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Estimation of the Chemosensitizing Activity of Modulators of Multi-drug Resistance via Combined Simultaneous Analysis of Sigmoidal Dose–Response Curves

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Abstract

The potency of modulators which re-establish sensitivity of resistant tumour cells to cytotoxic drugs is not usually described by ED50 values, even though such values are needed for comparison of modulator activity.

Various methods are reported for the determination of ED50 values of propafenone-type modulators of multi-drug resistance in cytotoxicity assays. Best results were obtained by using a combined simultaneous analysis of dose–response curve families. This approach enables calculation of statistically highly significant ED50 values without any data reduction directly from the original data points obtained in daunomycin cytotoxicity assays.

The method also enables extrapolation of the ED50 values of compounds with low activity or poor solubility, or both.

Cellular resistance to a broad spectrum of cytostatic drugs is an increasing problem in cancer therapy. The phenomenon of multi-drug resistance (MDR) confers resistance to a large variety of structurally and functionally diverse drugs, such as anthracyclines, epipodophyllotoxins, actinomycin D, vinca alkaloids, colchicine and taxol (Bradley et al 1988). The cross-resistance profile is usually accompanied by a reduction in drug accumulation in resistant cells and it has been shown that this decrease in intracellular drug levels is often mediated by the expression of P-glycoprotein. This trans-membrane protein functions as an ATP-driven efflux pump for a wide variety of cytotoxic drugs (Horio et al 1988). Several substances have been identified which are able to re-establish sensitivity of resistant tumour cells to cytotoxic drugs in-vitro (Ecker & Chiba 1995).

Different approaches are used to define modulatory potency. Potency is not usually described by ED50 values; most authors determine the MDR ratio, or MDR-fold reversion, which is expressed as the ratio of the ED50 value of a toxin in the absence of modifier and the ED50 value of the toxin in the presence of a fixed concentration of modulator (Ford et al 1989; Dhainaut et al 1992). A comparison of modulator potencies is attempted under the assumption that modulator efficacies are equal. Some papers give changes in the ED10 or ED50 values of modifiers at fixed toxin concentrations (Dodic et al 1995). For rational modifier design, which requires the search for quantitative structure–activity relationships, there is, however, a need for precise and comparable modifier activity data, e.g. ED50 values, for modulating activity. In this paper, various methods based on simultaneous analysis of dose–response curve families are reported for the determination of modulator ED50 values using cytotoxicity assay data.

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Materials and Methods

Chemistry

Compounds **1a-k** and **2a-e**, the chemical structures of which are given in Fig. 1, were prepared as described elsewhere (Ecker et al 1994, 1995, 1996; Chiba et al 1996).

Cell lines and culture conditions

The CCRF-CEM T lymphoblast cell line and the resistant line were obtained as described previously (Chiba et al 1996). Cells were kept in RPMI1640 medium supplemented with 10% foetal calf serum under standard culture conditions. The resistant CCRF vcr 100 cell line was kept in the continuous presence of 100 ng mL^{-1} vincristine. The selecting agent was washed out at least 1 week before the experiments. The cell line used in our studies was selected in the presence of increasing doses of vincristine without prior mutation. This cell line has been chosen on basis of distinct P-glycoprotein expression and does not show the mutation at codon 185. In addition, no significant contribution of other factors to MDR could be observed (unpublished data).

Cell cytotoxicity assay

The assay is dependent on the cellular reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St Louis, MO) to water-insoluble formazan in the mitochondria of viable cells. The assays were performed in 96-well plates essentially as described by Mosmann (1983), with the exception that water-insoluble formazan granules were dissolved in 0·04 M HCl in 2-propanol. The absorbance was read spectrophotometrically using an EL311 Biotek microtiter plate reader (Biotek Instruments, Highland Park, VT).

The chemosensitizing effect of the propafenone-type modulators relative to daunomycin was determined using CCRF-CEM vcr 100 cells. One independent experiment represents the evaluation of the toxicity of daunomycin in CCRF-CEM vcr

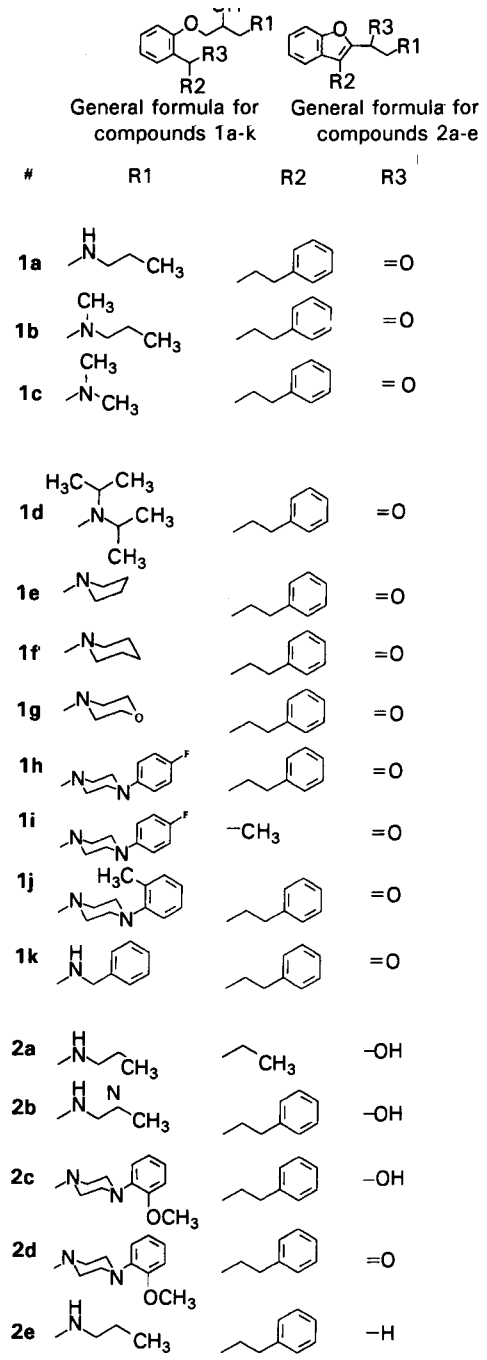

FIG. 1. The chemical structures of the compounds investigated.

100 cells in the presence of six different concentrations of a modifier. Each experiment also includes the measurement of a dose–response curve for daunomycin without modifier (VCR-control) and of one curve of the non-resistant, wild-type CCRF-CEM line (WT-control). Six different concentrations of toxin were usually used for each dose–response curve (Fig. 2).

In a first step, these experiments were analysed conventionally by fitting each dose–response curve of daunomycin according to equation 1:

$$\%S = 100 - 100[T]^a/(ED50_T{}^a + [T]^a) \quad (1)$$

where %S is the percentage survival, [T] the concentration of the toxin daunomycin, $ED50_T$ the ED50 value of daunomycin for a given concentration of modulator and a the Hill coefficient of daunomycin-toxicity curves. This model leads to eight ED50 values for daunomycin and the corresponding Hill coefficients. A plot of $ED50_T$ values against log (modulator concentration) leads to a sigmoidal dose–response curve which enables estimation of the ED50 value of the respective modulator according to equations 2a and 2b. To describe this dose–response curve a Minmax function was used with the minimum effect (MIN) being expressed as the ED50 value of toxin in the absence of modulator and the maximum possible effect (MAX) corresponding to complete reversion, represented by the ED50 value of toxin in the parental CCRF-CEM line (Fig. 3):

$$ED50_T = MIN - (MIN - MAX)\,[M]^b/(ED50_M{}^b + [M]^b) \quad (2a)$$

$$ED50_T = ED50_{VCR} - (ED50_{VCR} - ED50_{WT})\,[M]^b/(ED50_M{}^b + [M]^b) \quad (2b)$$

$ED50_T$ represents the ED50 value of daunomycin observed in the presence of a given concentration of modulator, $ED50_{VCR}$ and $ED50_{WT}$ are the ED50 values of daunomycin for the resistant CCRF-CEM vcr 100 control and the sensitive CCRF-CEM wild-type control (to generate more precise results these values are used individually for each experiment), [M] is the concentration of modulator, $ED50_M$ is the ED50 value of the modulator and b represents the Hill coefficient of the dose–response curve of the modulator. ED50 values of the modulators are listed in Table 1 (column-named single fit).

According to De Lean et al (1978), efficient data analysis should involve simultaneous description of all dose–response curves, rather than fitting them individually. A simultaneous analysis of daunomycin toxicity curve families was, therefore, performed according to equation 1 using a previously described algorithm for non-linear least squares fit (Schaper et al

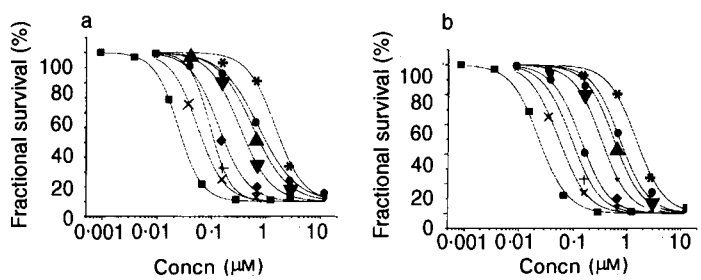

FIG. 2. Toxicity of daunomycin to CCRF-CEM vcr 100 cells in the presence of various concentrations of **1a**: (■) wild type CCRF-CEM control, (●) 0·31 μM, (▲) 0·63 μM, (▼) 1·25 μM, (◆) 2·5 μM, (+) 5·0 μM, (×) 10·0 μM, and (*) CCRF-CEM vcr 100 control: a. single fit of each dose–response curve, b. simultaneous fit of all dose–response curves.

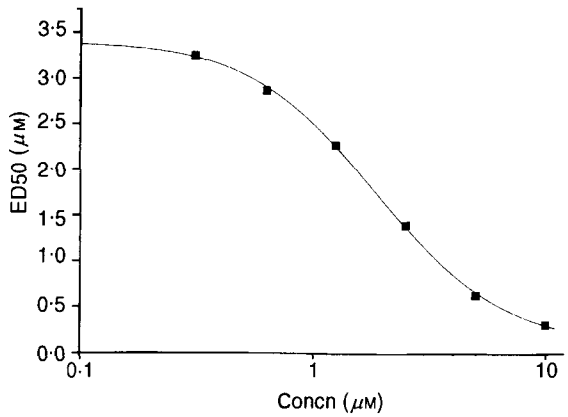

FIG. 3. Modulatory activity of compound **1g** obtained using daunomycin cytotoxicity assay. The dose–response curve was calculated using the single–fit procedure (ED50 = 1·85 μM).

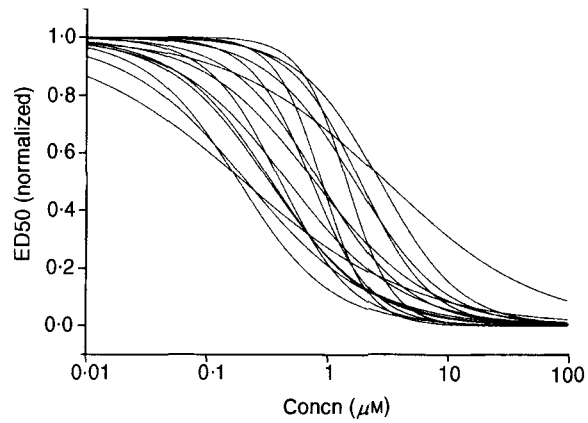

FIG. 4. Dose–response curves of all modulators tested. Potency for all modulators was normalized to values 0–1 using the minmax function.

1995). This analysis leads to eight $ED50_T$ values for daunomycin and only one Hill coefficient, a, for each cytotoxicity experiment with one modulator tested at eight concentrations (Fig. 2b). Using this procedure for estimation of $ED50_T$ values, modulator $ED50_M$ values were subsequently derived from these $ED50_T$ values by use of equation 2. The $ED50_M$ values are also presented in Table 1 (column-simultaneous toxin fit).

According to Schaper et al (1995) it is possible to analyse the dose–response curves of different drugs simultaneously under the assumption that all derivatives have the same mechanism of action within a given test model and, therefore, also have the same Hill coefficient. As previously demonstrated, this is so for the homologous series of propafenone-type MDR-modulators presented in this investigation (Chiba et al 1996). Further evidence is presented in Fig. 4, which shows that most of the dose–response curves of modulators obtained by plotting $ED50_T$ values (estimated by the simultaneous toxin fit procedure) are almost parallel. Curves showing different Hill coefficients, b, were derived from incomplete data. Thus, dose–response curves observed for all modulators were simultaneously analysed according to equation 2. This approach, which consists of two steps (step 1: simultaneous fit of all toxin dose–response curves (equation 1) leading to a set of $ED50_T$ values; step 2: simultaneous and independent fit of all modulator dose–response curves (equation 2) using $ED50_T$ values of step 1) leads to a further set of $ED50_M$ values of modulators (Table 1, column-double fit) and only one Hill coefficient, b, for all modulator dose–response curves.

By estimating eight $ED50_T$ values from 48 experimentally determined data points followed by estimation of one $ED50_M$ value the simultaneous toxin-fit approach results in an enormous concentration of information content within the available data. We therefore combined the two independent simultaneous-fit procedures (double-fit approach) by use of equation 3. This new approach enables determination of $ED50_M$ values of modulators directly from the original daunomycin toxicity

Table 1. The pharmacological activity of [ED50 (μM)] compounds **1a-k** and **2a-e**.

Compound	Single fit	Simultaneous toxin fit	Double fit	Combined simultaneous fit	Efflux
1a	0·33 ± 0·08	0·33 ± 0·08	0·37 ± 0·14	0·34 ± 0·08	3·55
1b	0·22 ± 0·21	0·21 ± 0·21	0·29 ± 0·22	0·37 ± 0·08	1·36
1c*	0·11 ± 1·85	0·20 ± 2·41	0·93 ± 0·91	0·81 ± 0·24	7·12
1d	0·81 ± 0·31	0·74 ± 0·28	0·69 ± 0·30	0·29 ± 0·07	0·61
1e*	1·46 ± 0·69	1·43 ± 0·73	0·85 ± 0·97	0·52 ± 0·16	2·55
1f*	0·51 ± 0·17	0·47 ± 1·85	0·23 ± 0·54	0·21 ± 0·06	1·65
1g	1·85 ± 0·08	1·85 ± 0·05	1·87 ± 0·27	1·43 ± 0·38	13·56
1h	0·18 ± 0·03	0·34 ± 0·03	0·29 ± 0·13	0·18 ± 0·03	0·16
1i*	0·74 ± 4·09	0·78 ± 4·09	1·21 ± 0·76	0·89 ± 0·25	8·61
1j	0·18 ± 0·03	0·18 ± 0·03	0·08 ± 0·05	0·03 ± 0·01	0·06
1k	0·98 ± 0·26	0·89 ± 0·26	0·39 ± 1·18	0·19 ± 0·05	0·41
2a	2·60 ± 3·80	2·96 ± 3·83	2·35 ± 1·85	4·19 ± 1·61	69·60
2b*	1·85 ± 0·13	1·67 ± 0·13	1·80 ± 1·10	1·59 ± 0·52	13·61
2c	0·42 ± 0·05	0·41 ± 0·05	0·40 ± 0·13	0·21 ± 0·05	0·46
2d*	2·68 ± 2·62	2·66 ± 2·62	2·76 ± 1·24	2·02 ± 0·58	4·96
2e	0·82 ± 0·49	0·86 ± 0·49	1·26 ± 1·01	0·87 ± 0·24	2·90

Values are ED50 values (± 95% confidence interval) of all modulators obtained in the MTT assay by various fit procedures. *ED50 values obtained using only three different modulator concentrations. ED50 values obtained using Rh-123 efflux represent the mean of at least two independently performed experiments. Standard deviations were usually below 15%.

data sets

$$\%S = 100 - 100[T]^a/\{[ED50_{VCR} - (ED50_{VCR} - ED50_{WT})\,[M]^b/(ED50_M{}^b + [M]^b)]^a + [T]^a\} \quad (3)$$

The abbreviations used are the same as before. $ED50_M$ values of modulators calculated by this approach are listed in Table 1 (column-combined simultaneous fit). The combined fit algorithm (equation 3) used for the simultaneous calculation of several $ED50_M$ values is a slight modification of the algorithm described recently (Schaper et al 1995). The previous number of two independent variables is increased by 1 (i.e. [M]). The Hill coefficient, a, was obtained from simultaneous analysis of all toxin dose–response curves using equation 1.

Analysis of incomplete data sets

For compounds with low activity or poor solubility, or both, it is sometimes not possible to obtain complete dose–response curves. In this circumstance the simultaneous analysis of dose–response curve families of a homologous series of drugs enables calculation of statistically significant ED50 values of these substances (Schaper et al 1995). To check the accuracy of this approach, we included a series of cytotoxicity assays performed using only three different concentrations of modifier (indicated with an asterisk in Table 1).

Rhodamine-123 efflux studies

The rhodamine-123 assay is a well documented direct and extremely reproducible functional assay for measuring P-glycoprotein-dependent efflux (Drach et al 1992). ED50 values of modulators obtained by this method were, therefore, used as reference values. We measured the ability of several propafenone analogues to inhibit P-glycoprotein-mediated rhodamine-123 efflux in resistant CCRF-CEM vcr 100 cells.

Rhodamine efflux studies were performed by analogy with previously published methods (Chiba et al 1996). Cells were pelleted, the supernatant was removed by suction and the cells were resuspended at a density of 1×10^6 mL^{-1} in RPMI1640 medium containing rhodamine-123 (Sigma) at a final concentration of 0·2 μg mL^{-1}. Cell suspensions were incubated at 37°C for 15 min. Tubes were chilled on ice and pelleted at 500 *g* in an Eppendorf 5403 centrifuge (Eppendorf, Germany). Supernatant was removed and the cell pellet was resuspended in medium which was pre-warmed to 37°C and contained either no modulator or chemosensitizer at various concentrations ranging from 16 nM to 500 μM, depending on the solubility and expected potency of the modifier. Eight concentrations (serial dilution 1 : 2·5) were tested for each modulator. After 30, 60, 90 and 120 s samples of the incubation mixture were transferred to tubes containing an equal volume of ice-cold stop solution (RPMI1640 medium containing verapamil at a final concentration of 10 μg mL^{-1}). Zero time points were obtained by immediately pipetting rhodamine-123 pre-loaded cells into ice-cold stop solution. Non-P-glycoprotein-expressing parental CCRF-CEM cells were used as controls for simple plasma-membrane diffusion, whereby initial rhodamine-123 fluorescence levels were adjusted to be equal to initial levels observed in resistant cells. Samples drawn at the respective time-points were kept in an ice-water bath and measured within 1 h on a Becton Dickinson Facscalibur flow cytometer (Becton Dickinson, Vienna, Austria). Viable cells were gated on the basis of forward and side scatter. Five thousand gated events were accumulated for the determination of mean fluorescence values. The time-dependent decrease in mean fluorescence values was linear for at least 2 min and was expressed as the percentage of the values of the corresponding zero time points, to enable comparison of independent experiments.

The time-dependent linear decrease in mean fluorescence in the presence of various concentrations of modifier was estimated and the initial efflux rates were calculated by linear regression analysis. Correction for simple diffusion was achieved by subtracting the efflux rates observed in the parental, non-P-glycoprotein-expressing line. ED50 values of all modifiers were determined from dose–response curves of initial efflux rates against modifier concentration and are given in Table 1. Data points from at least two independently performed experiments were fitted according to equation 4, where y is the initial rate of efflux determined as a function of modifier concentration, c, y_i is the initial efflux rate in absence of modulator and ME is the modulatory efficacy:

$$y = y_i - ME \cdot c/(ED50 + c) \quad (4)$$

Fit procedures

The fit of dose–response equations to single curves was performed using the software package Microcal Origin, installed on an IBM-compatible computer (468 DX2). The simultaneous analysis of dose–response curves was performed on a personal computer using an in-house software package (K.-J. Schaper, Borstel, Germany).

Results and Discussion

To prove the accuracy of our new method, we estimated the MDR-modulating activity of a series of propafenone analogues using an MTT-based cytotoxicity assay. The rhodamine-123 efflux assay, which is a highly reproducible functional assay for measuring P-glycoprotein-dependent efflux, was used as a reference method. As previously demonstrated, the CCRF-CEM vcr cell lines exhibit the MDR phenotype owing to overexpression of P-glycoprotein. No other reasons for resistance could be detected. Thus, cytotoxicity data obtained with CCRF-CEM vcr cells should show a stringent correlation with rhodamine-123 efflux data.

A gradual improvement in the correlation between MTT-assay and the efflux experiments can be observed by stepwise inclusion of data points in the simultaneous fitting procedure: (a) fit of dose–response curves of toxins at individual modifier concentrations (single fit); (b) simultaneous fit of eight dose–response curves for toxin at eight different concentrations of one modulator (simultaneous toxin fit); (c) simultaneous fit of all toxin curves and simultaneous (but independent) fit of all modulator dose–response curves (double fit); and (d) combined simultaneous fit of original cytotoxicity data points for all modulators (combined simultaneous fit). $ED50_M$ values obtained by fit procedures a-d and analogous values obtained in rhodamine-123 efflux studies were compared by linear regression analysis of the corresponding log (1/ED50) values. In this analysis the compounds with incomplete dose–response curves were also included. The best correlation was obtained by using the combined simultaneous fit analysis for data pro-

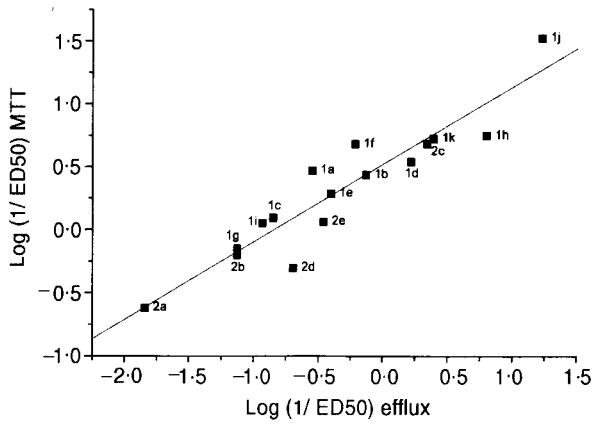

FIG. 5. Correlation of modulatory potencies (expressed as log (1/ED50) values; ED50 in μM) obtained in daunomycin cytotoxicity assays (MTT assay; values obtained using combined simultaneous-fit procedure) and rhodamine-123 efflux studies ($r = 0{\cdot}93$).

cessing ($r = 0{\cdot}93$, $n = 16$; Fig. 5). Regression analysis of the efflux data with the $ED50_M$ values obtained by the double-fit, simultaneous-toxin-fit or single-fit procedure resulted only in r values of 0·84, 0·63 and 0·58, respectively. Thus, using a combined simultaneous analysis of dose–response curve families of homologous drugs for analysis of cytotoxicity assays leads to highly significant ED50 values for modulators, which should enable the derivation of more informative and significant equations for quantitative structure–activity relationships.

The analysis of incomplete data sets by use of the combined simultaneous fit-curve algorithm shows that this is also a versatile method for analysing the activity of only slightly active or poorly soluble compounds (compounds marked with an asterisk in Table 1).

Conclusions

The combined simultaneous analysis of dose–response curve families of homologous drug series is a powerful method for the estimation of the pharmacological activity of MDR-modulators using cytotoxicity assays. This method enables the calculation of statistically highly significant Hill coefficients and ED50 values. ED50 values can, furthermore, be estimated for compounds which are poorly soluble or only slightly active, or both, with incomplete data sets. Each additional data set contributes to a statistically more precise estimation of variables (i.e. Hill coefficients) that are equal for all modulators. This information is lost when using individual fit procedures. By using a combined simultaneous-fit procedure, this information is conserved and can be used to facilitate the derivation of more informative and significant equations for quantitative structure–activity relationships.

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