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Nancy J. Millenbaugh · M. Guillaume Wientjes
Jessie L.-S. Au

A pharmacodynamic analysis method to determine the relative importance of drug concentration and treatment time on effect

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Abstract *Purpose*: The pharmacodynamics of most drugs follow the empirical relationship, $C^n \times T = h$, where C is drug concentration, T is exposure time and h is drug exposure constant. The value of n indicates the relative importance of C and T in determining the effect. An n value greater than 1.0 indicates that for two infusions that produce the same $C \times T$, a short infusion that delivers high concentrations over a short duration will produce a greater $C^n \times T$ and therefore a greater effect, compared to a long infusion that delivers lower concentrations. The reverse is true for an n value less than 1.0 and would support the use of a slow infusion. Hence, it is important to determine the n values and whether the n value significantly differs from 1.0. This report describes a three-step method for this purpose. *Methods*: First, we obtained experimental data on the relationship between drug concentration, treatment time and effect, and analyzed the data with a three-dimensional surface response method to obtain the pharmacodynamic model parameters and the magnitude of data variability. The experiments used mitomycin C and two human cancer cell lines, i.e. bladder RT4 and pharynx FaDu cells. The n values obtained from four experiments ranged from 1.04 to 1.16 for FaDu cells and from 1.14 to 1.46 for RT4 cells. The variability in the effect data decreased from 11.9% at 0% effect to 6.14% at 100% effect. Second, these results were used with Monte Carlo simulations to generate 100 concentration-time-effect data sets, which contained randomly and normally distributed data variability comparable to the experimentally observed variability, for each experimentally determined n value. This is analogous to performing 100 experiments under the same experimental conditions. Third, we analyzed the simulated data sets to obtain 100 estimated n values. The frequency with which these estimated n values fell above or below 1.0 indicated the probability that the experimentally determined n value used in the Monte Carlo simulations was truly different from 1.0. We defined this frequency for individual experiments as F_{one}, and calculated the overall probability for multiple experiments ($F_{multiple}$). A probability of greater than 97.5% (i.e. $P < 0.05$ for a two-tailed test) was considered statistically significant. *Results*: Analysis of the mitomycin C pharmacodynamic data yielded F_{one} and $F_{multiple}$ of 99% to 100% for FaDu and RT4 cells, indicating that the n values for these cells were significantly higher than 1.0. A comparison of the statistical significance of the n value analyzed by the three-step pharmacodynamic analysis method, a conventional statistical method such as the Student's *t*-test and nonlinear regression analysis, indicated two advantages for the pharmacodynamic method: fewer experiments were required (theoretically only one experiment with three replicates would be sufficient) and a higher statistical significance of the n value was obtained. *Conclusions*: In summary, the three-step pharmacodynamic study design and analysis method can be used to define the relative importance of drug concentration and treatment time on drug effect.

Key words Pharmacodynamic analysis · Monte Carlo simulations · Resampling techniques · Cancer chemotherapy

N.J. Millenbaugh · M.G. Wientjes · J.L.-S. Au (✉)
College of Pharmacy, The Ohio State University,
500 W. 12th Ave., Columbus, OH 43210, USA
e-mail: Au.1@osu.edu
Tel.: +1-614-2924244; Fax: +1-614-6883223

M.G. Wientjes · J.L.-S. Au
Comprehensive Cancer Center, The Ohio State University,
Columbus, OH 43210, USA

Introduction

Identification of optimal dosing regimens is an important field of study in cancer chemotherapy. The empirical pharmacodynamic relationship between drug concentration (C), treatment duration (T) and drug exposure constant (h), $C^n \times T = h$, where h is a constant

related to the drug effect, can be applied in in vitro experiments to determine the effect of treatment duration. The pharmacodynamic exponent n indicates the relative importance of C and T in determining the pharmacologic effect [1–4]. We have shown that analysis of the C vs T plot for a given effect (e.g. 50% inhibition of cell proliferation) provides the n value [3, 4]. When the n value equals 1.0, the relationship collapses to $C \times T = h$, and C and T are inversely related and contribute equally to the effect. In this case, treatment schedules that produce the same $C \times T$, regardless of the shape of the concentration-time profile (e.g. short exposure to high concentrations or long exposure to low concentrations), will result in identical effects. When the n value is greater than 1.0, the C^n term contributes more than T to the effect. In this case, a short infusion that delivers high concentrations will produce a greater effect than a long infusion that delivers the same $C \times T$ but lower concentrations. The reverse is true when n is less than 1.0.

It is noted that when the n value deviates from 1.0, even to a relatively minor extent, different treatment schedules can result in large differences in the $C^n \times T$ product. For example, two hypothetical concentration-time profiles, i.e. 10 μg/ml for 1 h or 1 μg/ml for 10 h, would yield identical $C^n \times T$ of 10 $\mu g^n \cdot h/ml^n$ at an n value of 1.0 but significantly different $C^n \times T$ values of 17.0 and 10.0 $\mu g^n \cdot h/ml^n$, respectively, at an n value of 1.23. Hence, the n value as well as the statistical significance of the n value (i.e. whether the estimated n is significantly different from 1.0) are important for deciding on the treatment schedules that produce the highest effect.

The relationship between the variability in the n value and the variability in the effect data is not known. As an example, if the effect data show a standard deviation (SD) of 20%, is the precision of the estimated n value also 20% and is an n value of 1.2 significantly different from 1.0? In a typical pharmacodynamic study, the drug effects at multiple treatment times are determined using several replicates at each time-point. These data provide a measurement of the variability in the effect data (e.g. SD of multiple observations), but not the variability in the n value because n is a calculated parameter derived from analysis of the concentration-effect relationship. A conventional method to determine the variability of the n value is to perform multiple experiments, analyze the data to obtain the n value for each experiment, determine the average value and variance of n, and analyze the data using statistical methods such as the Student's *t*-test to determine whether the average n value deviates from 1.0. The major disadvantage of this conventional method is the need for a large number of experiments, which may not be feasible, especially for studies using clinical materials such as patient tumor specimens.

We report here an alternative method to evaluate the statistical significance of the n value using the principle of resampling techniques. This method determines the variability in the n value based on the variability in the effect data by the use of Monte Carlo simulations. Resampling techniques have been applied in biomedical research in radiology [5], pharmacokinetics [6] and genetics [7], to evaluate variability in parameter estimates. Use of this method has the advantage that relatively few experiments (e.g. one to three experiments) are needed to estimate the n value and the variability in n, and to determine whether the estimated n is statistically different from 1.0.

Materials and methods

Experimental plan

The pharmacodynamic analysis method consists of several steps, as follows: (a) obtain pharmacodynamic data experimentally using replicate samples, (b) analyze the data with Eq. 1 (see below) to obtain the values of pharmacodynamic model parameters (E_0, h, m, n), (c) determine the magnitude of variability in the effect data from the replicate samples, (d) use Monte Carlo simulations, together with the experimentally determined pharmacodynamic model parameters and Eq. 1 to generate variability-containing pharmacodynamic data (100 data sets for each of the experimentally determined n values), (e) analyze the simulated data to obtain 100 n estimates (referred to as estimated n values), and (f) establish the fraction (i.e. frequency) of the estimated n values that deviated from 1.0. A frequency of >97.5% was considered statistically significant.

To generate experimental pharmacodynamic data, mitomycin C was used as the test drug. Two human cancer cell lines were used because previous studies have shown that the n values depend on the cell line [1, 2].

Chemicals and supplies

Mitomycin C was a gift from Bristol Myers Squibb Co. (Wallingford, Ct.). Cefotaxime sodium was purchased from Hoechst-Roussel (Somerville, N.J.), and sulforhodamine B (SRB) from Sigma Chemical Co. (St. Louis, Mo.). All other cell culture media and supplies were purchased from Gibco Laboratories (Grand Island, N.Y.). All chemicals and reagents were used as received.

Cell culture conditions

Human bladder RT4 and pharynx FaDu cells were obtained from the American Type Culture Collection (Rockville, Md.). RT4 cells were maintained in McCoy's medium containing 10% fetal bovine serum, and FaDu cells in minimum essential medium and 10% fetal bovine serum. All culture media were supplemented with 2 mM L-glutamine, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime sodium. Cells were incubated with complete medium at 37 °C in a humidified atmosphere of 5% CO_2 in air. For experiments, cells were harvested from subconfluent cultures using standard trypsinization procedures and resuspended in fresh medium before plating. The viability of cells was greater than 90%, as determined by trypan blue exclusion. The doubling times in exponentially growing cells were 33 h for RT4 cells and 22 h for FaDu cells.

Drug treatment

The stock solution of mitomycin C was prepared in culture medium at 750 μg/ml. Cells were seeded in 96-well microtiter plates at 5000 cells per well and allowed to attach to the well by growing in drug-free medium for 18 to 24 h. Afterwards, cells were incubated with mitomycin C at 0.001 to 100 μg/ml for five treatment times ranging from 1.5 to 48 h. Each experiment used six replicates. At the end of the treatment, drug-containing medium was removed and the culture plates were rinsed one to three times with drug-free medium.

Cells were then incubated with 200 µl of drug-free medium. All samples were processed for drug effect measurement at 48 h after addition of mitomycin C, regardless of the treatment time. Wells that contained only the tissue culture medium were included to serve as blanks.

Measurement of drug effect

Cell numbers were determined using the SRB assay. SRB stains for cellular proteins [8]. Briefly, after removing the culture medium, cells were rinsed once with 200 µl PBS. The cells were then fixed by incubating with 200 µl trichloroacetic acid at 4 °C for 1 h. The plates were washed five times with distilled water, allowed to air dry, and then stained with 50 µl 0.4% SRB solution. Afterwards, plates were washed five times using 1% acetic acid and allowed to air dry. Tris-HCl (200 µl, 10 m*M*) was added to each well to dissolve the protein-bound SRB, which was measured by absorbance at 490 nm using an EL 340 microplate biokinetics reader (Bio-Tek Instruments, Winooski, Vt.). A standard curve was constructed to determine the linear range of the absorbance as a function of the SRB concentration.

Pharmacodynamic model

Analysis of pharmacodynamic data was performed using the three-dimensional surface response as described by Eq. 1 [4].

$$E = E_0\left(1 - \frac{C^m}{\left(\frac{h}{T}\right)^{\frac{m}{n}} + C^m}\right) \quad (1)$$

where

$$\left(\frac{h}{T}\right)^{\frac{1}{n}} = IC_{50} \quad (2)$$

E_0 is the baseline value in the absence of drug and is the SRB reading for the untreated controls. E is the SRB reading for drug-treated samples. E_0 and E are expressed as percentage of the control value. Because E_0 is a fitted parameter, its value can exceed 100%. n is the pharmacodynamic exponent in the empirical relationship of $C^n \times T = h$. h is the drug exposure constant and is proportional to $(IC_{50})^n$ when T remains constant; a high h value indicates a high IC_{50} and therefore a lower tumor sensitivity [4]. m describes the shape of the surface response and a lower value of m is associated with a more shallow concentration-effect relationship. Equation 1 was derived by combining the empirical equation $C^n \times T = h$ and the sigmoid E_{max} model that describes a concentration response curve [4]. The assumption for the model described in Eq. 1 is that a given drug exposure will produce a pharmacologic effect of up to 100% (as opposed to an incomplete effect of <100%). We have shown that analysis of data by this surface response relationship provides more accurate and precise estimates of n, compared to a two-step method in which the IC_{50} values are first calculated for each exposure time and then fitted with the $C^n \times T = h$ equation to solve for the n value [4].

Monte Carlo simulations

The previously described Monte Carlo method [4] was used with Eq. 1 to generate the concentration-time-effect data sets. A two-step procedure was used to generate effect data which contain variability (E_{var}) that was representative of the variability observed experimentally. First, error-free effect data with no variability (E_{calc}) were generated. The other parameters described in Eq. 1, i.e. E_0, h, m, and n, were identical to those obtained experimentally (see Table 1). Second, a variability term, SD (standard deviation), was added to E_{calc} to generate the variability-containing E_{var} data using Eq. 3.

$$E_{var} = E_{calc} + SD * Rannor(x) \quad (3)$$

Rannor(x) values are normally distributed random numbers with a mean of 0 and a standard deviation of 1. The SD was derived from the experimental data obtained for RT4 and FaDu cells treated with mitomycin C. As shown in the Results, the coefficients of variation of E are linearly and negatively correlated with E. For the simulations, we used the average values, which ranged from 6% to 12%, to calculate the SD as a function of the E value (Eq. 4). As shown in the Results, simulations using these SD resulted in comparable variability as in the experimental data.

$$\text{Coefficient of Variation} = \frac{SD}{E_{mean}} \times 100\% = (\text{slope} * E + \text{intercept}) \quad (4)$$

where E_{mean} is the mean E value. Hence,

$$SD = \frac{(\text{slope} * E + \text{intercept}) * E_{mean}}{100\%} \quad (5)$$

Equations 3 and 5 were used to simulate E_{var}. For each experimentally determined n value, 100 concentration-response data sets with six replicates at each concentration were generated. The simulated data sets were then analyzed using Eq. 1 to obtain the estimated n values.

Determination of statistical significance of n values using the simulated data

The fraction of the 100 n estimates falling above or below 1.0 was determined for each experimentally determined n value that was used to generate the 100 n estimates. This fraction indicates the frequency of correctly identifying n values as greater or less than 1.0, and therefore the statistical significance of the experimentally determined n value. We defined the frequency of the n value in a single experiment to be either greater or less than 1.0 as F_{one}, and the frequency of the opposite event as (100% − F_{one}). Accordingly, the overall frequency for multiple experiments ($F_{multiple}$) was calculated as the (difference between 100% and the product of (100% − F_{one}) of individual experiments). The latter is analogous to determining the probability of having three female offspring consecutively (i.e. 50% × 50% × 50%). The null hypothesis that the n value was not different from 1.0 was rejected when the frequency was >97.5% (i.e. a >5% level of significance for a two-tailed test).

Standard method for determination of statistical significance of n values

A two-tailed Student's *t*-test was the standard statistical method used to determine whether the estimated n value was significantly different from 1.0 at a 5% level of significance. This analysis uses the variance in the n estimates and therefore involves determination of multiple n values from multiple sets of concentration-time-effect data. By this method, the minimum number of experiments (N) required to detect a difference, δ, between a mean n value and the value 1.0, at a 5% level ($\alpha = 0.05$) of significance with power of $1 - \beta = 0.90$, is described by Eq. 6.

$$N = \frac{SD^2}{\delta^2}(t_{\alpha(2)} + t_{\beta(1)})^2 \quad (6)$$

where SD is the standard deviation of the estimated n values and SD^2 is the sample variance calculated with N − 1 degrees of freedom, $t_{\alpha(2)}$ and $t_{\beta(1)}$ are the critical values of Student's *t* distribution for the α (two-tailed) and β (one-tailed) levels of significance, respectively [9].

Simulation and data analysis

Simulation of data sets and nonlinear estimation of pharmacodynamic parameters were performed using SAS (SAS Institute, Cary, N.C.) on an IBM model 3090 mainframe computer or a pentium-

Table 1 Pharmacodynamic parameters of mitomycin C. RT4 and FaDu cells were treated with mitomycin C for 1.5 to 48 h and the drug effect was measured by the SRB assay. The parameters were estimated by fitting Eq. 1 to the concentration-time-effect data using nonlinear least squares regression

Cell	Experiment	E_0 (%)	m	h	n
RT4	1	98.9	0.703	5.16	1.29
	2	100	0.633	4.28	1.22
	3	100	0.650	3.53	1.14
	4	100	0.665	2.29	1.46
	Mean ± SD	99.7 ± 0.55	0.662 ± 0.030	3.82 ± 1.22	1.28 ± 0.14
FaDu	1	101.9	1.19	6.63	1.04
	2	99.4	1.09	7.69	1.16
	3	97.0	0.962	5.84	1.16
	4	98.1	0.873	5.79	1.10
	Mean ± SD	99.1 ± 2.1	1.03 ± 0.14	6.49 ± 0.89	1.12 ± 0.06

based personal computer. Marquardt's method or the Multiple Variate Secant method (DUD) was used for all nonlinear estimations of parameters. Both methods gave the same results. Iteration for all simulated data sets converged. Equal weighting of the data was used (weight = 1). Statistical analysis of the difference between the selected n values and the estimated n values was performed by the two-tailed Student's *t*-test. The normality of frequency distributions was evaluated with the SAS UNIVARIATE procedure; deviation from normality was expressed by the Kolmogorov D statistic.

Results

This study was designed to develop a pharmacodynamic analysis method to determine the value of n and the statistical significance of the n values, and to compare the ability of the pharmacodynamic analysis method to determining the statistical significance of the n values compared to that of conventional methods.

Development of a pharmacodynamic analysis method

Figure 1A shows the representative classical two-dimensional sigmoidal concentration-response relationship in mitomycin C-treated FaDu cells. The results show a concentration-dependent effect and that nearly complete depletion of cells was achieved at higher drug concentrations, thus satisfying the assumptions used in Eq. 1. Similar results were obtained for RT4 cells (data not shown). Figure 2A shows the analysis of the data obtained for RT4 cells by the three-dimensional surface response method (Eq. 1). Similar data were obtained for FaDu cells (data not shown). Table 1 summarizes the pharmacodynamic parameters, E_0, m, h, and n, obtained in four experiments for each of the two cell lines.

The effect data were analyzed for the magnitude of variability. Figure 3 shows the plot of (distribution of the coefficients of variation of E) as a function of (E), for the data in RT4 cells and FaDu cells. The coefficients of variation of E were linearly and negatively correlated with E. A plot of residuals from the regression versus E showed no apparent deviations from linearity (data not shown). The average data variability, ranging from 11.9% at 0% effect to 6.14% at 100% effect, was used with Eqs. 3 and 5 to simulate variability-containing data.

Figures 1B and 2B show randomly selected examples of the simulated concentration-time-effect data. The simulated results are comparable to the experimental data, as indicated by the similar curve shape (Figs. 1 and 2) and the similar variability in the effect data (Fig. 3). Analyses of the simulated results by the conventional sigmoidal relationship and by the surface response method provided pharmacodynamic parameters that are comparable to the parameters used to perform the simulations (e.g. see legends for Figs. 1 and 2). The agreement between the experimental data and the simulated data confirms the ability of the Monte Carlo simulations to generate concentration-effect-data sets that are representative of the experimental data.

The simulation results were used to determine the probability that the n values for RT4 and FaDu cells were significantly different from (i.e. greater than) 1.0. For both cell lines, the values of F_{one} and $F_{multiple}$ for each of the four experiments exceeded 97.5% (i.e. 99% to 100%, Table 2), indicating that the n values were significantly greater than 1.0.

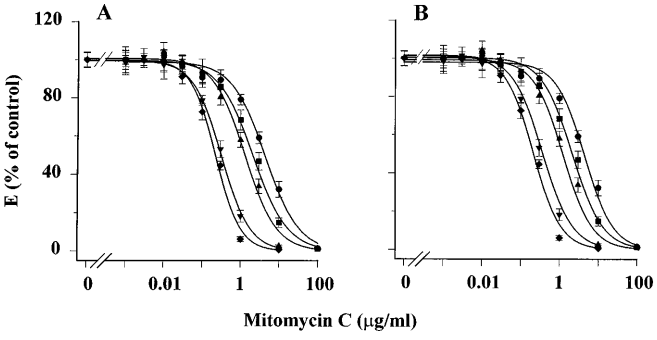

Fig. 1 Sigmoidal concentration-effect relationship for mitomycin C-treated cells. **A** Experimental data. FaDu cells were treated with 0.001 to 100 μg/ml mitomycin C for 1.5 h (●), 3 h (■), 6 h (◆), 24 h (◆), and 48 h (◆), and processed for the determination of drug effect by the SRB assay at 48 h. The drug effect was measured in terms of the remaining cell number and is expressed as percentage of the number in untreated controls. The data represent the mean ± SD of six replicates of a representative experiment. **B** Simulated data. Simulations were performed using the pharmacodynamic parameters derived from the experimental data (E_0 = 99.3%, m = 1.11, h = 7.86, n = 1.15). The simulated data set yielded comparable pharmacodynamic parameters (E_0 = 100.1%, m = 1.16, h = 7.67, n = 1.19). The data represent mean ± SD of six replicates of a randomly selected simulation. Symbols are the same as for the experimental data shown in **A**

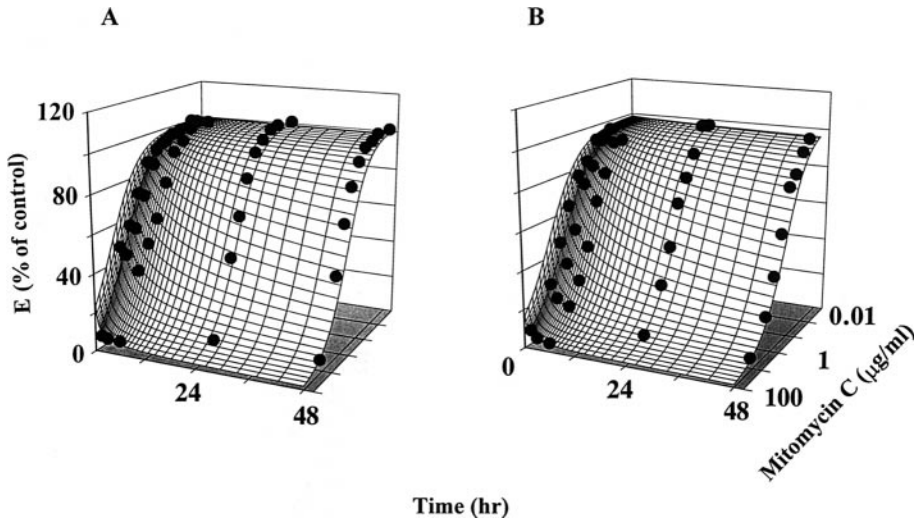

Fig. 2A,B Surface response analysis of data for mitomycin C-treated cells. **A** Experimental data. RT4 cells were treated with 0.001 to 100 μg/ml mitomycin C for 1.5 to 48 h, and processed for the determination of drug effect by the SRB assay at 48 h. The drug effect was measured in terms of the remaining cell number and is expressed as percentage of the number in untreated controls. The data represent the mean ± SD of six replicates of a representative experiment. The mesh surface was obtained from nonlinear parameter estimation according to Eq. 1. The resulting parameter estimates are: $E_0 = 100.0\%$, $m = 0.633$, $h = 4.28$, $n = 1.22$. **B** Simulated data. Simulations were performed using the pharmacodynamic parameters derived from the experimental data. The data represent the mean ± SD of six replicates of a randomly selected simulation. The mesh surface was obtained from nonlinear parameter estimation according to Eq. 1. The resulting parameter estimates are: $E_0 = 99.6\%$, $m = 0.67$, $h = 4.13$, $n = 1.30$

Comparison of pharmacodynamic analysis method with conventional Student's *t*-test for determination of statistical significance of n values

One major difference between the two methods is the number of experiments required to assess the precision of the estimation of n, or to demonstrate that n is significantly different from 1.0. For the pharmacodynamic analysis method, this could be accomplished by determining the n value and the effect data variability estimated from as few as one experiment with at least three replicate effect measurements. The replicates are needed to estimate the variability in the measurement of the drug effect. In contrast, the conventional statistical method requires the variance of the n value and therefore multiple experiments. As shown in the example in the Introduction, a 23% difference in n can cause a 70% difference in $C^n \times T$. Hence it is critical to achieve a high accuracy in the determination of the n value. We have arbitrarily selected an accuracy of 10% as a desirable goal; a 10% difference in n results in a 26% difference in $C^n \times T$. To obtain this degree of accuracy in the determination of n, which is equivalent to showing that an n value of 1.1 is significantly greater than 1.0, we calculated from Eq. 6 that 18 experiments would be required for a standard deviation of 0.14, as was observed in RT4 cells (Table 1).

Table 2 compares the ability of the two methods to determine the statistical significance of the n values. When the probability of the n values being different from 1.0 was analyzed either as F_{one} or $F_{multiple}$, the pharmacodynamic analysis method identified the n values as being greater than 1.0 with 99% to 100% frequency (i.e. $P < 0.01$) in each of the four experiments for both cells. Hence, one experiment was sufficient to establish the statistical significance of the n values in these cells. In contrast, the Student's *t* test analysis did not provide F_{one}, due to the nature of the data analysis, and required the results of three (RT4) or four (FaDu)

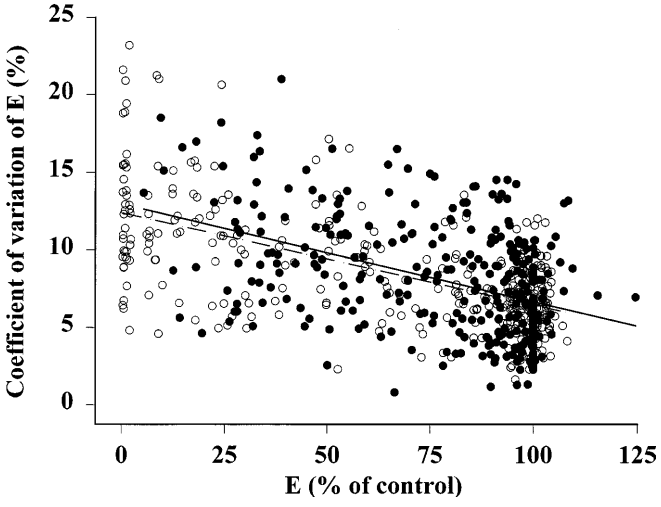

Fig. 3 Coefficient of variation of E as a function of E. RT4 and FaDu cells were treated with 0.001 to 100 μg/ml mitomycin C for 1.5 to 48 h, and processed for the determination of drug effect by the SRB assay at 48 h. Solid symbols are the coefficients of variation of E for six replicates for the experimental data. The *solid line* is the least squares linear regression line for the experimental data: $y = 13.0\% - 0.063\ E$ ($r^2 = 0.19$, $P < 0.0001$). *Open symbols* are the coefficients of variation of E for six replicates for the simulated data. The *dotted line* is the least squares linear regression line for the simulated data: $y = 12.0\% - 0.052\ E$ ($r^2 = 0.21$, $P < 0.0001$)

Table 2 Analysis of statistical significance of n estimates. Calculation of the frequency that the n value is greater than 1.0 by the pharmacodynamic analysis method requires only the n value and the variability of the effect data. Hence, this method provides the frequency for individual experiments (F_{one}) and the frequency for multiple experiments ($F_{multiple}$). In this method, $F_{multiple}$ was calculated as the difference between 100% and product of (100% − F_{one}) of individual experiments. Student's *t*-test requires the variance in the n values to calculate the overall frequency and therefore provides only the $F_{multiple}$, which, because at least three-n values are needed to calculate the variance value, was obtainable with at least three experiments. Note that the null hypothesis is rejected when F_{one} or $F_{multiple}$ is >97.5%, i.e. a *P*-value of <0.05 for a two-tailed test (*NA* not applicable)

Cell	Experiment	n	Pharmacodynamic analysis		Student's *t*-test	
			F_{one} (%)	Cumulative $F_{multiple}$ (%)	Cumulative variance	Cumulative $F_{multiple}$ (%)
RT4	1	1.29	100	NA	NA	NA
	2	1.22	100	100	NA	NA
	3	1.14	100	100	0.0058	96
	4	1.46	100	100	0.0192	97
FaDu	1	1.04	99	NA	NA	NA
	2	1.16	100	100	NA	NA
	3	1.16	100	100	0.00480	90
	4	1.10	100	100	0.00330	97

experiments to establish $F_{multiple}$ of >95% (i.e. $P < 0.05$).

Relationship between the variability in n values obtained from pharmacodynamic analysis and from asymptotic standard error of nonlinear regression

Another method to obtain variability in the n value is the SAS nonlinear regression procedure which provides the asymptotic standard error of the estimated n. We compared the variabilities in the pharmacodynamic parameter estimates provided by the pharmacodynamic analysis method and the nonlinear regression method, in FaDu cells (Table 3). The asymptotic standard errors in the n value obtained by nonlinear regression analysis were, on average, 30% higher than the standard deviation obtained for 100 Monte Carlo simulations based on the same data set. A similar comparison for the other estimated parameters (i.e. m, h, E_{max}) showed that the standard error from the nonlinear regression analysis ranged between 95% and 200% of the variability obtained from the pharmacodynamic analysis.

Relationship between variability in experimental effect data and in the value of n

The above results on the variability in n were generated using simulations where the coefficient of variation of the effect was allowed to change with the value of the effect, as observed experimentally. We also determined the variability in n for situations where the coefficient of variation of the effect stayed constant, independent of the effect level. The resulting coefficients of variation in n for effect data with 10%, 20% and 30% coefficient of variation were 2.4%, 4.9%, and 7.5%, respectively. These data indicate a several fold lower variability in the n value compared to the variability in the effect data.

Table 3 Comparison of variability in pharmacodynamic parameter (n, h, m, E_{max}) estimates obtained by pharmacodynamic analysis and nonlinear regression analysis. Results were obtained from experiments using FaDu cells. Ratio represents the ratio between the standard error from the nonlinear regression analysis and the standard deviation from the pharmacodynamic analysis

Parameter	Experiment	Pharmacodynamic analysis (mean ± SD)	Nonlinear regression analysis (mean ± asymptotic SE)	Ratio
n	1	1.038 ± 0.022	1.039 ± 0.034	1.49
	2	1.154 ± 0.030	1.156 ± 0.027	0.92
	3	1.163 ± 0.034	1.165 ± 0.032	0.94
	4	1.099 ± 0.020	1.100 ± 0.026	1.27
h	1	6.640 ± 0.224	6.630 ± 0.305	1.36
	2	7.697 ± 0.273	7.688 ± 0.259	0.95
	3	5.848 ± 0.234	5.835 ± 0.240	1.03
	4	5.796 ± 0.149	5.787 ± 0.205	1.38
m	1	1.196 ± 0.038	1.192 ± 0.058	1.53
	2	1.087 ± 0.030	1.086 ± 0.032	1.07
	3	0.962 ± 0.025	0.961 ± 0.030	1.21
	4	0.874 ± 0.014	0.873 ± 0.022	1.57
E_{max}	1	99.96 ± 0.717	101.90 ± 0.787	1.10
	2	99.99 ± 0.391	99.38 ± 0.485	1.24
	3	99.99 ± 0.303	96.96 ± 0.549	1.81
	4	99.99 ± 0.245	98.12 ± 0.489	2.00

Distribution of estimated parameters

In the simulations, the errors added to the effect measurements were generated with a random error generator, with a normal distribution about the mean. We sought to determine whether the resulting distributions of the estimated parameters, n, m, h, E_{max}, also approached normality. Using the parameter estimates from 1000 simulations, we found normal distributions for all four parameters. In all cases, skewness and kurtosis of the distributions were less than ± 0.26 ($P > 0.15$).

Discussion

The value of the pharmacodynamic exponent n in the empirical relationship, $C^n \times T = h$, is important for determining the relative importance of drug concentration and treatment duration on the treatment outcome. For example, an n value of greater than 1.0 indicates a more important role for drug concentration than for treatment duration. Similarly, the drug concentration plays a more important role at an n value of 1.5 compared to an n value of 1.2. As shown in the present study, the value of n and whether the n value significantly deviates from 1.0 can be determined by three methods.

First, the n value and the variance in the n value can be determined by performing multiple experiments. These values are then analyzed by conventional statistical methods to determine whether the mean n value deviates from 1.0. This method, because of the need for multiple experiments, is not convenient nor cost-effective, and, in the case where the availability of human tissues is limited, cannot be accomplished. Second, the experimental data can be analyzed by nonlinear regression to obtain the n value and its asymptotic standard error. The n value is considered to be significantly different from 1.0 if 1.0 falls outside the 95% confidence intervals. Third, we can obtain the n value by analyzing the experimental data and using the experimentally determined pharmacodynamic parameters together with Monte Carlo simulations to generate additional n values. The n values are then analyzed to determine the frequency at which the n values deviate from 1.0. A frequency of >97.5% is considered significant (equivalent to 5% level of significance by a two-tailed test).

The advantage of the nonlinear regression method is its relative ease. In comparison, the new pharmacodynamic analysis method requires more steps. The variability in the n value obtained from the nonlinear regression analysis was similar to that from the pharmacodynamic analysis (Table 3). An advantage of the pharmacodynamic analysis method is that this computer simulation-based method allows us to examine the consequences of changes in the experimental data, whereas the nonlinear regression analysis does not provide this flexibility. This capability is especially useful for designing experiments. For example, we can use the pharmacodynamic analysis method to simulate whether and to what extent an increase in the variability in the effect data will alter the estimate in the n value, which in turn allows us to develop guidelines on the acceptability of the experimental results (e.g. whether a 20% variability in the effect data is acceptable). This in turn provides the basis to select between a more cost-effective but less precise assay (e.g. SRB assay) as opposed to a more labor-intensive but more precise assay (e.g. cell count), and provides the basis to select drug concentrations and treatment durations that would provide the most precise results. Furthermore, the use of the Monte Carlo simulations circumvented the need for defining the mathematical relationship between the variability in the effect data and the n values. With a pentium-based personal computer, this new pharmacodynamic analysis method including the generation of 100 data sets and the subsequent analysis can be completed within 10 min.

The following summarizes the three-step new pharmacodynamic analysis method and our recommendations. (a) Determine the drug effect at various drug concentrations and treatment times, and analyze the data using the surface response method described in Eq. 1 to obtain the n value and model parameters. Three or more replicates are needed to provide a measurement of variability in the effect data, as a function of effect (i.e. variability usually decreases as effect increases, see Fig. 3). Although one experiment is theoretically sufficient, we recommend performing three independent experiments to obtain a measurement of intra- or interday variation in data and to rule out outliers. A greater number of observations provided by multiple experiments with multiple replicates, as compared to fewer observations, are more likely to provide accurate data variability, which in turn is important for simulating the variability-containing data sets. (b) Use Monte Carlo simulations, together with the experimentally derived model parameters, to generate multiple concentration-time-effect data sets for each experimentally determined n value. The number of simulated data sets used in the present study was 100. A greater number could also be used. (c) Analyze the simulated data sets to obtain multiple estimated n values, determine the precision of estimation of n by comparing the estimated n value with the experimentally determined n value used to generate the simulated data sets, and determine the frequency with which the estimated n values deviate from 1.0. A frequency of >97.5% is considered statistically significant.

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