to produce key parameters to summarize the responses and they can be used to quantify the effect of different chemicals. Since there are many model alternatives able to fit reasonably well the cytotoxicity in TCLA, a selection process is needed to determine the model that most efficiently describes and predicts the dose–response effects of agents.[59] The results of the comparison of several mathematical models for describing the experimental profiles highlighted the fitting and description capacities. The Weibull model was selected and tested using experimental results obtained for validation purposes and results from other authors under different conditions. Accurate and statistically consistent fittings were obtained in all the cases.

From the Weibull model, a set of parameters was proposed as criteria to aid the comparison of the cytotoxic results between agents (LD_{50}, D_A, LD_λ, LD_K or any desired LD_n values). All these parametric values are based on the first principles or with clear geometric and physical meaning, which describe the cytotoxic characteristics completely. However, as demonstrated, on their own none of them can produce reliable results and all parametric analyses need to be presented in conjunction to be able to compare the inhibitory activity of agents.

In consequence, the results prove that: (1) the preference for apparently simple quantification procedures, routinely applicable with minimal calculation requirements, is not very justifiable in our days, given the availability of computational applications and microplate readers, whose combination provides adequate tools to work with data sets that allow to perform accurate evaluations with non-linear mathematical models; (2) after a detailed statistical analysis of the dose–response models typically applied to a selected data set, the most efficient mathematical model for the description of the cytotoxic responses of TCLA methods is selected; (3) the reduction to study the dose–response only at LD_{50} values could lead frequently to unreliable results; and (4) other additional parametric values such as the maximum and average rate process, lag-phase period values, among others, should be presented along with the LD_{50} values in order to properly describe the cytotoxic effects of agents. Such results can be easily extrapolated to the other *in vitro* and *in vivo* assays generally used to determine the mortality patterns of tumor cells.

However, as stated by many authors before, living systems are exposed to agents and any particular effect may be expected to be a function of both the dose in the external surroundings and the exposure time, in a bivariate form.[58,72] This present work deals with the responses of *in vitro* TCLA assays performed in the end-point format, pinpointing the controversy around the analyses of the dose–response effects. We are aware that the traditional end-point studies suffer from an essential defect by supposing that at any time measured, the same dose–response parametric values would be produced.[5,9,10,15,16] However, this only occurs in an ideal case scenario, when all time-course dose–responses show the first-order profiles, and in all other scenarios the dose–response parametric values will change at different time analysis.[14] Therefore, this study is the first approach to establish, in a standard format, the basis for future work including the time-course effects in a multivariable model allowing to compute jointly time-dose dependent responses.[13,14,73]

Abbreviations

General notations

TCLA	Tumor cell line assays
ATP	Adenosine triphosphate
DMSO	Dimethyl sulfoxide
TCA	Trichloroacetic acid
HBSS	Hanks' balanced salt solution
Tris	Tris(hydroxymethyl)aminomethane, $(HOCH_2)_3CNH_2$
TrypLE express	Replacement of trypsin from animal origin
FBS	Fetal bovine serum
R^2	Linear correlation coefficient
R	Response of the % of inhibition

Tumor cell lines

NCI-H460	Human cell line of non-small cell lung carcinoma
HeLa	Human cell line of cervical carcinoma
MCF-7	Human cell line of breast adenocarcinoma
HepG2	Human cell line of hepatocellular carcinoma

Most frequent techniques for the evaluation of cell proliferation in cytotoxicity assays

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
SRB	Sulforhodamine B

Inhibitor agents of tumor cell growth

ME	Methanolic extract of *Achillea millefolium* L. plant
WE	Water extract of *Achillea millefolium* L. plant
EL	Ellipticine

VP-16 Etoposide
CDDP Cisplatin

Parametric values to describe the dose–response effect of agents

K Asymptote value of the response (% of death cells)
LD_{50} Dose units at which the 50% of R is achieved
a Sigmoid shape parameter (dimensionless)
LD_{λ} Dose units at which the minimum R is detected
D_A Dose with the maximum affinity in the response reaction (R per dose unit)
LD_K Dose units at which the maximum R is detected

Model selection criterion

AIC Akaike information criterion
AICc Akaike information criterion corrected
BIC The Schwartz or Bayesian information criterion
RIC Residual information criterion
Cp Mallows criteria
R^2_{adj} Adjusted correlation coefficient of multiplied determination
FPE Akaike's final prediction error
MSIC Model selection criterion
MA^2 Square model analysis

Mathematical models used

Michaelis–Menten	Eqn (2)
Bertalanffy	Eqn (3)
Verhulst	Eqn (4)
Gompertz	Eqn (5)
Weibull	Eqn (6)
Hill	Eqn (7)
Verhulst modified	Eqn (8)
Gompertz modified	Eqn (9)
Richards–Chapman	Eqn (10)

Conflicts of interest

There are no conflicts to declare.

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