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ORIGINAL ARTICLE

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In vitro cell growth pharmacodynamic studies: a new nonparametric approach to determining the relative importance of drug concentration and treatment time

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Abstract *Purpose*: The effect of an anticancer treatment on tumor cell proliferation in vitro can be described as a three-dimensional surface where the inhibitory effect is related to drug concentration and treatment time. The analysis of this kind of response surface could provide critical information: for example, it could indicate whether a prolonged exposure to a low concentration of an anticancer agent will produce a different effect from exposure to higher concentrations for a shorter period of time. The parametric approach available in the literature was not flexible enough to accommodate the behavior of the response surface in some of the data sets collected as part of our research programs. Therefore, a new, general, nonparametric approach was developed. *Methods*: The response surface of the inhibition of cell-based tumor growth was described using a radial basis function neural network (RBF-NN). The RBF-NN was trained using regularization theory, which provided the initialization of a constrained quadratic optimization algorithm that imposes monotonicity of the surface with respect to both concentration and exposure time. *Results*: In the two analyzed cases (doxorubicin and flavopiridol), the proposed method was accurate and reliable in describing the inhibition surface of tumor cell growth as a function of drug concentration and exposure time. Residuals were small and unbiased. The new method improved on the parametric approach when the relative importance of drug concentration and exposure time in determining the overall effect was not constant across the experimental data. *Conclusions*: The proposed RBF-NN can be reliably applied for the analysis in cell-based tumor growth inhibition studies. This approach can be used for optimizing the administration regimens to be adopted in vivo. The use of this methodology can be easily extended to any cell-based experiment, in which the outcome can be seen as a function of two experimental variables.

Keywords Pharmacodynamic analysis · Cell-based in vitro testing · Neural networks · Response surface

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Introduction

In cell-based experiments which seek to define the antitumor effect of new drug candidates, tumor cells are typically exposed to a concentration of the tested compound for a certain time. A range of different concentrations and exposure times are explored in a series of experiments and the inhibition of cell proliferation caused by the drug is evaluated. The results of this kind of experiment can be represented using a three-dimensional response surface (inhibition surface), in which the inhibitory effect on tumor cell growth is reported as a function of drug concentration and exposure time (see Figs. 4A and 5A). From these data, the relative importance of drug concentration and exposure time can be evaluated. For example, whether the exposure of tumor cell lines to high concentrations of the anticancer drug for a short period produces a greater antitumor effect than a more prolonged exposure to lower concentrations can be determined. This provides critical information on the optimal drug administration mode (at least from a qualitative point of view, e.g. for testing whether long infusions are better than bolus injections).

A pharmacodynamic model that expresses the relationship between drug concentration (C), exposure time

(t), and the resulting effect (E) for a chemotherapeutic agent has been reported [8]. It assumes that the effect is a function E(h) of a drug exposure constant h, which, in turn, depends on C and t through the relationship: $C^n \times t = h$. In this model, n is a parameter indicating the relative importance of C and t in determining the effect. When n is equal to 1 an increase in concentration (e.g. a doubling) will give the same antitumor effect if it is compensated for by an inversely proportional decrease (e.g. a halving) in exposure time. When n is greater than 1, the concentration plays a more important role than the exposure time: to keep the effect constant, an increase in C must be compensated for by a more than inversely proportional decrease in exposure time. On the contrary, when n is less than 1, t is more important than C. An example of the application of this model to mitomycin data is reported in reference 9.

This approach is parametric in the sense that it depends on the application of a specific mathematical relationship $E = f(C,t)$. The advantage is the ability to summarize the experimental outcomes with a small number of biologically relevant parameters (n, in this case). Nevertheless, we observed that the parametric model was not flexible enough to accommodate the behavior of the response surface in some of the data sets collected as part of our research programs. We thought this was related to the violation of the constancy of the parameter n throughout the range of concentrations and exposure times experimentally adopted. Therefore, an alternative nonparametric approach (whose mathematical formulation was not dependent on biological parameters), based on radial basis function neural networks (RBF-NN), is proposed to estimate the response surface. NNs are being used with increasing success in the analysis of biomedical data as multivariate tools for classification, pattern recognition and function approximation [2]. In the present context they were used to solve a bivariate function approximation problem. In essence, the inhibition surface is approximated as the superposition of monotonic smooth functions that are obtained by integrating bell-shaped functions with radial symmetry (radial basis functions, RBFs).

Materials and methods

Chemical supplies

Doxorubicin was from Pharmacia; flavopiridol was synthesized by Pharmacia. Other reagents were from Sigma (Milan, Italy).

Cell culture conditions

The human ovarian adenocarcinoma A2780 cell line was obtained from the European Center of Cell Cultures. Cells were cultured under standard condition (37°C, atmosphere containing 5% CO_2) using RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum and 2 m*M* glutamine.

Experimental

Experiments were performed according to previously described methods [11, 12] in a 96-well plate format. Approximately 5000 cells/cm^2 were incubated in RPMI 1640 medium. Each experimental condition was tested using eight replicates. Doxorubicin was used at the following concentrations: 5, 13, 33 and 82 n*M* and 0.205, 0.512, 1.28, 3.2, 8 and 20 μ*M*. Flavopiridol concentrations were 13, 33 and 82 n*M* and 0.205, 0.512, 1.28, 3.2, 8, 20 and 50 μ*M*. The following exposure times were evaluated for both compounds: 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 48 and 72 h. At the end of the treatment, the drug-containing medium was removed and the culture plates were rinsed with drug-free medium. In all the experiments, cell viability was evaluated at 72 h. At this time, cells were fixed adding 50 μl ice-cold trichloroacetic acid (50%) for 1 h. Plates were washed five times with distilled water and dried. Then 100 μl 0.4% sulforhodamine B acetic (1%) solution was added to each well for 30 min. Plates were rinsed five times with 1% acetic acid solution and dried again. Bound dye was solubilized with 100 μl 10 m*M* TRIS and plates were read at 490/540 nm. Replicates of blanks for background measurement (culture medium without cells) and controls (cells without treatment) were also included in each experiment. All data are expressed as percentage inhibition effect (E) compared to controls.

Nonparametric estimation of the response surface

Our goal was to estimate from noisy data the inhibition surface $E = g(C,t)$ making minimal assumptions on its shape. The only assumption was that the inhibition surface was monotonic and non-decreasing with respect to both exposure time and concentration (i.e. when either concentration or exposure time increased, inhibition could not decrease). The nonparametric approach was implemented in two steps. In the first step, RBF-NNs were used to filter the noise in the observed data to obtain a smoother surface without imposing the monotonicity constraint. Then, the monotonic surface that best approximated the smoothed surface was calculated. The subsequent application of NNs and constrained optimization was useful from a computational point of view: the first step was fast and brought the estimated surface close to the solution, thus reducing the run time of the second step.

The nonparametric approach based on RBF-NNs is more effective if the surface to be estimated does not exhibit trends. This is not the case for $E = g(C,t)$ that has an increasing trend. We solved the problem by considering as an unknown function the second derivative with respect to C and t (i.e. $f(C,t) = \partial^2 g(C,t)/\partial C \partial t$), which is indeed free of trends. Once f has been estimated the inhibition surface is immediately obtained by integrating f(C,t) along the C and t axes. In detail, in the first step the f(C,t) surface was described as the linear combination of a number of radially symmetric bell-shaped functions. The value of f(C,t) for a given concentration and exposure time was therefore calculated as the sum of all the RBFs contributing in the given point (see Fig. 1). In analytical form, the estimate $\hat{f}(C,t)$ of f(C,t) can be expressed as:

$$\hat{f}(C,t,\vartheta) = \sum_{k=1}^{M} \vartheta_k \cdot r_k(C,t) \qquad (1)$$

In Eq. 1, the functions $r_k(C,t)$ are two-dimensional Gaussian functions of constant width ϕ, centered in (C_k,t_k):

$$r_k(C,t) = e^{-\frac{(C-C_k)^2+(t-t_k)^2}{2\cdot\phi^2}}$$

Once the centers (C_k,t_k) have been fixed, the free parameters of the model are the M heights ϑ_k and the width ϕ of the Gaussian functions (neurons).

In the actual implementation, C and t values were log-transformed [$r_k = r_k(\log C, \log t)$] because the grid formed by the observations was more uniform in the log-log plane [the plane (logC,logt)] than in the plane (C,t). The position of the centers was not estimated but laid on a fixed uniform grid on the (logC,logt)

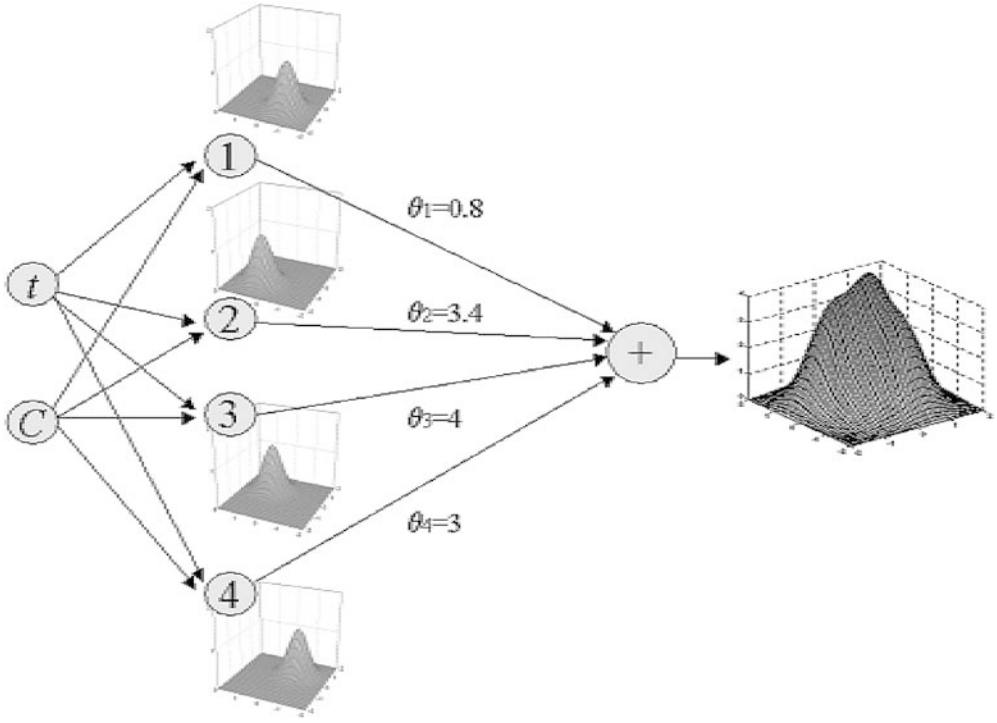


Fig. 1 Structure of an RBF-NN with four (RBF) neurons (M = 4). After the training, the four coefficients θ_1, θ_2, θ_3, θ_4 defined the heights of the Gaussian functions

plane that extended beyond the smallest experimental values of C and t in order to avoid border problems. For the application presented here a 21×21 grid proved satisfactory. Such a grid corresponded to a network with M = 441 neurons. Note that, because the number of unknown parameters largely exceeded the number of observations, this NN model was characterized by an extreme flexibility, but it could not be trained according to the standard least-squares paradigm. In order to address the model over-parameterization we resorted to regularization [10], a technique that is largely used in the field of ill-posed and inverse problems [1]. In particular, for a given ϕ the NN was trained by finding the weights ϑ_k that minimized the cost function:

$$J = SSR + \gamma \sum_{k=1}^{M} \vartheta_k^2 \tag{2}$$

where SSR is the sum of squared residuals and γ is a positive parameter to be properly tuned, which controls the compromise between least squares fit and smoothness of the surface (if γ is large the optimal weights will be small yielding a smoother surface) [3]. In this method the values ϑ_k were not free to assume any possible value, but rather they were constrained towards low values by the fact that the cost function J must be minimized (an increase in the sum of the squared parameters ϑ_k is obviously detrimental to the cost function). In our specific problem, the penalty on the parameters caused the second derivative of the surface to shrink towards zero, so that irregular profiles (typical of over-parameterized models) were avoided. This reduced the degrees of freedom of the model, thus making the estimation problem feasible. Notably, if we fix the centers, the width ϕ and the parameter γ, the weights ϑ_k that minimize Eq. 2 can be efficiently calculated solving a system of linear equations [6, 10]. Once the weights were computed, the goodness of fit was assessed by the generalized cross-validation (GCV) criterion [7] that provides an objective method for finding the optimal values of the parameters ϕ and γ, which are iteratively tuned until GCV is minimized. The GCV criterion is a numerically efficient approximation of the leave-one-out cross-validation procedure. It is used to assess how well a model will predict data points not used in the estimation stage. Overfitted models predict very well the points used for estimation but exhibit high GCV values because they are not able to predict accurately when applied to new data.

Note that in the practical implementation, no numerical derivation of the data was needed: the inhibition surface was represented just by a NN whose basic functions were obtained by integrating twice (along the t and C axis) the bivariate Gaussians r_k.

As a result of the first step, a smooth surface was obtained, but a violation of the monotonicity constraints could not be excluded. In order to enforce the constraints, in the second step the estimated RBF-NN was resampled on the grid of the centers. Then, the "final" estimated inhibition values $\left(\hat{E}_{i,j}\right)$ were computed on this grid as the closest ones (in a least squares sense) to the regularized inhibition values $\left(\tilde{E}_{i,j}\right)$ subject to the satisfaction of monotonicity with respect to both C and t:

$$\begin{aligned} &\sum_{i,j} \left(\hat{E}_{i,j} - \tilde{E}_{i,j}\right)^2 \\ &\tilde{E}_{i+1,j} \geq \tilde{E}_{i,j} \\ &\tilde{E}_{i,j+1} \geq \tilde{E}_{i,j} \end{aligned} \tag{3}$$

Equation 3 represents a typical quadratic problem that shows constraint limitations in the partial derivatives of the function with respect to concentration (index i) and time (index j). To solve this problem, well-established techniques are available, from among which we chose the so-called active set method (also known as a the projection method) [4, 5]. The nonparametric method described above has been applied using MATLAB (v 6.1; MathWorks, Natick, Mass.), which includes a built-in implementation of the active set method described above.

Parametric approach

The outcome of the nonparametric approach was compared with the parametric model reported in the literature [8]. Using this approach, the inhibition effect E for a given exposure time was considered to be dependent on the drug concentration C according to the Hill equation:

$$E = E_{\max} \frac{C^m}{IC_{50}^m + C^m}$$

where E_{max} is the maximum inhibition level (in our case $E_{max} = 100$), $IC_{50}(t)$ represents the concentration value at which the

effect is 50% of E_{max}, and m determines the steepness of the concentration-effect curve. In turn, the dependency of the effect on exposure time t was empirically described using a hyperbolic relationship $h = C^n \cdot t$ [8], where h is a constant related to the effect and n indicates the relative importance of C and t.

Overall, letting h_{50} be the value corresponding to $\frac{E_{max}}{2}$, the dependence of E on the exposure time t was described by:

$$IC_{50} = \left(\frac{h_{50}}{t}\right)^{1/n}$$

so that:

$$E(C,t) = \left(\frac{E_{max}}{\left(\frac{h_{50}}{t \cdot C^n}\right)^{m/n} + 1}\right) = E(h) \quad (4)$$

Nonlinear least squares (WINNONLIN professional, v. 3.1; Pharsight Corporation, Mountain View, Calif.) were used to estimate the three parameters of the model m, n and h_{50}, given the set of N observed data $z_\rho = EC(C_\rho, t_\rho) + v_\rho$, $\rho = 1,...,N$ where the measurement errors v_ρ are zero-mean and mutually uncorrelated with $Var[v_\rho] = \sigma^2$.

Results

First we compared the performance of the new methodology with that of the parametric one in a case in which the parametric approach was intrinsically to be preferred, as it was used to simulate the observations (simulation study). Both methods were then applied to real data.

Simulation study

Using the parametric model, a data set was simulated to evaluate the performance of the nonparametric method. The parameters were: $m = 1$, $h_{50} = 14.3$, $n = 2$. The concentration and the exposure time grid were the same as for the doxorubicin data. A Gaussian random error with zero mean and $\sigma^2 = 9$ was added. The performance of the estimate was assessed using the root mean square error (RMSE):

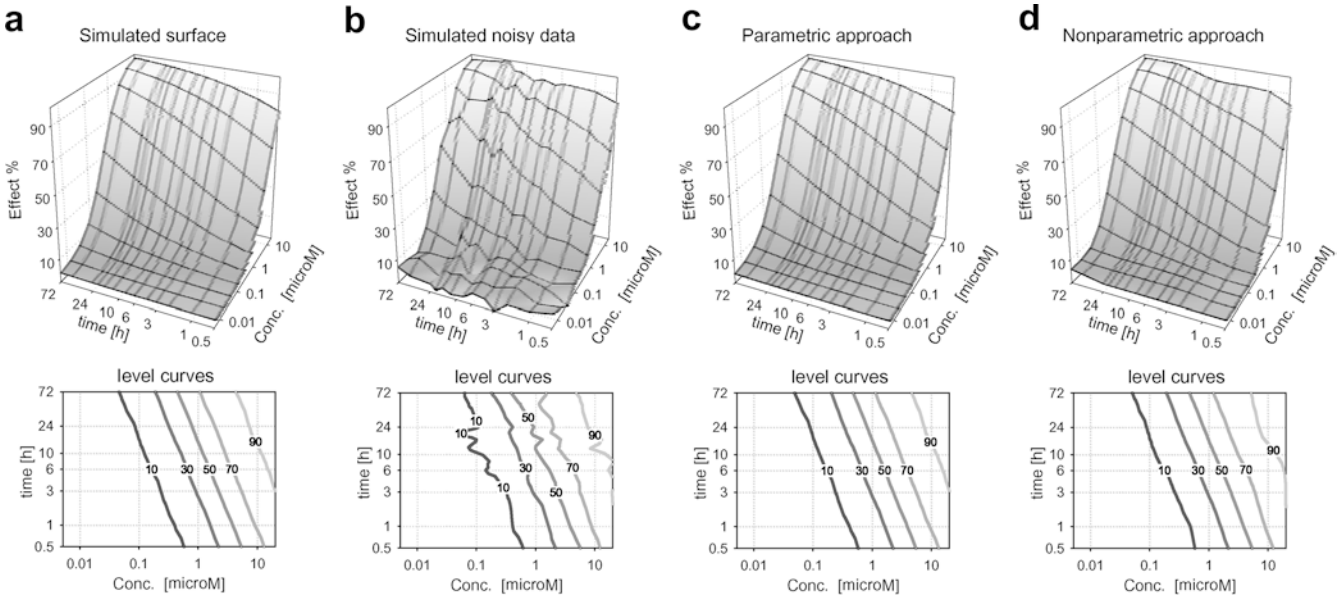
$$RMSE = \sqrt{\frac{1}{N}\sum_{i,j}^{N}\left(\hat{E}_{i,j} - E_{i,j}\right)^2} \quad (5)$$

where $E_{i,j}$ are the simulated inhibition data without noise addition, $\hat{E}_{i,j}$ are the estimates and N is the number of generated data.

The inhibition surfaces estimated using the two approaches are shown in Fig. 2c, d. As expected, the parametric method gave a low RMSE (0.58) and an estimate of SD (2.83) consistent with the σ^2 used for the simulation. Using the nonparametric approach, the RMSE was 1.23, still indicating a very good performance of the methodology (the RMSE value must be evaluated recalling that the simulated data represented percentage inhibition, ranging between 0 and 100). Analysis of the distribution of residuals (Fig. 3) showed that the residuals were uniformly distributed around 0 with an SD of 2.54. It must be born in mind that in the simulated example the parametric approach has the definite advantage of knowing the ''true model''.

It is interesting to note that the inhibition surface could be described also using a contour plot with level curves describing points on the surface characterized by the same inhibition effect. In the parametric model, the parameter n was associated with the slope of the level curves, which were parallel straight lines in the plane (logC,logt). It is remarkable that the level curves obtained using the nonparametric approach were close to the true level lines even if the model structure was not exploited (Fig. 2a, d, lower panel).

Fig. 2a–d Simulation study: **a** generated data ($n = 2$, $m = 1$, $h_{50} = 14$); **b** raw data (including a random error); **c** parametric estimate ($n = 2.04$, $m = 1.01$, $h_{50} = 15.7$); **d** nonparametric estimate (*upper panels* inhibition surfaces, *lower panels* level curves)



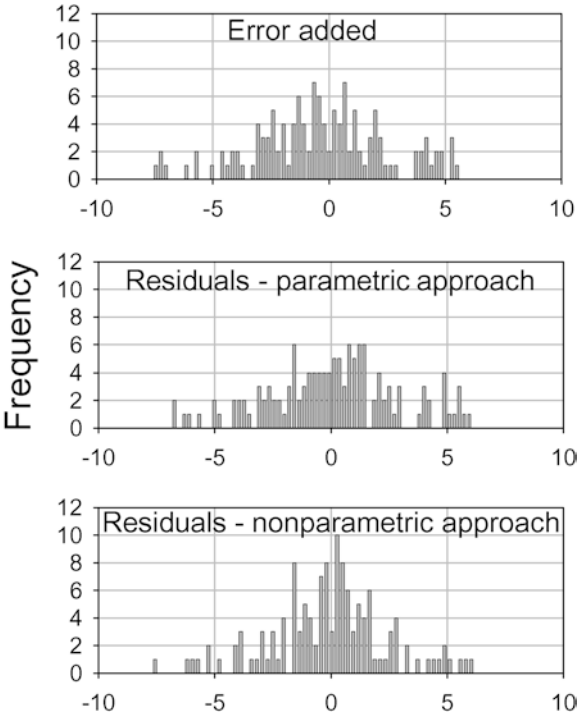


Fig. 3 Simulation study: distribution of the generated noise added to the simulated data (*upper panel*); distribution of the residuals obtained with application of the parametric approach (*central panel*); distribution of the residuals obtained with nonparametric approach (*lower panel*)

Real data

The nonparametric and parametric approaches were applied to analyze doxorubicin and flavopiridol cell-based tumor inhibition data. The results are shown in Figs 4, 5 and 6. In Fig. 7 the observed data and fitted lines are shown for selected exposure times for doxorubicin and flavopiridol, respectively. For doxorubicin, the residuals ranged between −15.4 and 14.7, (mean −3.2, SD 4.7) with the parametric approach, whereas with the nonparametric approach the residuals ranged between −5 and 5 (mean 0.0054, SD 1.15). For flavopiridol the parametric approach gave residuals ranging between −7.7 and 37.6, (mean 6.1, SD 8.6), whereas with the nonparametric approach the residuals ranged between −4.6 and 7.7 (mean 0.064, SD 2.00). In contrast to the parametric approach, the nonparametric approach yielded small and unbiased residuals.

Another difference between the two methods is highlighted by the level curves of the estimated inhibition surfaces. A level curve was formed by all points (C,t) that shared the same inhibition effect. The level curves of the surfaces estimated by the nonparametric method were not constrained to be parallel straight lines with slope −n (where n indicates the relative importance of concentration and exposure time, see Introduction), as holds for the parametric model. However, by computing the local slope of the level curve at a given point (C_ρ, t_ρ) it was possible to work out a local estimate for n, corresponding to that calculated using the parametric approach:

$$n(C_\rho, t_\rho) = \frac{\frac{\partial g(\log C_\rho, \log t_\rho)}{\partial(\log C)}}{\frac{\partial g(\log C_\rho, \log t_\rho)}{\partial(\log t)}} \tag{6}$$

In contrast to the parametric method the index n was not constrained to be constant, but rather it depended on C and t. This means that for a certain drug, there may be a region in the plane (C,t) in which it is more convenient to increase C in order to enhance inhibition, whereas in another region it may be more convenient to increase t.

In the case of doxorubicin, an antitumor agent with known cytotoxic characteristics, the estimated n(C,t) was always greater than 1 (in Fig. 4 the level lines are

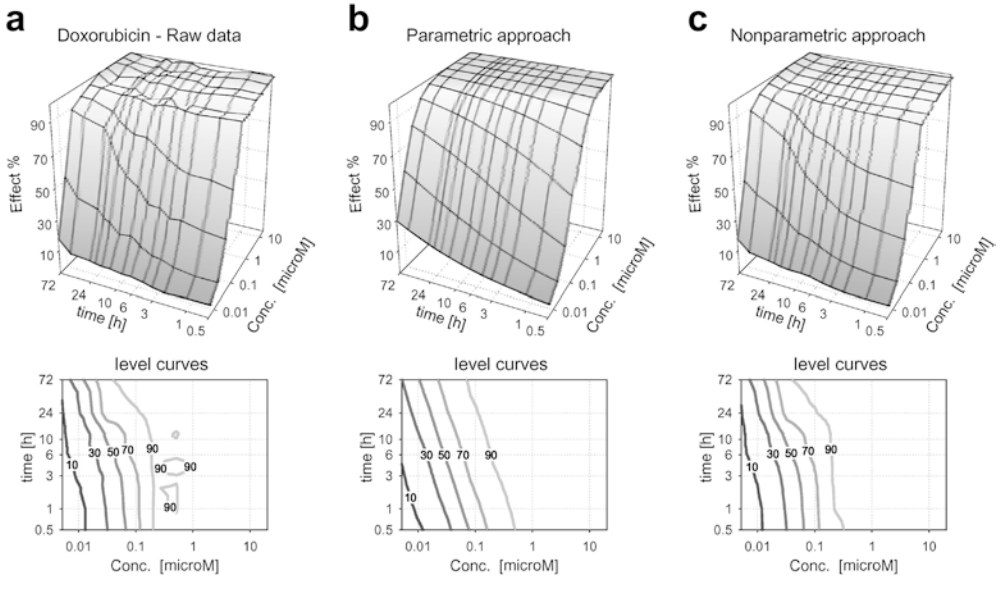


Fig. 4a–c Doxorubicin: **a** raw data; **b** parametric estimate ($n = 2.5$, $m = 1.2$, $h_{50} = 0.00084$); **c** nonparametric estimate (*upper panels* inhibition surfaces, *lower panels* level curves)

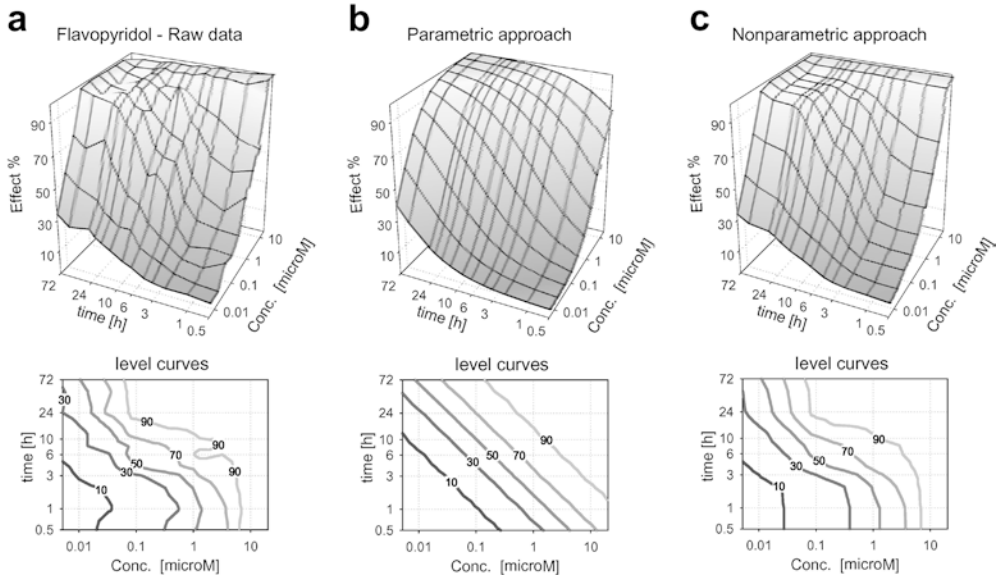


Fig. 5a–c Flavopiridol: **a** raw data; **b** parametric estimate ($n = 0.8$, $m = 0.81$, $h_{50} = 1.6$); **c** nonparametric estimate (*upper panels* inhibition surfaces, *lower panels* level curves)

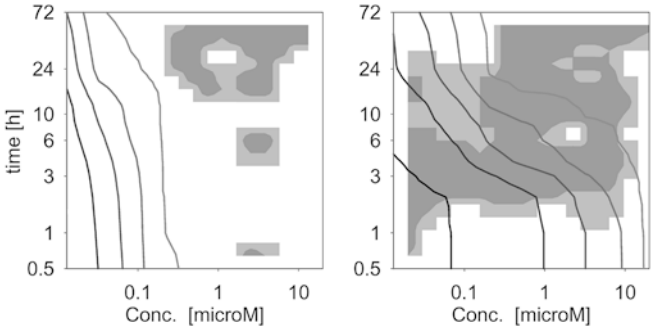


Fig. 6 Doxorubicin (*left*) and flavopiridol (*right*): plot of level lines and local n values (*dark gray* $n < 0.75$, *light gray* $0.75 \le n \le 1.5$, *white* $n > 1.5$)

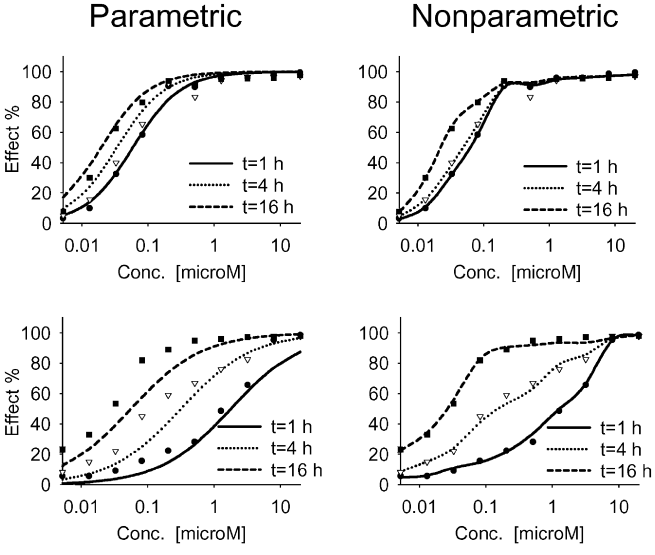


Fig. 7 Doxorubicin data (*upper*) and flavopiridol data (*lower*): observed and fitted cell-based tumor inhibition curves at selected exposure times

more "vertical" than lines with unitary slope). Therefore, for this sort of drug a "pulsed" exposure (high concentration and short exposure time) should be more efficient in optimizing the therapeutic response than a prolonged exposure to low concentrations. Note that both the parametric and nonparametric approaches gave similar results, because the actual level curves were reasonably approximated by straight lines.

Flavopiridol is a compound with a different mechanism of action (cell-cycle specific agent). In this case, the slope of the level curves defined three different regions in the (C,t) plane (Fig. 5). This characteristic was well described by the nonparametric approach where for exposure times shorter than 2 h and longer than 24 h the values of n(C,t) were greater than 1, indicating a behavior similar to that of doxorubicin. In contrast, for intermediate times, n(C,t) was less than 1, suggesting that in this region it would be more efficient to increase exposure times rather than concentrations to optimize the inhibition of tumor cell growth (Fig. 6). The parametric method, in this case, was not able to adapt the estimated response to the three different regions.

Discussion

The parametric model [8], although easy to apply, may be not flexible enough to provide a satisfactory description of the whole response surface. The new nonparametric approach fitted the cell-based tumor growth inhibition data with less constraint. This new method gave an estimated response surface similar to that obtained using the parametric approach, or even better in the case of complexities (i.e. the case where the relative importance of drug concentration and exposure time was not constant for all the concentration and exposure time values).

In the present implementation, the RBFs of the NN were constrained to have the same width ϕ (to be estimated) and to be centered on a predetermined grid. It goes without saying that the method can be generalized relaxing these constraints. Also, some consideration may be given to the choice of the number M of basis functions to be used: in our current version no major differences in the estimated surfaces were seen using M ranging from the number of experimental points to four times higher than that.

The differences observed between the features of the inhibition surfaces of doxorubicin and flavopiridol may indicate critical differences in terms of mode of action. Based on the recognition of the patterns, it is also possible to give suggestions concerning the administration modes to be adopted in vivo for optimizing the antitumor effect at the same dose intensity. In this respect, for cell-specific drugs, the available data indicate that conditions may exist in which exposure to relatively low concentrations for prolonged exposure times should be beneficial, whilst for a cytotoxic agent such as doxorubicin a pulsed exposure (high concentrations for short times) should typically be the most effective administration mode.

To have a full understanding of the applicability of this methodology, some further investigations should be done on the experimental setting used. For example, although improbable, it has to be considered that the patterns observed could be due to the choice of the experimental conditions adopted, e.g. constant duration of the experiments with variable recovery. A more comprehensive set of experimental conditions should therefore be explored (for example a set of constant recovery times).

It is interesting to note that this approach can be applied to any cell-based in vitro testing, in which observations depend on any couple of experimental variables. Assuming that the surfaces describing both efficacy and toxicity are available, the problem of safety margins could be addressed in vitro as a two-objective optimization problem. Again, this approach could be beneficial in designing the in vivo administration schedule able to optimize efficacy while minimizing toxicity.

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