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Original Paper

Serum Can Inhibit Reversal of Multidrug Resistance by Chemosensitisers

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The purpose of this study was to evaluate to what extent the ability of various chemosensitisers (CS) to reverse P-glycoprotein-associated multidrug resistance (MDR) is reduced when tested in physiological serum protein concentrations. Utilising drug sensitivity and accumulation assays, the CS were tested in medium containing 10% fetal bovine serum and in 100% horse or human serum. Two RPMI 8226 human myeloma sublines were used which express different levels of P-glycoprotein. The CS were tested at various concentrations, including clinically achievable blood levels. When using the CS at high doses, wide differences were observed in the extent CS activity was diminished by serum. Verapamil, cyclosporin A and quinine were not affected, quinidine and medroxyprogesterone acetate were moderately inhibited, and amiodarone and trifluoperazine were largely inactivated. When the CS were used at concentrations achievable in humans, the activity of all agents except quinine was markedly reduced by serum. With respect to the extent to which CS activity was diminished by serum, good statistical correlation ($r > 0.90$, $P < 0.001$) was found between the use of cytotoxicity and drug accumulation assays, horse and human serum or cell lines with high and low levels of P-glycoprotein, respectively. These studies demonstrated that physiological serum protein concentrations can profoundly diminish the MDR reversing activity of particular CS. Some drugs, such as amiodarone and trifluoperazine, are largely inactivated by serum when used at a wide range of concentrations. Other agents, such as verapamil and cyclosporin A, are essentially unaffected when used at high doses but markedly inhibited at concentrations achievable in humans. These data suggest that *in vitro* studies of CS in medium containing low serum protein concentrations can result in misleading conclusions regarding the potential clinical activity of such agents. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

P-GLYCOPROTEIN (Pgp)-associated multidrug resistance (MDR) is an extensively studied experimental phenomenon [1] that seems to have clinical relevance. Overexpression of MDR1/Pgp has been detected in a variety of human cancers [2, 3] and in certain malignancies, such as acute leukaemias and various childhood cancers, MDR1/Pgp-positivity has been found to correlate with a lower response rate to chemotherapy and shorter relapse-free and overall survival [4–6]. Hence, effective clinical reversal of MDR may be able to improve chemotherapy efficacy in such cancers.

A number of agents have been found capable of overcoming MDR in preclinical models [7]. Such chemosensitisers (CS) are thought to function by competitive inhibition of Pgp-mediated efflux of cytotoxic drugs. In recent years, a variety of CS have been used in clinical studies aimed at overcoming MDR, including verapamil (VER), cyclosporin A (CSA), quinidine (QD), quinine (Q), amiodarone (AMD), trifluoperazine (TFP) and tamoxifen (TAM) [8–16]. In most of these studies, CS have failed to show activity in overcoming clinical chemotherapy resistance [17, 18]. Thus, better preclinical models for assessing potential clinical activity of CS appear to be needed.

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In vitro studies of CS are usually performed in medium containing low serum concentrations. In such conditions, a large proportion of any agent, irrespective of its degree of serum protein binding, is present as a free, non-protein-bound drug capable of blocking Pgp function. Clinically, however, CS are exposed to high concentrations of drug-binding proteins, both in blood and tissues. Depending on the degree of serum protein binding, this can significantly reduce bioavailability of CS, which may diminish MDR reversing activity. The goal of the present study was to evaluate to what extent the activity of various first-generation CS are inhibited when used in physiological serum protein concentrations. Further objectives were to assess whether the order of equimolar potency of these agents differs when determined in low versus high serum protein concentrations and to devise a suitable model for analysing serum effects on CS.

MATERIALS AND METHODS

Cells

The 8226/DOX6 and DOX40 lines, which were derived from the RPMI 8226 human myeloma cell line and exhibit all the characteristics of MDR as previously described [19], were used as MDR models in these studies. The 8226/DOX6 cells express Pgp at a level similar to myeloma cells from patients with drug-refractory disease [20]. The amount of Pgp present in 8226/DOX40 variants is higher than usually detected in clinical tumour samples. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine (1% v/v). Cell cultures were maintained at 37°C in humidified air supplemented with 6% CO_2. Prior to experiments, 8226/DOX cells were grown without doxorubicin (DOX) for a minimum of 1 week. Using the Hoechst dye 33256, cell lines were proven to be free of *Mycoplasma* infection prior to experiments [21].

Drugs and sera

The formulations available for clinical i.v. use were utilised as sources of DOX, daunorubicin (DNR), VER, CSA, AMD, TFP and medroxyprogesterone acetate (MPA). QD, Q and TAM were purchased in powder form from Sigma (St. Louis, Missouri, U.S.A. and Buchs, Switzerland). The source of CSA was Sandimmun® IV, which contains Cremophor® EL as vehicle for the water-insoluble CSA. Cremophor® EL has been previously found to be capable of reversing MDR *in vitro* [22].

FBS was purchased from Irvine Scientific (Santa Ana, California, U.S.A.) or GIBCO BRL (Life Technologies AG, Basel, Switzerland). Horse serum was obtained from Irvine Scientific or Inotech AG (Dottikon, Switzerland), human serum from healthy volunteers. Only those lots of horse serum which were shown by biochemical analysis to have a serum protein profile (total protein, albumin, globulin subfractions) similar to normal human serum were used. The concentration of alpha-1-acid glycoprotein (AAG) in the human sera was in the normal range. Owing to the lack of species-specific reagents, we were not able to measure AAG concentrations in horse serum.

In vitro *sensitivity testing*

CS effects on DOX sensitivity were tested by using a two-layer, soft-agar colony assay [23]. Exponentially growing cells were exposed to drug(s) for 1 h at 37°C in either RPMI 1640 medium containing 10% FBS or in 100% horse or human serum. Cells were then washed twice with ice-cold phosphate-buffered-saline (PBS) and plated in triplicate at a concentration of 10000 cells per 35 mm tissue culture dish. Tumour cell colonies of 60 μm in diameter or greater were enumerated 14–21 days after plating using an automated image analysis instrument optimised for tumour colony counting (FAS II Omnicon, Bausch and Lomb, Rochester, New York, U.S.A.) [24]. The per cent survival was determined from the plating efficiencies of treated versus control cells. The IC_{50} for DOX, alone or in combination with CS, was defined as the DOX concentration which reduced colony formation to 50% of untreated controls. Sensitisation factors (SFs) were determined by dividing the IC_{50} for DOX alone by the IC_{50} for DOX in the presence of a particular CS.

Drug accumulation studies

CS effects on cellular DNR accumulation were analysed by flow cytometry (FACScan or FACStar, Becton Dickinson, San Jose, California, U.S.A.). DNR rather than DOX was used as the index anthracycline because of its superior fluorescence profile and, thus, good correlation of intracellular drug concentrations as measured by flow cytometry and other methods, such as high-pressure liquid chromatography [25]. Preliminary experiments indicated that the fluorescence spectra of the CS did not interfere with the fluorescence emitted by DNR.

Exponentially growing cells were incubated at a concentration of 500000 cells/ml at 37°C with DNR, in the absence or presence of a CS. DNR concentrations were 1.5 and 3.0 μM when using 8226/DOX6 and DOX40 cells, respectively. After drug exposure for 1 h in either medium containing 10% FBS or 100% horse or human serum, cells were washed twice with ice-cold PBS, resuspended in ice-cold PBS, and kept on ice until analysis of cellular DNR content. The excitation and emission wavelengths used were 488 and 585 nm, respectively. Ten thousand cells were analysed for each histogram generated. In preliminary experiments, cellular uptake of DNR was found to be diminished by approximately 75% when using 100% serum rather than culture medium supplemented with 10% FBS.

Statistical methods

The cytotoxicity data were analysed assuming a Poisson distribution for cell colony growth. Maximum likelihood estimates of the regression parameters were computed using a Newton–Raphson algorithm in the statistical language S [26]. Estimates of the standard errors for the parameters were obtained using asymptotic likelihood theory [27]. Standard errors for IC_{50}s, SFs and reduction ratios were obtained by applying the delta method [28]. Means and standard errors for DNR accumulation were computer-based on logarithmic transformation of the data. Correlations between SFs for different types and concentrations of sera and between the reduction ratios for DOX cytotoxicity and DNR accumulation were calculated using Pearson's correlation coefficient. *P* values were based on the one-sample *t*-test for correlation coefficients [29]. Statistical comparison of SFs as achieved by CS in 100% serum versus culture medium was performed by using the non-parametric Wilcoxon test.

RESULTS

First, we evaluated the effects of horse serum (with protein composition similar to human serum) on the ability of CS

Table 1. Chemosensitiser effects on doxorubicin IC_{50}(μM) *in 8226/DOX40 cells in culture medium containing 10% fetal bovine serum versus in 100% horse serum*

Drug	Culture medium			100% horse serum		
	Estimate*	95% confidence interval	Mean SF†	Estimate*	95% confidence interval	Mean SF†
Doxorubicin alone	3.86	3.62–4.09		15.45	14.63–16.55	
+ Verapamil‡	0.37	0.33–0.42	10.4	1.42	1.32–1.51	10.9
+ Cyclosporin A	0.32	0.29–0.35	12.0	1.30	1.16–1.42	11.9
+ Amiodarone	0.17	0.15–0.20	22.2§	4.56	4.36–4.70	3.4§
+ Quinidine	0.71	0.62–0.77	5.4§	3.95	3.77–4.11	3.9§
+ Quinine	0.88	0.78–0.97	4.4	2.97	2.82–3.11	5.2
+ Trifluoperazine	0.30	0.26–0.33	12.9§	7.72	7.43–8.01	2.0§
+ Tamoxifen	2.63	2.44–2.80	1.5	10.89	10.31–11.26	1.4
+ Medroxyprogesterone acetate	0.45	0.39–0.53	8.5§	2.39	2.07–2.67	6.5§

*Estimates and 95% confidence intervals derived from two independent experiments performed in triplicate. †SF, sensitisation factor. ‡Each chemosensitiser used at 20 μM. §$P = <0.005$.

to enhance anthracycline cytotoxicity and accumulation in 8226/DOX40 cells. Table 1 shows the effects of eight CS on DOX sensitivity when used at 20 μM in medium containing 10% FBS or in 100% horse serum. CS were non-toxic to the cells when used alone. CS effects on DNR accumulation are illustrated in Figure 1. In both assay systems, the use of 100% serum almost completely inactivated AMD and TFP, resulted in moderate inhibition of QD and MPA, and had no effect on VER, CSA and Q. Accordingly, the order of equimolar CS activity changed significantly in 100% versus 10% serum. Excellent agreement was found between flow cytometry and clonogenic assay with respect to the extent of CS inhibition produced by serum (Figure 2).

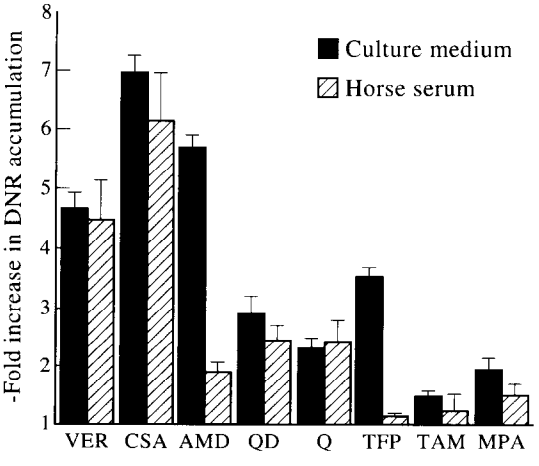

Figure 1. Effects of chemosensitisers on daunorubicin accumulation in 8226/DOX40 cells when used in culture medium containing 10% FBS or in 100% horse serum. Cells were incubated with 3 μM DNR alone or in the presence of a CS at 20 μM. Each column represents mean ± SEM of three independent experiments each performed in triplicate. VER, verapamil; CSA, cyclosporin A; AMD, amiodarone; QD, quinidine; Q, quinine; TFP, trifluoperazine; TAM, tamoxifen; MPA, medroxyprogesterone acetate.

Escalating concentrations of AAG have been recently found to render VER inactive in cells with high but not with low levels of Pgp [30]. Accordingly, the 8226/DOX6 cell line was used in subsequent experiments, which expresses low amounts of Pgp. To further enhance clinical relevance, horse serum was replaced by human serum. Table 2 and Figure 3, respectively, show the effects of 100% serum on the ability of CS to enhance DOX cytotoxicity and DNR accumulation. The pattern of CS inhibition was similar to that observed with horse serum. However, in human serum, the activity of all CS was diminished to some extent. In these experiments, CS concentrations of 5.0 rather than 20 μM, and 8226/DOX6 rather than DOX40 cells, were used. Thus, it was not clear whether the observed differences in the extent of CS inhibition were due to the use of human serum, lower CS doses or cells with lower amounts of Pgp. To answer that question,

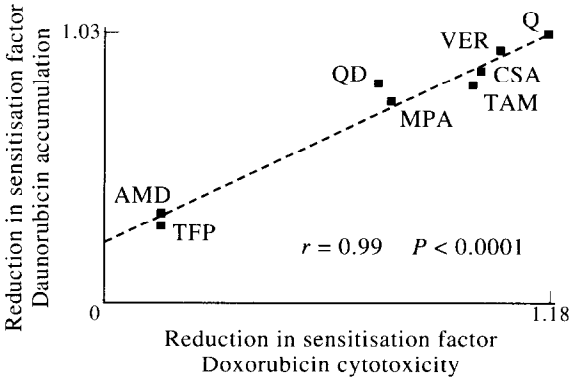

Figure 2. Correlation of reduction in chemosensitiser activity on anthracycline cytotoxicity versus accumulation, as produced by horse serum. CS were used at 20 μM. Reduction ratios were derived from mean sensitisation factors as calculated for CS in 100% serum versus culture medium. Symbols represent mean reduction ratios of at least three independent experiments each performed in triplicate. See Figure 1 legend for abbreviations.

Table 2. Chemosensitiser effects on doxorubicin IC_{50}(μM) *in 8226/DOX6 cells in culture medium containing* 10% *fetal bovine serum versus in* 100% *human serum*

Drug	Culture medium			100% human serum		
	Estimate*	95% confidence interval	Mean SF†	Estimate*	95% confidence interval	Mean SF†
Doxorubicin alone	0.25	0.22–0.29		0.60	0.52–0.68	
+ Verapamil‡	0.05	0.04–0.06	5.1§	0.21	0.18–0.24	2.8
+ Cyclosporin A	0.03	0.03–0.04	7.7	0.17	0.15–0.20	3.5
+ Amiodarone	0.04	0.04–0.05	6.2	0.47	0.41–0.55	1.3
+ Quinidine	0.08	0.07–0.09	3.3	0.39	0.34–0.45	1.5
+ Quinine	0.08	0.07–0.09	3.3	0.33	0.28–0.38	1.8
+ Trifluoperazine	0.06	0.05–0.07	4.2	0.58	0.50–0.66	1.0
+ Medroxyprogesterone acetate	0.06	0.05–0.07	4.1	0.50	0.45–0.56	1.2

*Estimates and 95% confidence intervals derived from two independent experiments performed in triplicate. †SF, sensitisation factor. ‡Each chemosensitiser used at 5 μM. §For each chemosensitiser tested, difference of SF in human serum versus culture medium was significant ($P = <0.005$).

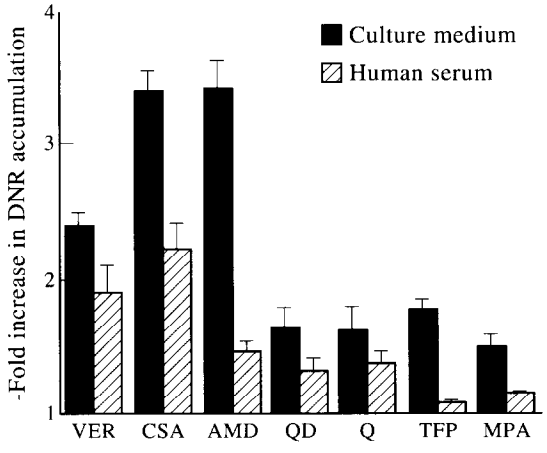

Figure 3. Effects of chemosensitisers on daunorubicin accumulation in 8226/DOX6 cells when used in culture medium containing 10% FBS versus in 100% human serum. Cells were incubated with 1.5 μM DNR alone or in the presence of CS at 5 μM. Each column represents mean ± SEM of at least two independent experiments each performed in triplicate. See Figure 1 legend for abbreviations.

clonogenic as well as flow cytometry studies were repeated by using 8226/DOX40 versus DOX6 cells, CS at 5.0 versus 20 μM, and 100% human versus horse serum. Similar levels of serum inhibition were found when using 8226/DOX40 or DOX6 cells and horse or human serum, with correlation coefficients of >0.90 and P values <0.001 (data not shown). In contrast, diminution of CS activity was greater when the agents were used at 5.0 rather than 20 μM. This particular observation appeared important because for most of these CS the serum levels achievable in humans are lower than the concentrations used in these studies. Thus, we evaluated the extent of inhibition by human serum when using VER, Q and AMD at graded concentrations, including clinically achievable blood levels (Figure 4). While VER and Q were not affected by 100% serum when used at 20 μM, an increasing and eventually profound reduction in reversing activity was observed when used at lower doses. Conversely, AMD was profoundly inhibited by serum at each dose level tested.

DISCUSSION

Among researchers working in the field of MDR reversal, there is a general feeling that better preclinical models are needed for assessing the potential clinical effectiveness of CS. This notion is based on the fact that while many agents seem capable of effectively reversing MDR in preclinical systems, they are frequently unable to overcome clinical drug resistance. Obviously, the biological basis of drug resistance is often poorly understood in clinical cancers where overexpression of MDR1/Pgp may be but one of many factors or even irrelevant. However, there are obvious differences between *in vitro* and clinical CS studies which can be addressed. Two such factors are the concentrations of CS, which in experimental studies often are much higher than achievable in humans, and the concentrations of drug-binding proteins that CS are exposed to, which *in vitro* are usually much lower than present in the body. As a result of both these factors, the amount of free CS available in clinical MDR reversal studies may be significantly lower than *in vitro*.

Our data indicate that some CS can be significantly inhibited by serum. AMD and TFP were largely inactivated even when used at doses which were highly effective in overcoming MDR when tested in medium containing low serum protein concentrations. VER, CSA and Q, in contrast, were little affected by serum when used at high doses. The differential extent of inhibition by serum significantly changed the order of MDR reversing potency when CS were used in high versus low serum concentrations. The particular differences observed in serum inhibition could not have been predicted from the degrees of serum protein binding reported for the various CS, which are around 99, 96, 98 and 90% for TFP [31], AMD [32], CSA [33] and VER [34], respectively. Furthermore, for various drugs, the published data on protein binding differ depending on the particular type of analytic method used. For example, the degrees of serum protein binding reported for QD and Q vary from 50 to 95% and from 70 to 91%, respectively [35, 36]. The concentration of free CS present at the various experimental conditions employed in these studies was not analysed. However, it seems quite likely that the loss in activity observed for particular CS in high serum concentrations was due to protein-binding of the agents.

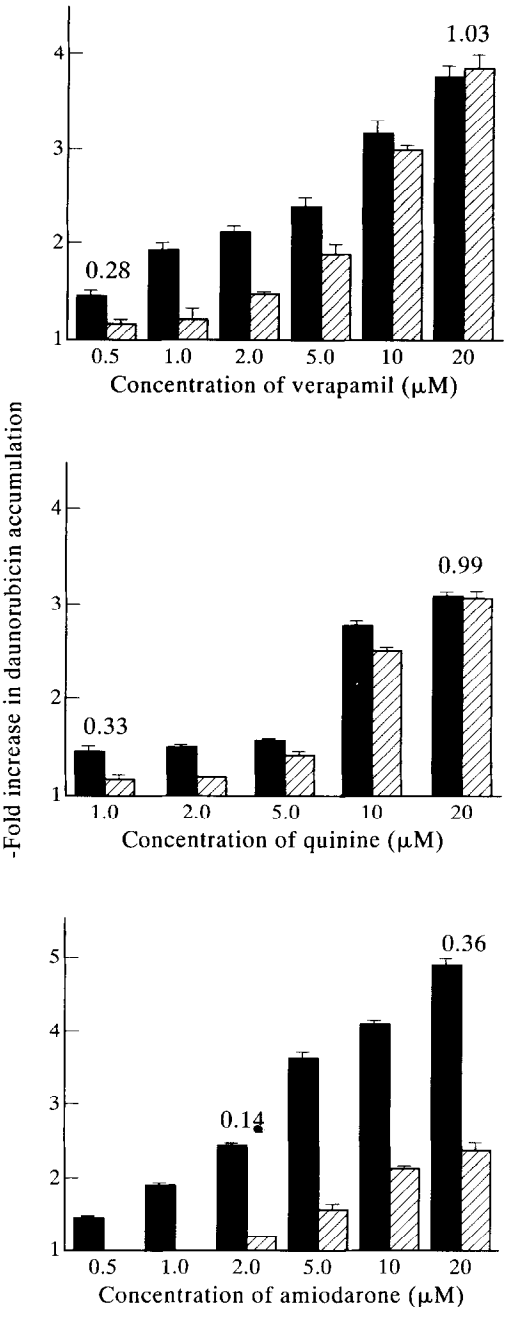

Figure 4. Effects of graded concentrations of verapamil, quinine and amiodarone on DNR accumulation in 8226/DOX6 cells. Cells were incubated with 1.5 μM DNR alone or in the presence of CS at the indicated concentrations in culture medium containing 10% FBS (solid columns) or in 100% human serum (hatched columns). The numbers shown on top of various pairs of columns represent mean reduction ratios in 100% serum versus culture medium containing 10% serum as calculated for the particular CS concentrations. Amiodarone at 0.5 and 1.0 μM was inactive when used in 100% human serum. Columns represent mean ± SEM of at least two independent experiments each performed in triplicate.

Broxterman and associates have previously found VER activity to be reduced by 40–50% when tested in 4% bovine serum albumin (BSA) or 4% human plasma versus 1% BSA [37]. A good correlation between cytotoxicity and drug accumulation assays was observed with respect to the degree of CS inhibition by protein binding, which is in agreement with our findings. The *in vitro* effects of rat serum on the ability of CS to enhance DOX uptake in a Pgp-positive rat colon cancer cell line have been reported by Genne and colleagues [38]. The study focused on *cinchona* alkaloids, including quinine and quinidine. However, amiodarone and verapamil were also tested. In agreement with our observations, amiodarone activity was profoundly diminished by rat serum. Quinine and quinidine were little affected, while verapamil activity was reduced by approximately 50%. Recently, the effects of AAG on the MDR reversal activities of VER and toremifene have been reported. Increasing AAG concentrations have been found to diminish progressively the ability of VER and toremifene to reverse resistance in a MDR cell line expressing high levels of Pgp [30, 39]. At an AAG concentration of 2 mg/ml, a level which can be present in human serum, VER activity was fully abrogated in the MDR subline, while it was reduced by only 20% in the wild-type cells which express low levels of Pgp. In the present study, no differences were found in the degree of CS inhibition by serum when using 8226/DOX40 and DOX6 cells, two MDR lines which express high and low amounts of Pgp, respectively. Most CS are hydrophobic bases at physiological pH. Such agents are bound in serum to various types of proteins including albumin, alpha-2-globulin and AAG [35]. Thus, we believe that testing of CS in whole serum is better able to approximate clinical conditions compared with using medium supplemented with particular serum proteins such as albumin or AAG.

The extent of inhibition of CS activity was similar when using horse or human serum and flow cytometry or clonogenic assay, respectively. Thus, flow cytometry and horse serum appear to be valid tools for evaluating serum effects on CS. A distinct advantage of flow cytometry over drug sensitivity assays is that results are available within a few hours. The advantage of horse serum over commercially available human serum are the much lower costs. It should be noted that wide variations in protein concentrations were found in various lots of horse serum, which resulted in differing degrees of CS inhibition compared with human serum (data not shown). Thus, analysis of protein concentrations prior to use of horse serum in such studies is critical.

When used at low concentrations, each of the tested CS was profoundly inhibited by serum. It is to be recognised that for most of the agents evaluated in the present study, the maximum achievable serum levels in humans are in the range of 2–5 μM or even lower, that is, in a range of concentrations where serum diminished MDR reversal activity of either drug. For instance, maximum achievable blood levels of racemic VER have been previously found to be around 2 μM [40]. At this particular dose level, serum diminished VER activity by approximately 50%. By contrast, Q is able to readily yield blood levels of 20 μM [41], a concentration at which Q activity was not impaired by serum. When tested in 100% human serum, Q at 20 μM was approximately twice as effective as VER at 2 μM in increasing DNR accumulation in 8226/DOX6 cells. Whether this suggests Q is more likely than VER to be capable of overcoming clinical drug resistance remains to be proven, and these *in vitro* data must be interpreted with great caution with respect to their clinical application.

We recognise that any kind of experimental model falls short of truely mimicking the complex clinical scenario. How-

ever, we believe that *in vitro* models can be developed which approximate clinical conditions more closely than the assays which are routinely applied, for example, by testing the CS at clinically relevant concentrations in an environment containing high serum concentrations. Studies along these lines are currently in progress and we hope that such pharmacologically guided *in vitro* modelling will prove capable of better predicting the potential clinical effectiveness of CS.

1. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993, **62**, 385–427.
2. Goldstein LJ, Galski H, Fojo A, *et al.* Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst* 1989, **81**, 116–124.
3. Noonan KE, Beck C, Holzmayer TA, *et al.* Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc Natl Acad Sci USA* 1990, **87**, 7160–7164.
4. Arceci RJ. Clinical significance of P-glycoprotein in multidrug resistance malignancies. *Blood* 1993, **81**, 2215–2222.
5. Chan HSL, Thorner PS, Haddad G, *et al.* Immunohistochemical detection of P-glycoprotein. Prognostic correlation in soft tissue sarcoma of childhood. *J Clin Oncol* 1990, **8**, 689–704.
6. Chan HSL, Haddad G, Thorner PS, *et al.* P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N Engl J Med* 1991, **325**, 1608–1614.
7. Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* 1990, **42**, 155–199.
8. Salmon SE, Dalton WS, Grogn TM, *et al.* Multidrug-resistant myeloma. Laboratory and clinical effects of verapamil as a chemosensitizer. *Blood* 1991, **78**, 44–50.
9. Miller TP, Grogan TM, Dalton WS, *et al.* P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high-dose verapamil. *J Clin Oncol* 1991, **9**, 17–24.
10. Sonneveld P, Durie BGM, Lokhorst HM, *et al.* Modulation of multidrug-resistant multiple myeloma with cyclosporin. *Lancet* 1992, **340**, 255–259.
11. List AF, Spier C, Greer J, *et al.* Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. *J Clin Oncol* 1993, **11**, 1652–1660.
12. Wishart GC, Bissett D, Paul J, *et al.* Quinidine as a resistance modulator of epirubicin in advanced breast cancer. Mature results of a placebo-controlled randomized trial. *J Clin Oncol* 1994, **12**, 1771–1777.
13. Solary E, Caillot D, Chauffert B, *et al.* Feasibility of using quinine, a potential multidrug resistance-reversing agent, in combination with mitoxantrone and cytarabine for the treatment of acute leukemia. *J Clin Oncol* 1992, **10**, 1730–1736.
14. Van der Graaf WTA, de Vries EGE, Uges DRA, *et al. In vitro* and *in vivo* modulation of multi-drug resistance with amiodarone. *Int J Cancer* 1991, **48**, 616–622.
15. Miller RL, Bukowski RM, Budd GT, *et al.* Clinical modulation of doxorubicin resistance by the calmodulin inhibitor, trifluoperazine. A phase I/II trial. *J Clin Oncol* 1988, **83**, 105–110.
16. Millward MJ, Cantwell BMJ, Lien EA, *et al.* Intermittent high-dose tamoxifen as a potential modifier of multidrug resistance. *Eur J Cancer* 1992, **28A**, 805–810.
17. Sikic BI. Modulation of multidrug resistance. At the threshold. *J Clin Oncol* 1993, **11**, 1629–1636.
18. Lehnert M. Reversal of P-glycoprotein-associated multidrug resistance. The challenge continues. *Eur J Cancer* 1993, **29A**, 636–638.
19. Dalton WS, Durie BGM, Alberts DS, *et al.* Characterization of a new drug-resistant human myeloma cell that expresses P-glycoprotein. *Cancer Res* 1986, **46**, 5125–5130.
20. Dalton WS, Grogan TM, Rybski JA, *et al.* Immunohistochemical detection and quantitation of P-glycoprotein in multiple drug-resistant human myeloma cells. Association with level of drug resistance and drug accumulation. *Blood* 1989, **73**, 747–752.
21. Chen TR. *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp Cell Res* 1977, **104**, 255–262.
22. Woodcock DM, Jefferson S, Linsenmeyer ME, *et al.* Reversal of the multidrug resistance phenotype with Cremophor EL, a common vehicle for water-insoluble vitamins and drugs. *Cancer Res* 1990, **50**, 4199–4203.
23. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science (Wash, DC)* 1977, **197**, 461–463.
24. Salmon SE, Young L, Leibowitz J, *et al.* Evaluation of an automated image analysis system for counting human tumor colonies. *Int J Cell Cloning* 1984, **2**, 142–160.
25. Speth PAJ, Linssen PCM, Boezeman JBM, *et al.* Quantification of anthracyclines in human hematopoietic cell subpopulations by flow cytometry correlated with high pressure liquid chromatography. *Cytometry* 1985, **6**, 143–150.
26. Becker RA, Chambers JM, Wilks AR. *The New S Language.* Pacific Grove, Wadsworth & Brooks/Cole, 1988.
27. Lehmann EL. *Theory of Point Estimation.* New York, Wiley, 1983.
28. Billingsley P. *Probability and Measure*, 2nd edn. New York, Wiley, 1986.
29. Rosner B. *Fundamentals and Biostatistics*, 3rd edn. Boston, PWS-Kent, 1990.
30. Chatterjee M, Robson CN, Harris AL. Reversal of multidrug resistance by verapamil and modulation by α1-acid glycoprotein in wild-type and multidrug-resistant chinese hamster ovary cell lines. *Cancer Res* 1990, **50**, 2818–2822.
31. Freedberg KA, Innis RB, Creese I, *et al.* Anti-schizophrenic drugs—differential plasma protein-binding and therapeutic activity. *Life Sci* 1979, **24**, 2467–2473.
32. Latini R, Tognoni G, Kates RE. Clinical pharmacokinetics of amiodarone. *Clin Pharmacokinet* 1984, **9**, 136–156.
33. Vozeh S, Schmidlin O, Taeschner W. Pharmacokinetic drug data. *Clin Pharmacokinet* 1988, **15**, 254–282.
34. Keefe DL, Yee YG, Kates RE. Verapamil protein binding in patients and in normal subjects. *Clin Pharmacol Ther* 1981, **29**, 21–26.
35. Lindup WE. Plasma protein binding of drugs—some basic and clinical aspects. In Bridges JW, Chasseaud LF, Gilson GG, eds. *Progress in Drug Metabolism.* London, Taylor & Francis Ltd, 1987, 141–185.
36. Jahnchen W, Muller WE. Stereoselectivity in protein binding and drug deposition. In Breimer DD, Speiser P, eds. *Topics in Pharmaceutical Sciences.* New York, Elsevier Science, 1983, 109–117.
37. Broxterman HJ, Kuiper CM, Schuurhuis GJ, *et al.* Daunomycin accumulation in resistant tumor cells as a screening model for resistance modifying drugs. Role of protein binding. *Cancer Lett* 1987, **35**, 87–95.
38. Gene P, Dimanche-Biotrel MT, Mauvernay RY, *et al.* Cinchonine, a potent efflux inhibitor to circumvent anthracycline resistance *in vivo*. *Cancer Res* 1992, **52**, 2797–2801.
39. Chatterjee M, Harris AL. Enhancement of Adriamycin® cytotoxicity in a multidrug resistant chinese hamster ovary (CHO) subline, CHO-Adr^r, by toremifene and its modulation by alpha$_1$ acid glycoprotein. *Eur J Cancer* 1990, **26**, 432–436.
40. Pennock GD, Dalton WS, Roeske WR, *et al.* Systemic toxic effects associated with high-dose verapamil infusion and chemotherapy administration. *J Natl Cancer Inst* 1991, **83**, 105–110.
41. Hofheinz W, Merkli B. Quinine and quinine analogues. In Peters W, Richards WHG, eds. *Antimalarial Drugs II, Current Antimalarials and New Drug Developments.* Berlin, Springer, 1984, 61–81.

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