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Lu-Bin Lan · Suhail Ayesh · Elena Lyubimov
Irina Pashinsky · Wilfred D. Stein

Kinetic parameters for reversal of the multidrug pump as measured for drug accumulation and cell killing

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Abstract We determined the kinetic parameters that describe the effect of 20 different modulators of the multidrug resistance pump on the reversal of cytotoxin accumulation in a resistant strain of P388 leukemia cells (P388/ADR), and on the reversal of cell killing for these cells. When measured by a direct comparison of the amplitude of the pertinent protocol (accumulation or cell killing), the K_i for reversal of accumulation was generally some four or five times larger than that for reduction of cytotoxicity. We showed that this was only an apparent discrepancy, since a full theoretical analysis of the two protocols allowed the intrinsic K_i to be obtained for the two procedures and these computed K_i values were then almost identical. We found that for six of the modulators studied (namely, cyclosporin A, quinidine, dipyridamole, propafenone, mefloquine, tamoxifen) the extent of pump reversal should be better than 90% at tolerated plasma levels culled from the literature.

Key words Multidrug resistance · Drug accumulation · Cell killing · Reversal · Kinetics

Introduction

Resistance to the cytotoxic action of cancer chemotherapeutic drugs is often associated with the manifestation of the multidrug resistance (MDR) pump, both in cells in culture and in cells taken from patients in the clinic [1–6]. The MDR pump is a well-characterised membrane-bound protein with demonstrable action as an ATPase [7–10]. Its activity results in a marked reduction in the intracellular concentration of a wide range of compounds, these being the substrates of the pump. Overcoming the action of this pump in a clinically acceptable protocol would be an important addition to our weapons against cancer. The action of the MDR pump in reducing intracellular drug concentrations can be reversed by numerous unrelated compounds, many of them being well-studied pharmaceuticals in clinical use for conditions other than cancer [11]. Some of these, such as verapamil [12], quinidine [13], cyclosporin A [14], and prochlorperazine [15, 16] have already been used in phase I and phase II clinical trials, in attempts to reverse drug resistance, but the high plasma levels of reversers that are needed have limited the usefulness of this approach. Structure–activity analysis of MDR pump reversers has led to the development of new drug candidates with an improved capacity to reverse MDR [17].

We have followed another approach in which combinations of known reversers can be used in an attempt to use the low specificity of the MDR pump to block it (Lyubimova et al., Anti Cancer Drugs, in press). For this approach, we needed to measure accurately the value of the kinetic parameter that describes the action of such reversers, namely the K_i for reversal, which we define as that reverser concentration that is required to bring about one-half the maximal degree of reversal. We performed such determinations for a wide range of reversers by measuring the effects of the reversers on drug accumulation and also on cytotoxicity. We found that the value of K_i defined in this way for the reversal of drug accumulation was always substantially larger than the value obtained by measurements of cell growth. Other workers have reported similar discrepancies between the effects of reversers on drug accumulation and on cell killing [18–20], and some have attempted to reconcile these findings [21–23]. In this report we show, in a full experimental and kinetic analysis of the effects of reversers on these two processes, that the two definitions of K_i have, indeed, different meanings. Their interrelation can be explicitly

L.-B. Lan · S. Ayesh · E. Lyubimov · I. Pashinsky · W. D. Stein (✉)
Biochemistry Department, Silberman Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel
Tel. (+ 972)-2-6585409; Fax (+ 972)-2-6585440

formulated so as to show that the demonstrated discrepancy is only an apparent one, and that these 20 modulators have exactly the same intrinsic effect on drug accumulation as on cytotoxicity.

Materials and methods

Chemicals

Vinblastine, amiodarone, amitriptyline, chlorpromazine, diltiazem, dipyridamole, fluoresceindiacetate, fluphenazine, progesterone, propafenone, β-propranolol, quinidine, reserpine, spironolactone, terfenadine, trifluoperazine, triflupromazine, and verapamil were obtained from Sigma Chemical Company (Petah Tikvah, Israel). Mefloquine, promethazine and tamoxifen were kind gifts from Dr.H.Ginsburg, while cyclosporin A was a kind gift from Dr. E.Shohami. Tritium-labelled compounds were: [G-^{3}H]-vinblastine (21 Ci/mmol) and [G-^{3}H]-thymidine (27 Ci/mmol) from Amersham Life Sciences (UK), and [G-^{3}H]-daunomycin (3.9 Ci/mmol) from NEN-DuPont Products, Boston, Mass.

Cell culture and treatments

The P388 lymphoma cells used were an adriamycin-resistant strain, a kind gift from Prof Avner Ramu of the Hadassah-Hebrew University Hospital [24]. They were grown in RPMI-1640 medium (Biological Industries, Kibbutz Beit Ha'emek, Israel) to which was added 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 10 μ*M* β-mercaptoethanol, and 0.2% (w/v) glucose, together with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Ha'emek, Israel), the whole being hereafter termed complete RPMI medium. Incubations were carried out in an atmosphere of humidified air containing 5% CO_2 at 37° C, in suspension culture in 260-ml plastic bottles (Nunc, Denmark). Before an experiment, cells were counted in a cell counter (model 134), Analys, (Stockholm, Sweden), and the cells resuspended in fresh medium at a concentration of 3.75 million cells per milliliter of medium for the subsequent transport assay. The cells were then incubated for at least 1 h at 37°C to regenerate their full MDR pump activity.

Measurement of drug uptake

For the transport studies for cells in suspension, 0.4 ml of a suspension of cells in complete RPMI medium were transferred to a siliconized microfuge tube, and 40 μl of the working solution of ^{3}H-labelled cytotoxin (with sufficient cytoxin to give a final concentration of vinblastine of 0.33 n*M*, or of daunomycin of 2 n*M*) containing the required concentration of reverser was added at time zero. Preliminary experiments showed that the most reproducible results were obtained after 40 min incubation at 25°C for vinblastine, and 60 min incubation at 37°C for daunomycin. Uptake of the cytotoxin was stopped by rapid centrifugation in a microfuge (Beckmann model 11) at maximum speed for 1 min. The supernatant layer was immediately quantitatively removed by aspiration and the cell pellet resuspended in 0.5 ml 10% Triton X-100. The cell suspension was then transferred to vials for radioactivity counting. In all cases, aliquots of cells were incubated for comparable times with no reverser present, and time zero uptakes performed by adding appropriate aliquots of precooled labelled vinblastine solutions to precooled cell suspensions. The latter determination served to measure the amount of label trapped between the cells during centrifugation. It was generally some 5–10% of the maximum amount of label that was taken up during 40 min in the presence of reverser. In all cases, aliquots of the loading solutions were taken for scintillation counting to enable the conversion of the data from disintegrations per minute per sample to femtomoles of cytotoxin accumulated per 10^5 cells.

Cytotoxicity assays

Resistant P388 cells were grown as described above, incubated in fresh complete RPMI medium for 1 h at 37°C then transferred to 24-well plastic dishes (Nunc, Denmark) in equal aliquots of 0.9 ml containing 1.5 million cells. To each well was added the desired concentration of vinblastine with or without the desired concentration of reverser. Dishes were incubated for 22–24 h at 37°C and then 0.5 μCi of radiolabelled thymidine was added and the cells further incubated for 1 h at 37°C. They were immediately transferred to ice and four aliquots taken from each well into a 96-well plate for radioactivity counting using a direct beta counter (Packard Matrix 96). Data were obtained as counts per minute per well. In some experiments, cell survival was measured using the fluorescein diacetate method [25] in which cells were grown as before and then the medium removed by flicking the plate. After one wash with phosphate-buffered saline, the cells were further incubated with fluorescein diacetate (10 μg/ml) for 1 h at 37°C and the fluorescence of the resulting fluorescein read in the Perkin-Elmer fluorometer.

Results

The effects of 20 different reversers chosen from a wide range of pharmacological classes on the accumulation of vinblastine and daunomycin into drug-resistant P388 leukemia cells were determined. Figure 1 shows six representative experiments. In each case, the amount of labelled vinblastine present in the P388 cells was measured after 40 min incubation at 25°C, with the cytotoxin at 0.33 n*M*. The data are plotted as a function of the concentration of the reverser added together with the vinblastine. The data were fitted using a modified form of the Michaelis-Menten equation:

$$D_i = D_r + (D_s - D_r) \cdot \frac{C}{K_{i(accum)} + C} \quad (1)$$

where D_i is the measured amount of cytotoxin (here vinblastine) accumulated at the reverser concentration C, while D_s and D_r are the amount of cytotoxin accumulation for fully reversed (equivalent to sensitive) cells and resistant cells, respectively. $K_{i(accum)}$ is the reverser concentration required for half-reversal of cytotoxin accumulation. The quantity (D_s-D_r) is the increment in cytotoxin accumulation brought about by the action of a maximal concentration of the reverser. In some experiments, as indicated in Table 1, the effect of the reverser on the accumulation of labelled daunomycin at 2 n*M* was measured during 60 min at 37°C. In other experiments, the value of $K_{i(accum)}$ for both substrates was measured (Table 2). No difference was found between the two measures. In Table 1 we list the values of $K_{i(accum)}$ for 20 reversers, together with the SE of the determination, each value of $K_{i(accum)}$ having been determined in a number of different experiments for either

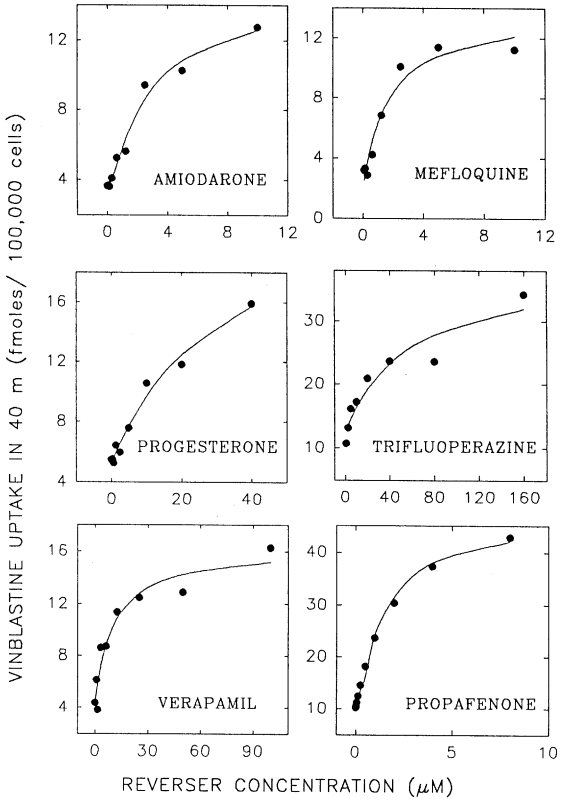

Fig. 1 Effect of six reversers on vinblastine accumulation in resistant P388/ADR leukemia cells. Cellular accumulation was measured after 40 min incubation at room temperature and a vinblastine concentration of 0.33 n*M*, and with the series of dilutions of the reversers indicated

vinblastine or daunomycin as noted. The $K_{i(accum)}$ values range over two orders of magnitude with cyclosporin A having the lowest value in our hands and amitriptyline having the highest. A number of compounds were found to have very little or no effectiveness (data not shown). These included testosterone, yohimbine, clodinine, hydrocortisone, cortisone, deoxycorticosterone, β-estradiol, theophylline, phloretin, and thiobarbituric acid.

We measured the effect of the 20 reversers in a cytotoxicity assay in which P388 cells in 24-well culture dishes were incubated for 22 h at 37°C with increasing concentrations of cytotoxin at various concentrations of reverser, and then for an additional 1 h with labelled thymidine to measure the inhibition of DNA synthesis. Figure 2A shows data for the action of propafenone, and Fig. 2b for the action of trifluoperazine. For each curve of cell survival against cytotoxin concentration, the data were fitted using the equation:

$$\text{Survival} = S_0 - (S_0 - S_e) \cdot \frac{C}{IC_{50} + C} \qquad (2)$$

in which Survival is the extent of survival of the cells at any cytotoxin concentration, C, S_0 is the survival at zero cytotoxin concentration, and S_e is its value at an infinitely high cytotoxin concentration. Thus (S_0-S_e) is the decrement in cell survival brought about by a maximal concentration of cytotoxin. Finally, IC_{50} is the computed value of the IC_{50}, i.e. the cytotoxin

Table 1 Kinetic parameters for reversers acting on the MDR pump of drug-resistant P388 cells. Listed are the values of K_i for reversal of drug accumulation, the calculated intrinsic K_i (see text) and the K_i for reversal of cell klling, and also the maximal tolerated drug levels in human subjects. For K_i (accumulation), the values were obtained using Eq. 1 and are given as the means ± SE with the number of observations in parentheses. For K_i (intrinsic), Eq. A5 of the Appendix was used. For K_i (cell killing), the values are all from single experiments with eight appropriate concentrations of modulator and the mean and SE were obtained from the curve-fitting program using Eq. 3 (all values are in units of micromoles)

Reverser	K_i (accumulation)	K_i (intrinsic)	K_i (cell killing)	Maximal clinical plasma level (reference)
Amiodarone	3.26 ± 0.52 (4)	0.88	1.11 ± 0.97	3.6 (27)
Amitriptyline	42.8 ± 7.3 (3)	7.53	5.40 ± 2.08	2.2 (28)
Chlorpromazine	17.0 ± 3.7 (4)	2.41	2.64 ± 0.86	0.32 (29)
Cyclosporin A	0.15 ± 0.02 (4)	0.038	0.013 ± 0.005	1.25 (30)
Diltiazem	19.1 ± 4.4 (4)	5.41	5.38 ± 4.12	1.4 (31)
Dipyridamole	2.27 ± 0.22 (3)	0.52	0.32 ± 0.14	4.6 (32)
Fluphenazine	20.0 ± 5.6 (9)	5.52	5.0 ± 4.36	0.0064 (33)
Mefloquine	2.07 ± 0.57 (5)	0.43	0.76 ± 0.33	5.4 (34)
Progesterone	21.2 ± 10.7 (4)	4.6	2.29 ± 0.95	4.1 (35)
Promethazine	12.2 ± 2.6 (10)	3.45	3.77 ± 0.99	0.22 (36)
Propafenone	2.31 ± 0.13 (3)	0.44	0.71 ± 0.21	5.3 (37)
β-Propranolol	15.2 ± 3.3 (3)	6.2	3.4 ± 0.91	0.50 (38)
Quinidine	5.47 ± 0.66 (4)	2.05	0.55 ± 0.15	5.46 (13)
Reserpine	0.62 ± 0.44 (3)	0.14	0.31 ± 0.07	–
Spironolactone	14.5 ± 3.3 (2)	4.14	3.66 ± 1.90	1.3 (39)
Tamoxifen	4.33 ± 0.60 (4)	1.39	0.49 ± 0.28	3.7 (40)
Terfenadine	2.37 ± 0.45 (3)	0.63	0.96 ± 0.33	2.6 (41)
Trifluoperazine	11.5 ± 0.5 (3)	3.8	1.22 ± 1.08	0.27 (42)
Triflupromazine	20.6 ± 3.8 (5)	3.48	4.45 ± 2.80	–
Verapamil	2.4 ± 0.5 (14)	0.69	1.41 ± 0.98	3.8 (12)

Table 2 Kinetic parameters for reversers acting on the MDR pump of drug-resistant P388 cells measured with vinblastine and daunomycin for cytotoxin accumulation and for cell killing (number of experiments in parentheses)

Reverser	K_i using vinblastine	K_i using daunomycin
Intrinsic K_i values for accumulation of cytotoxin		
Chlorpromazine[a]	1.61 ± 0.31 (2)	1.69 ± 0.50 (2)
Triflupromazine[a]	2.82 ± 1.03 (1)	2.51 ± 0.60 (1)
Promethazine[a]	2.57 ± 0.61 (3)	2.27 ± 0.80 (3)
Amiodarone[a]	0.94 ± 0.31 (3)	0.55 ± 0.15 (3)
Cyclosporin A	0.038 ± 0.005 (4)	0.04 ± 0.004 (4)
Verapamil	0.69 ± 0.11 (22)	0.30 ± 0.06 (4)
K_i values for cell killing		
Trifluoperazine	0.98 ± 0.42 (3)[b]	1.19 ± 0.21 (1)
Propafenone	0.71 ± 0.21 (1)	0.78 ± 0.41 (1)

[a] Values are weighted means of experiments done in parallel with the two cytotoxins
[b] Value is weighted mean of three determinations

concentration which gives 50% of the maximal possible decrement in cell survival. The value of IC_{50} so obtained at each concentration of reverser was then plotted against reverser concentration, as in the insets of Fig. 2. These plots were then fitted using the equation:

$$IC_{50} = IC_r - (IC_r - IC_s) \cdot \frac{C}{K_{i(kill)} + C} \quad (3)$$

where IC_{50} is the value of the IC_{50} at any reverser concentration C, while $K_{i(kill)}$, IC_r, and IC_s are the parameters derived by curve fitting for, respectively, the reverser concentration that gives a half-maximal decrement in IC_{50}, the value of IC_{50} for fully reversed cells, and its value for fully resistant cells. Thus, $(IC_r - IC_s)$ is the decrement in IC_{50} brought about by the action of the reverser. The values of $K_{i(kill)}$ obtained in this way for the 20 reversers are also listed in Table 1. To check whether the data were biased by the use of the thymidine uptake assay, the values of $K_{i(kill)}$ using the reversers cyclosporin A and dipyridamole were determined using fluoresceine diacetate. The values obtained (0.019 ± 0.001.4 μ*M* and 0.198 ± 0.060 μ*M*, respectively) were not significantly different from those shown in Table 1 using the thymidine incorporation assay.

It is apparent that K_i for drug accumulation was generally four or five times larger than that for cell growth. In order to explore the possible basis for this discrepancy we first tested whether the time of

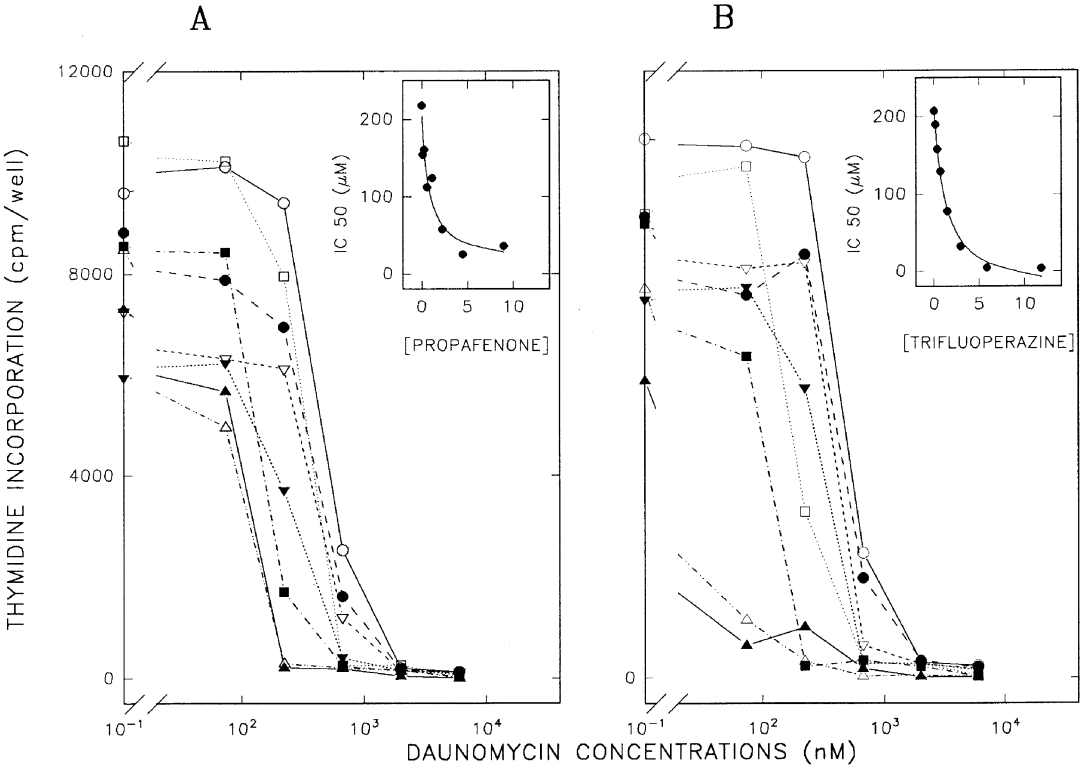

Fig. 2A, B Effect of propafenone (**A**) and trifluoperazine (**B**) on daunomycin cytotoxicity. Cells were incubated for 22 h at 37°C with a series of concentrations of daunomycin (0, 74, 222, 667, 2000, 6000 n*M*) containing 0 *μM* (○--○), 0.14 *μM* (●–●), 0.28 *μM* (▽--▽), 0.56 *μM* (▼--▼), 1.12 *μM* (□--□), 2.24 *μM* (■--■), 4.48 *μM* (△--△) or 8.96 μm; (▲--▲) propafenone, or 0 *μM* (○--○), 0.18 *μM* (●–●), 0.37 *μM* (▽--▽) 0.74 *μM* (▼--▼), 0.48 *μM* (□–□), 2.95 *μM* (■–■), 5.90 *μM* (△--△) or 11.8 *μM* (▲–▲) trifluoperazine. The IC_{50} values calculated from these data using Eq. 2 are displayed in the inset figures in each case. The *x*-axis of the insets represents the concentration of reverser (μ*M*) and the *y*-axis the computed IC_{50} values in (μ*M*). From these plots the $K_{i(kill)}$ values calculated using Eq. 3 were 0.71 ± 0.36 μ*M* for propafenone and 1.19 ± 0.21 μ*M* for trifluoperazine

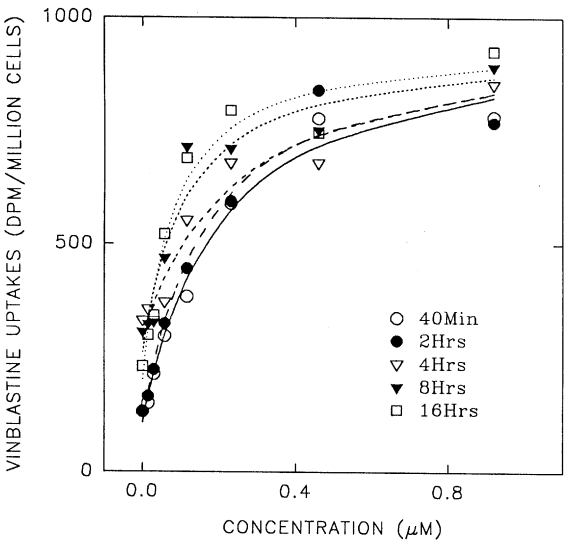

Fig. 3 Effect of cyclosporin A on vinblastine accumulation by resistant P388 leukemia cells during different time periods. Cellular accumulation was measured at a series of concentrations of cyclosporin A (0, 0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 0.96 μ*M*) with a vinblastine concentration of 0.33 n*M*, at room temperature. From each data set the appropriate value of $K_{i(accum)}$ was calculated using Eq. 1 of the text, obtaining for the experiments of 40 min, 2 h, 4 h, 8 h, and 16 h duration, respectively, 0.203 ± 0.086, 0.119 ± 0.054, 0.262 ± 0.143, 0.113 ± 0.042, and 0.082 ± 0.024 μ*M*

incubation with cytotoxin contributed to this difference. The incubation period used for the determination of cell growth was 22 h, while that for drug accumulation was 40 or 60 min. In Figure 3 shows an experiment in which cells were incubated with labelled vinblastine for times ranging from 40 min to 16 h. For each time, the extent of cytotoxin accumulation at the end of the incubation period was measured as a function of reverser concentration. The data according to Eq. 1 were plotted to obtain values for $K_{i(accum)}$ (shown in the legends to Fig. 3). Clearly, there was no systematic variation in $K_{i(accum)}$ with increasing times of incubation with cytotoxin.

In another experiment, we tested whether it was the concentration of cytotoxin that might be responsible for the discrepancy between $K_{i(accum)}$ and $K_{i(kill)}$. The uptake of labelled vinblastine was measured both at the concentration normally used for the accumulation assays (0.33 n*M*) and also at 243 n*M*, a concentration in the middle of the range of those used in the cell growth assays (data not shown). For each data set the appropriate value of $K_{i(accum)}$ was calculated using Eq. 1, obtaining for the data at 0.33 n*M*, 0.142 ± 0.054 μM and for the data at 243 n*M*, 0.550 ± 0.105 μ*M*, respectively. It can be seen that the $K_{i(accum)}$ was certainly not smaller at the higher concentration of cytotoxin; if anything, it was larger.

We then attempted to determine whether there was indeed some basic difference in the meaning of K_i in our two types of assay. A kinetic model was set up for the effect of reversers on drug pumping by the MDR pump (see Appendix). This analysis showed that $K_{i(kill)}$ was indeed exactly equal to the intrinsic K_i of the pump, i.e. that concentration of reverser that blocks its activity by one-half (compare Eq. A3 of the Appendix). In contrast, $K_{i(accum)}$ was not equal to the intrinsic K_i, but was equal to K_i multiplied by (using the terminology of Eq. 1) D_s/D_r, the ratio of the amount of drug accumulated at maximal concentration of reverser to that accumulated in the absence of reverser (compare Eq. A5 of the Appendix). This result is a simple kinetic consequence of the form in which Eq. 1 is written and used. The ratio D_s/D_r was available from the curve fitting results for each determination of $K_{i(accum)}$ listed in Table 1. It was therefore possible to calculate the appropriate value of the intrinsic K_i from each value of $K_{i(accum)}$. The derived values are also listed in Table 1, and Fig. 4A shows a plot of the values of the intrinsic K_i calculated from the values of $K_{i(accum)}$ for each reverser against the appropriate value of $K_{i(kill)}$. The regression line ($r = 0.870$) with slope $m = 1.08$ shows that, for most of the reversers, the two values of K_i coincide. It is therefore possible to argue that the discrepancy between the concentration dependence of reverser activity for drug accumulation and cell growth is more apparent than real.

In certain cases, we determined the parameters $K_{i(kill)}$ and $K_{i(accum)}$ also for the cytotoxin daunomycin. From $K_{i(accum)}$ we again calculated the appropriate intrinsic K_i. These values are also listed in Table 2. There was clearly no significant difference between the values of K_i determined using vinblastine and those determined using daunomycin.

Discussion

A number of authors have reported a similar discrepancy between the effects of reversers on drug accumulation and on cell killing [18–23], but we are unaware of a survey as quantitative and extensive as the present one. Thus, in the report by Schuurhuis et al. [21], it is clear from a comparison of their Figs 1 and 5 that far higher reverser concentrations are required to reverse doxorubicin accumulation in drug-resistant CH^RC5 cells than to reverse cell killing by 50% using this cytotoxin. Schuurhuis et al. [22], in a further study, found a marked discrepancy between the effects of verapamil and bepridil on the accumulation of doxorubicin (adriamycin) by SW-1573 lung cancer cells and MCF-7 breast cancer cells and their effects on cell killing. In both these studies [21, 22], the effects of the reversers on the distribution of cytotoxin between cytoplasm and nucleus were measured. In resistant cells and in the absence of reverser, the cytotoxin was shown to be largely confined to the cytoplasm while, with reverser present, the cytotoxin is within the nucleus. In sensitive cells, on the other hand, the cytotoxin is found

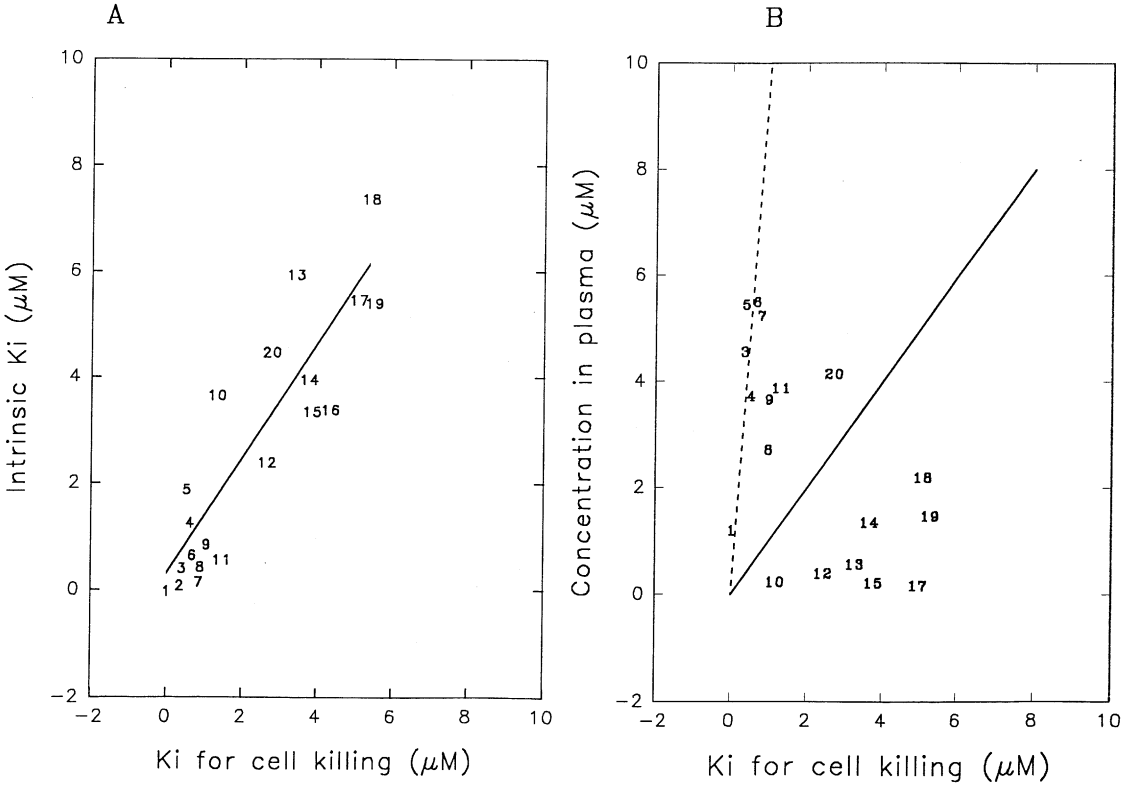

Fig. 4 The calculated K_i for the effect of 20 modulators on cell killing plotted against (**A**), the respective intrinsic K_i calculated from their effects on cytotoxin accumulation and (**B**) the maximal tolerated plasma levels of these modulators reported in the literature. The numbers represent the different modulators as follows: *1* cyclosporin A, *2* reserpine, *3* dipyridamole, *4* tamoxifen, *5* quinidine, *6* propafenone, *7* mefloquine, *8* terfenadine, *9* amiodarone, *10* trifluoperazine, *11* verapamil, *12* β-propranolol, *13* chlorpromazine, *14* promethazine, *15* spironolactone, *16* triflupromethazine, *17* fluphenazine, *18* amitriptyline, *19* diltiazem, *20* progesterone. The straight line in **A** is the regression through the points with an intercept of 0.315 μ*M*, a slope of 1.08, and a regression coefficient of 0.87. The solid line in **B** has unit slope, the dashed line a slope of 10

in the nucleus and its distribution is unaffected by the presence of the reversing agents. These authors suggested that these results imply an effect of the reverser on the distribution of cytotoxin within the cell, and that this putative dual effect of reversers might explain the killing/accumulation discrepancy. However, careful study of Fig. 5 of reference 21, which plots both the distribution ratio of cytotoxin between nucleus and cytoplasm and the IC_{50} as a function of reverser concentration, shows that the effect for the change in drug distribution is half-maximal at a high reverser concentration, compatible with the effect of the reverser on drug accumulation rather than on cell growth. The reverser certainly affects the distribution of toxin as between nucleus and cytoplasm but this effect does not provide a quantitative explanation of the discrepancy in the concentration dependence of our two experimental paradigms.

Pereira and Garnier Suillerot [23] made a significant advance by determining the effect of a dozen reversers on the accumulation of 4′-*O*-tetrahydropyranyl-adriamycin in K562 erythroleukemia cells and on the effect of these reversers on the growth of cells affected by this cytotoxin. They studied cytotoxin accumulation as a function of reverser concentration and the effect of different concentrations of reverser on cell killing. A plot of the decrement of cell killing at a single concentration of reverser (5 μ*M*) against the increment in the extent of drug accumulation for each of these reversers gave a good fit to the line of identity, strongly suggesting that the two phenomena are closely correlated. They were not able, however, to compare quantitatively the kinetic parameters characterizing the two phenomena and they studied only a narrow range of reverser types, namely quinidine, quinine, verapamil and a series of phenothiazines. Our data fully confirm their pioneering study, and our analysis provides the necessary conceptual basis for understanding the apparent discrepancy reported in the literature.

In essence, the theoretical analysis in the Appendix shows that, in measuring the effect of reversers on cell killing, the internal concentration of cytoxin is always the same at the IC_{50} while it is the external concentration of cytotoxin that varies with the reverser concentration. In this situation, the appropriate K_i — the reverser concentration for a half-maximal effect,

$K_{i(kill)}$ — measures directly what we have called the intrinsic K_i, the reverser concentration needed to half-saturate the MDR pump. In contrast, in measuring the effect of reverser on drug accumulation, it is the external concentration of cytotoxin that is held constant and the internal concentration that varies with reverser concentration. The kinetic analysis in the Appendix shows that in this situation, the appropriate K_i, $K_{i(accum)}$, contains a factor which is the ratio of the cytotoxin concentration in the presence of a saturating concentration of reverser to that in the absence of reverser, namely, D_r/D_s. Thus, in order to obtain the intrinsic K_i, it is necessary to multiply $K_{i(accum)}$ by D_s/D_r. With this correction the effects of a reverser on drug accumulation are predictable from its effects on cell growth (as in Fig. 2A) and there is no need to assume any dual effect of reverser on drug pumping and drug distribution.

The above considerations apply to the absolute values of the half-saturation concentrations K_i found for the 20 reversers, i.e. the intensity of their effects as modulators of the MDR pump. However, it is necessary to consider also the amplitude of the change that the modulators bring about. Comparing the experimental data points in Fig. 1 with those in the inset curves of Fig. 2, it can be seen that the amplitude of the change brought about by the reverser was not the same in these two experimental situations. For the reversal of drug accumulation the amplitude of the effect was some fivefold. For the effect on cell growth it can readily reach 100-fold. Why should this discrepancy occur? The appropriate intrinsic K_i values derived for the two phenomena were the same for particular reverser for all the reversers studied, and the K_i values were the same with daunomycin as with vinblastine, so the two phenomena have presumably the same molecular basis. Our findings are consistent with the sole effect of the modulators being at one molecular site, presumably the P-glycoprotein.

We suggest, following Schuurhuis et al. [21, 22], that the discrepancy might arise as a result of the sequestration of cytotoxin within intracellular vesicles brought about as a result of the presence of MDR pumps on those vesicles [26]. The phenomenon of cell killing depends only on the internal concentration of free cytotoxin, since it is this that is in equilibrium with the intracellular sites (whatever they may be) to which the cytotoxin binds when it brings about the killing of the cell. Drug accumulation, however, is a measure of all the cytotoxin within the cell, both free and bound. Any cytotoxin trapped within intracellular vesicles will appear as intracellular drug and will be counted in the uptake of labelled cytotoxin. As the reverser concentration is increased, the MDR pumps (both of the plasma membrane,which pump drug out of the cell, and of the vesicular membranes, which pump drug into the vesicle but not out of the cell) will be increasingly blocked. At saturating levels of the modulator, the internal and external drug concentrations will be the same (or at any rate will be given by a distribution law, e.g. dependent on the titratable nature of the cytotoxin and the transmembrane pH gradient, which is independent of the MDR pump). In the total absence of reverser, therefore, there may be a very large transmembrane gradient of free cytotoxin, but only a much smaller intracellular/extracellular concentration ratio of total drug, since part of the cytotoxin will be sequestered within vesicles. It should be noted that for this explanation to be applicable, it must be the pump itself that contributes to the intravesicular trapping of the cytotoxin. Otherwise, the degree of trapping will be proportional to the concentration of free cytoplasmic cytotoxin, and will change in parallel with the change in free cytotoxin, as the reverser concentration is raised. Thus the extent of the effect of the reverser will be the same for total as for free cytotoxin. This is, of course, not the case.

The hypothesis that the total amount of intracellular cytotoxin includes that trapped within cytoplasmic vesicles is supported by the cytochemical studies of Willingham et al. [26] who showed that daunomycin can be identified as being bound within lysosomes and in the perinuclear Golgi region in resistant KB-C4 drug-resistant cells but not in the parent KB-3–1 drug-sensitive cells, where it is predominantly bound in the nucleus. The hypothesis is also consistent with the study of Schuurhuis et al. [22] who showed that the addition of reverser affects the distribution of doxorubicin between cytoplasm and nucleus. In their drug-resistant 2780^{AD} cells, the doxorubicin was largely cytoplasmic present "as a diffuse Golgi-like cloud or in punctuate pattern throughout the cytoplasm". In the presence of increasing concentrations of reverser, the cytotoxin showed an increasing tendency to be preferentially localized to the nucleus. On the present hypothesis, in the presence of reverser, with the intracellular vesicular pumps blocked, cytotoxin should be free to bind to the nucleus, while in the absence of reverser the cytotoxin should be trapped within cytoplasmic vesicles and hence unable to bind to nuclear sites. This is exactly what Schuurhuis et al. [22] describe. Our data (Table 2) add to the strength of this conclusion by showing that the same differential effect of reversers on cytotoxin accumulation and cell killing is found for a drug which is bound to microtubules within the cytoplasm (vinblastine) as well as for a nuclear-bound cytotoxin (daunomycin). This suggests strongly that the fundamental issue is the partitioning of cytotoxin between sequestration in vesicles and free in the cytoplasm from where it is in equilibrium with either nuclear or cytoplasmic sites involved in the cell killing process.

The present study was initiated in a program to provide a set of reversers that could be used in combination to help overcome resistance to chemotherapy in patients. Having established that our measures of the effectiveness of the various reversers are equivalent and are equally appropriate to predict their possible

effectiveness in patients, we can now compare the various reversers as chemotherapeutic aids. Figure 4B shows, for most of the reversers in Table 1, the values found in a literature search for the maximal tolerated plasma level of each reverser in human subjects plotted against the $K_{(kill)}$, which our analysis showed to be equal to K_i. Figure 4B also shows the line of unit slope (solid) and the line of slope 10 (dashed). Points that fall below the latter line indicate reversers with not much chance of being effective in patients since, at maximum tolerated plasma levels, they bring about less than 90% inhibition of the pump. Points that lie on or above the line of slope 10 indicative reversers that do have a potential for effectiveness in clinical situations. The list of those potentially useful reversers, according to our analysis, includes cyclosporin A, mefloquine, propafenone, quinidine, tamoxifen and dipyridamole. Since all of these have almost the same ratio of the tolerated plasma level to the measured K_i, they should be almost equally effective as reversers, in spite of the fact that the doses of each vary over quite a range. Our study that shows that these reversers can act together additively in reversing the MDR pump (Lyubimov et al., Anti-Cancer Drugs, in press) suggests that combinations of these six reversers at less than tolerated levels might be effective in reversing MDR.

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Appendix

Interpretation of apparent K_i values for the action of chemosensitisers in reversing cell killing and drug accumulation

Consider a cell into which a cytotoxic drug is both entering and leaving by simple diffusion, while the drug is also being pumped out of the cell by a membrane-bound pump. Let p be the permeability coefficient for this drug/cell pair, and let the pump act on the drug with a maximum velocity of V per unit area and a Michaelis parameter (describing saturation of the pump) of K_m. Then if the concentration of cytotoxin outside the cell is D_e, while that within the cell is D_i, and the cell has surface area A, the rate of drug influx is $p \times D_e \times A$ and the rate of drug efflux is $p \times D_i \times A + (D_i \cdot V.A/(D_i + K_m))$. When the internal concentration of cytotoxin is low, i.e., when D_i is low in comparison with K_m, the efflux term simplifies to $(p + V/K_m) \times D_i \times A$. At the steady-state for drug accumulation, the rate of influx is equal to the rate of efflux and we can write:

$$\frac{D_i}{D_e} = \frac{p}{(p + V/K_m)} = \frac{1}{\left(1 + \frac{V}{K_m \cdot p}\right)} = \frac{1}{(1 + R)} \cdots . \quad (1)$$

where we write R in place of $V/K_m \cdot p$, this being the overall factor that determines the degree of resistance of the cell to the cytotoxin. Equation (1) gives the ratio of cytotoxin within the cell compared to that outside the cell and is the relevant parameter for investigating the effect of pump-reversing agents on both cytotoxin accumulation and cell killing, as we proceed to analyse. Thus, if R is zero, $D_i = D_e$ and the resistance is zero, while at any other value of R, the intracellular concentration of drug is reduced by the factor $1/(1 + R)$.

Consider now the effect of the addition of a chemosensitiser. Let this block the drug pump according to a Michaelis parameter (describing its ability to saturate the pump) of value K_i. Then, at a concentration C of this reverser and at a low concentration of cytotoxin, it will block a fraction of the pump equal to $C/(C + K_i)$, which we can write as $1/(1 + C')$, where C' is the "reduced concentration" of the reverser, i.e., its concentration divided by its appropriate Michaelis parameter. Now, this chemosensitiser can in principle block the cytotoxin's efflux either competitively or non-competitively. For both modes of inhibition, however, the effect of pump inhibition on cytotoxin efflux *at low cytotoxin concentrations* as compared with the cytotoxin's K_m, is to reduce the value of V/K_m in equation (1) by the factor $1/(1 + C')$. This follows since for competitive inhibition K_m is multiplied by $(1 + C')$, while for non-competitive inhibition V is divided by this same term. We can therefore write:

$$\frac{D_i}{D_e} = \frac{1}{\left(1 + \frac{R}{(1 + C')}\right)} = \frac{(1 + C')}{(1 + R + C')} \cdots . \quad (2)$$

The internal cytotoxin concentration at zero concentration of reverser (which we define as D_0) is given by setting C' equal to zero in Eq. (2), whence we have: $D_0 = 1/(1 + R)$, while the cytotoxin concentration at infinite concentration of reverser, D_∞, is equal to unity. (Put $C' = \infty$ in Eq. (2)).

We first handle the question of the reversal of cell killing. Let D_k be the internal concentration of cytoxin at which 50% of the cells in question are killed. Let IC_{50} be the external cytotoxin concentration at which 50% of cell killing is obtained. Then, in the absence of any resistance to the cytotoxin, $IC_{50} = D_k$. Also, at an infinitely high concentration of reverser, (where we define the IC_{50} as IC_∞) the same result will hold, since reversal of resistance will be complete. Similarly, we can define the IC_{50} at zero concentration of reverser as IC_0. Its value is $D_k \times (1 + R)$, from Eq. (1). We can obtain the value of IC_{50} at any other concentration of reverser by putting $IC_{50} = D_e$ and $D_k = D_i$ in Eq. (2), and then substituting IC_∞ for D_k and IC_0 for $D_k \cdot (1 + R)$ to get

$$\begin{aligned} IC_{50} &= D_k \frac{(1 + R + C')}{(1 + C')} = \frac{IC_0 + IC_\infty \cdot C'}{1 + C'} \\ &= \frac{IC_0 + IC_0 C' - IC_0 C' + IC_\infty C'}{1 + C'} \\ &= IC_0 - (IC_0 - IC_\infty) \cdot \frac{C'}{(1 + C')} \\ &= IC_0 - (IC_0 - IC_\infty) \cdot \frac{C}{K_i + C} \cdots . \end{aligned} \quad (3)$$

This gives an expression for how the IC_{50} at any concentration of reverser, C', varies with C' in terms of the IC_{50} at zero reverser and at a maximal concentration of reverser. The term $(IC_0 - IC_\infty)$ is the *decrement* in IC_{50} as the reverser concentration is raised from 0 to infinity. It should be noted that the expression on the extreme right-hand side of Eq. (3) is merely the common Michaelis form. Thus the dependence of IC_{50} on the concentration of reverser C gives directly the value of K_i for the reverser.

Similarly, for cytotoxin accumulation, if we write D_0 for the amount of accumulation at zero concentration of reverser, and D_∞ for the amount of accumulation at an infinite concentration of reverser, it will be obvious that $D_\infty = D_e$, while $D_0 = D_e/(1 + R)$. We can write the right-hand side of Eq. (2) in terms of partial fractions,

and substitute for the terms in D as follows:

$$\frac{D_i}{D_e} = \frac{(1+C')(1+R)}{(1+R+C')(1+R)} = \frac{1+R+C'+R\cdot C'}{(1+R)(1+R+C')}$$

$$= \frac{1}{1+R} + \frac{R}{1+R}\cdot\frac{C'}{1+R+C'}$$

Hence,

$$D_i = D_0 + (D_\infty - D_0)\cdot\frac{C'}{1+R+C'}$$

$$= D_0 + (D_\infty - D_0)\cdot\frac{C}{(1+R)K_i + C}\cdots. \quad (4)$$

This gives an expression for how the accumulation of cytotoxin depends on C', in terms of the accumulation at zero reverser and at a maximal concentration of reverser. The term $D_\infty - D_0$ is the *increment* in accumulation as the reverser concentration is raised from 0 to infinity.

The expression on the extreme right-hand side of Eq. (4) is again a Michaelis form, but the dependence of IC_{50} on the concentration of reverser C no longer gives directly the value of K_i for the reverser, but rather $(1 + R)$ times K_i. Thus the apparent K_i as derived from cytotoxin accumulation studies is higger than that derived from cell killing studies, by the factor $(1 + R)$. We can cast Eq. (4) into a form that is independent of R and contains only experimentally derivable parameters by substituting for $(1 + R)$ in Eq. (4), the equivalent term D_0/D_∞, obtaining:

$$D_i = D_0 + (D_\infty - D_0)\cdot\frac{C}{C + K_i\cdot(D_\infty/D_0)}\cdots. \quad (5)$$

from which D_0, D_∞, and K_i can be found from the amount of accumulation D_i as a function of C.

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