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Reversal of P-glycoprotein is greatly reduced by the presence of plasma but can be monitored by an *ex vivo* clinical assay

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The effects of nine reversers of P-glycoprotein on the uptake of daunomycin into *MDR1*-transfected P388 cells were quantitatively determined in undiluted human or mouse plasma and compared with their effects when measurements are made in a conventional cell culture medium (RPMI 1640) containing only 10% serum. Plasma diminished or greatly diminished the effectiveness of the reversers, reductions of up to 20-fold being found for reversers (cyclosporin A, prochlorperazine and amiodarone) that have been used in clinical trials, although quinidine was almost as effective in plasma as in cell culture medium containing 10% fetal calf serum. Human or bovine serum albumin could mimic the effect of whole plasma. When measurements of the effectiveness of the reverser cyclosporin A were made in an *ex vivo* assay, using these P388 cells, complete accord was found between such *ex vivo* determinations and cyclosporin A's effectiveness *in vivo*, as monitored by its ability to increase the accumulation of vinblastine in mouse kidney tissue. The *ex vivo* assay was shown to be suitable to monitor the effectivity of reversers present in plasma taken from patients receiving quinidine and cyclosporin A in routine clinical treatment.

Key words: *Ex vivo* assay, multidrug resistance, P-glycoprotein, plasma.

Introduction

P-glycoprotein, the product of the *MDR1* gene in humans or *mdr1* and *mdr3* in mice,[1,2] is at least in part responsible for some of the resistance that cancers develop towards chemotherapy.[3–5] Other membrane-located drug pumps are known that may also contribute to chemotherapy resistance[6–9] and cytoplasmic components can also contribute to overall drug resistance.[10–12] For all these components, intensive efforts are being made, on a worldwide scale, to find ways to overcome their effects. For P-glycoprotein in particular, these efforts have been put into finding ancillary drugs that will counteract P-glycoprotein by blocking its pumping action on the cytotoxins.[13–15] Very many reversers of P-glycoprotein's action have been discovered and numbers of these have been put into clinical trial.[16–21] As of now, these efforts have been disappointing with no agent yet being sufficiently effective and non-toxic to be fully accepted as an auxiliary for the conventional chemotherapeutic agents. Reversers cannot be given in doses that patients can tolerate but are yet high enough to block P-glycoprotein *in vivo*. At least one possible reason for this phenomenon is that the effectiveness of the known reversers has generally been measured in cell culture media contain no or low concentrations of serum whereas, *in vivo*, components in the patient's plasma might compete against P-glycoprotein for binding to the reverser, thus lowering its effectiveness. A few reports have appeared[22–24] of the ability of serum or serum components to antagonize the effects of P-glycoprotein reversers. In our own studies of the reversal of P-glycoprotein *in vivo* in mice,[25] in which our assay was the accumulation of a cytotoxin in the tissues of the living mice, we found that plasma concentrations of cyclosporin A, which would be effective reversers in a saline medium, were ineffective in the mouse. We postulated that this might be due to the binding of cyclosporin A to serum proteins *in vivo*. In the present paper we subject this hypothesis to a quantitative test. We show, by measuring the effect of reversers on the accumulation of daunomycin into cells containing human P-glycoprotein (a technique that we have shown[26] gives a valid measure of reverser effects of reversers also on cell killing), that

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the quantitative kinetic parameters that describe the effectiveness of many reversers are affected by the presence of serum or serum components. Different reversers are affected to different extents, cyclosporin A and tamoxifen being some of the most affected, their effectiveness being reduced some 20-fold by serum. In contrast, quinidine is almost unaffected by the presence of serum. We have developed an *ex vivo* assay to monitor *in situ* the effectiveness of a reverser in serum, an assay which should enable the clinician to study, on a real-time basis, the ability of a particular reverser regime to overcome the action of P-glycoprotein.

Methods

Chemicals

Amiodarone, dipyridamole, prochlorperazine, propafenone, quinidine and verapamil were obtained from Sigma (Petah Tikvah, Israel). Mefloquine and tamoxifen were kind gifts from Dr H Ginsburg, while cyclosporin A was a kind gift from Dr E Shohami. Tritium-labeled [G-^{3}H]daunomycin (3.9 Ci/mmol) was from NEN-DuPont (Boston, MA). [G-^{3}H]vinblastine (21 Ci/mmol) was from Amersham Life Sciences (UK) or from Rotem (Israel) at 52 Ci/mmol.

Cell culture, animals and treatment

The cells used were a drug-resistant strain of P388 lymphoma cells, having been transfected with the human *MDR1* gene, a kind gift of Dr MM Gottesman of the National Cancer Institute (Bethesda, MD). They were grown in RPMI 1640 medium (Biological Industries, Kibbutz Beit Ha'emek, Israel) to which were added penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), 10 μM β-mercaptoethanol and 0.2% (w/v) glucose, together with 10% fetal calf serum (Biological Industries), the whole being hereafter termed complete RPMI medium, in 5% CO_2 humidified air at 37°C, in suspension culture, in 260 ml plastic bottles (Nunc, Denmark). The mice were of the Sabra strain and were maintained in the animal house of the Institute of Life Sciences. All experiments with these mice were performed in accordance with the Animal Care Guidelines and approved by the Institute's Animal Care Committee. Human blood was collected in the Department of Endocrinology of the Sharay Tzedek Hospital, Jerusalem and handled in accordance with the ethical standards of that institution. Human plasma was prepared by centrifuging outdated transfusion blood. Mouse plasma was prepared by bleeding mice from the femoral vein into a tube containing sufficient EDTA to yield a final concentration of 5 mM, after sacrifice by cervical rupture. Before an experiment, cells were counted in a hemocytomer, and the cells resuspended in fresh medium at 1.5×10^6 cells per 1 ml medium or human plasma, or, for the mice experiments, at 300 000 cells in 0.08 ml medium or mouse plasma for the subsequent transport assay. Either 1.0 or 0.08 ml of a suspension of cells in complete RPMI, human plasma or mouse plasma were transferred to a microfuge tube, and 30 or 4 μl of the working solution of ^{3}H-labeled cytotoxin (with sufficient cytoxin to give a final concentration of daunomycin at 2 nM) containing the required concentration of reverser was added at zero time. Incubation was for 60 min at 37°C. Uptake of the cytotoxin was stopped by rapid centrifugation in the Beckmann model 11 microfuge 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 liquid scintillation counting using Quiksafe A flurophor (Zinser Analytic, UK), in a Beckman 4S 600TA liquid scintillation counter. In all cases, aliquots of cells were incubated for comparable times with no reverser present and zero time uptakes performed by adding appropriate aliquots of pre-cooled labeled daunomycin solutions to pre-cooled 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 60 min in the presence of reverser. This zero time value was subtracted from the counts obtained during the timed incubations.

Cytotoxins were assayed in human plasma samples using commercially available clinical systems, the TDX-FLX system of Abbott Inc. for quinidine and the Inc-Star Co. kit for cyclosporin A.

Data analysis

To obtain the appropriate value of the Michaelis parameter, K_i, for the effectiveness of a chemosensitiser on counteracting the action of P-glycoprotein on the accumulation of daunomycin, we used equation (5) of the Appendix of Lan *et al.*[26] (rewritten as equation (1) of the Results section of the present paper). By fitting this equation to the data of D_i

against C, using standard curve-fitting procedures, the appropriate values of K_i, D_0 and D_e can be found.

Results

P388 lymphoma cells, transfected with the human *MDR1* gene, were incubated for 1 h with 2 nM daunomycin at 37°C, in the presence of various concentrations of reversers of P-glycoprotein, either in the conventional accumulation medium (RPMI 1640) or in human plasma. Figure 1 depicts the accumulation of the labeled daunomycin by the P388 cells in the presence of four different, well-established reversers of P-glycoprotein. In every case, the presence of the plasma reduces the effectiveness of the reverser, drastically so in the

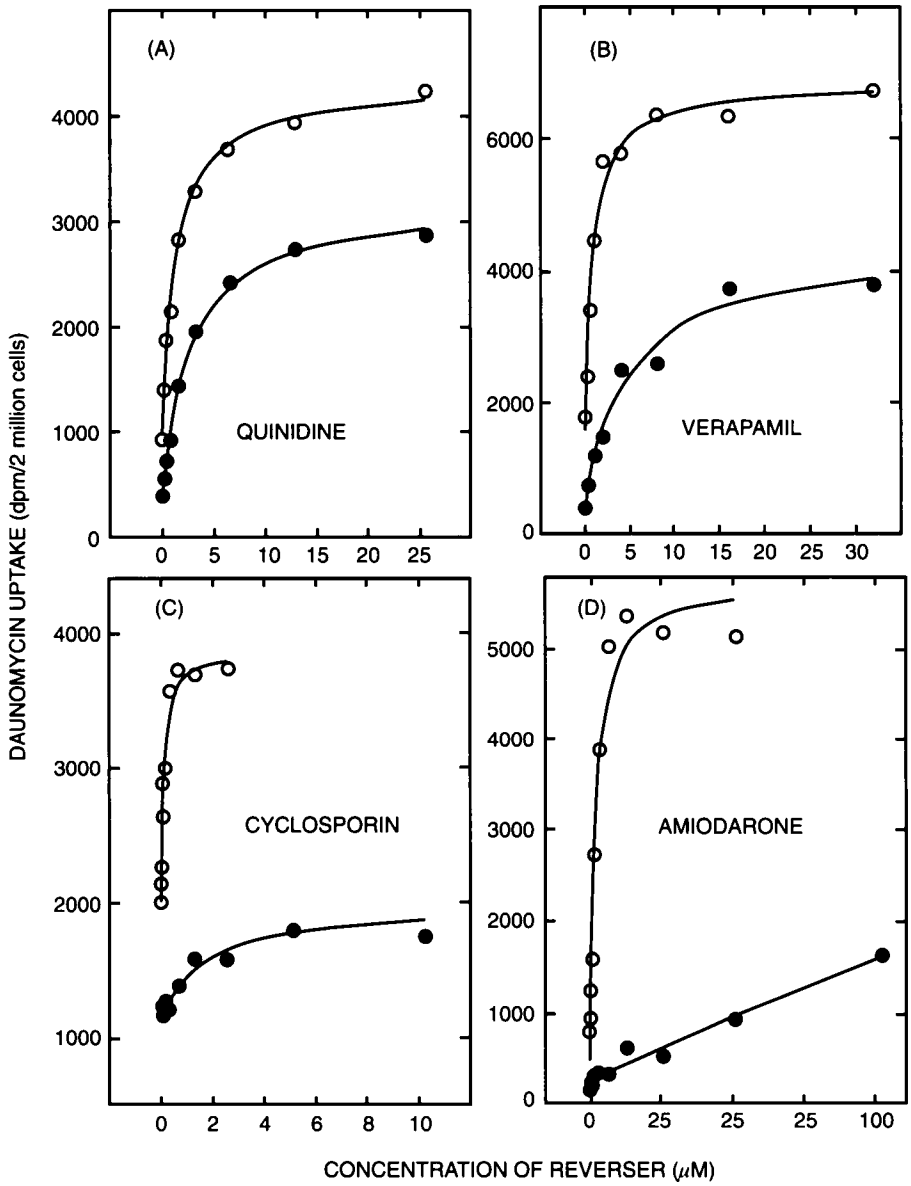

Figure 1. Effect of four reversers on the accumulation of daunomycin into multidrug-resistant P388 lymphoma cells, transfected with the human *MDR1* gene, suspended in conventional RMPI culture medium containing 10% fetal calf serum (open circles) or in undiluted human plasma (filled circles). Cellular accumulation was measured during 60 min incubation at 37°C, from a daunomycin concentration of 2 nM and with a series of dilutions of reverser as indicated. The solid lines are fitted by a form of the Michaelis–Menten equation (see text and Lan[26]) which enables extraction of the parameter K_i that measures the ability of the reverser to block P-glycoprotein (appropriate values being listed in Table 1).

cases of cyclosporin A or amiodarone (Figure 1c and d, respectively). The data of Figure 1 were fitted by a modified form of the Michaelis–Menten question (equation (5) of the Appendix of Lan *et al.*[26]):

$$D_i = D_0 + (D_e - D_0) * C/(K_i * (D_e/D_0) + C) \quad (1)$$

where D_i is the uptake of daunomycin at a concentration C of the reverser in equation, D_0 is the uptake in the absence of reverser, D_e is the uptake at a maximal concentration of reverser, while $K_i * (D_e/D_0)$ is that concentration of reverser at which one-half the maximal effect is attained, K_i being the *intrinsic* affinity of the reverser for P-glycoprotein (see Lan *et al.*[26] for a discussion of apparent and intrinsic inhibition constants for P-glycoprotein; the instrinsic inhibition constant is the appropriate parameter when cell killing by the accumulated cytotoxin is being considered). Values of the parameter K_i, which characterizes the effectiveness of the reverser in reversing P-glycoprotein's effect on cell killing by cytotoxins, are collected in Table 1 for these four reversers and four others that we studied.

Figure 2 depicts such a study in which the effect of the reverser mefloquine is determined in the presence of the RPMI medium, of human plasma and of human plasma albumen in amounts equal to that in a corresponding volume of human plasma (Figure 2a) or in RPMI or bovine serum albumin or defatted bovine serum albumin in Figure 2(b), again in amounts equal to that of albumen in human plasma. Clearly, the albumin component of the blood plasma makes an overwhelming contribution to the capacity of plasma to reduce the effectiveness of this reverser of P-glycoprotein, and it matters little if the albumin is, or is not, defatted.

In an earlier study of some 20 reversers,[26] we compared the effectiveness of the reversers (measured by the appropriate inhibition constant, K_i, to

Table 1. The inhibition parameter, K_i, describing the reversal of P-glycoprotein, measured by the effect of reversers on the accumulation of daunomycin (see text) in RPMI medium or in human plasma (measured at 25°C)

Reverser	K_i in RPMI (μM)	K_i in plasma (μM)
Amiodarone	0.37 ± 0.26	11.5 ± 4.4
Cyclosporin A	34 ± 9 (nM)	560 ± 230 (nM)
Dipyridamole	1.15 ± 0.29	8.14 ± 0.80
Mefloquine	0.54 ± 0.36	4.2 ± 1.5
Propafenone	0.24 ± 0.07	0.78 ± 0.05
Quinidine	0.26 ± 0.03	0.32 ± 0.02
Tamoxifen	1.56 ± 0.87	31 ± 11
Verapamil	0.30 ± 0.10	0.91 ± 0.39

K_i is obtained from curve-fitting data such as depicted in Figures 1 and 2 and are reported as the derived value of K_i ± SE. For the theory behind such curve-fitting see Lan *et al.*[26]

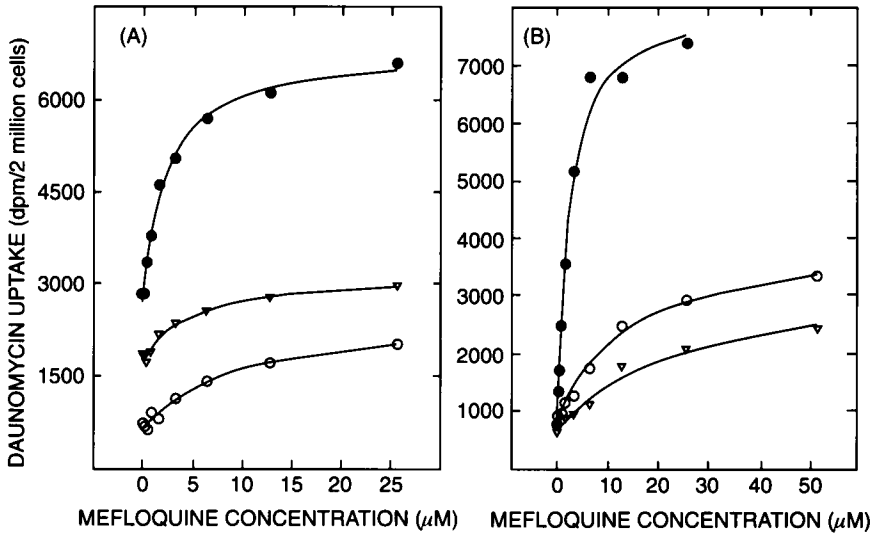

Figure 2. Effect of mefloquine on the accumulation of daunomycin into multidrug-resistant P388 lymphoma cells transfected with the human *MDR1* gene. Cellular accumulation determined as in Figure 1. (a) In the presence of a RPMI-based medium (solid circles) or in undiluted human plasma (open triangles) or in human plasma albumin in RPMI medium (empty circles). The appropriate values of the parameter K_i, by curvefitting, are 0.90 ± 0.16, 2.67 ± 0.77 and 2.81 ± 0.79 for these three cases, respectively. (b) In the presence of a RPMI-based medium (solid circles) or in bovine plasma albumin (open triangles) or in fat-free bovine serum albumin in RPMI medium (empty circles). The appropriate values of the parameter K_i, but curvefitting, are 0.18 ± 0.08, 5.66 ± 2.72 and 2.95 ± 0.55 for these three cases, respectively.

the maximal levels of these reversers that have been reported in human plasma in patients receiving those reversers, either for attempted reversal of P-glycoprotein in clinical trials or else in pharmacodynamic studies of these pharmaceuticals. We showed that for only a few of these compounds was the reported maximal plasma level sufficient to give a substantial reversal of the MDR pump *when the kinetic parameter* K_i *was measured in the RPMI medium*. Figure 3 depicts, for the eight reversers that we studied in the present work, the ratio of the maximal reported plasma level of each reverser to the measured K_i as measured in RPMI (open bars) or in the presence of undiluted plasma (hatched bars). Note that for all these reversers the effectiveness of the reverser is lowered, in most cases substantially so, and for only for two of them, quinidine and propafenone, is the maximal plasma level sufficient to give substantial inhibition of the MDR pump (i.e. more than 80%). Thus, for example, cyclosporin A and tamoxifen, which in saline medium are very effective reversers, are very little effective when measured in undiluted plasma and, at the maximum doses that have been reported in clinical trials of their effectiveness as blockers of P-glycoprotein, would not be expected to give much reversal.

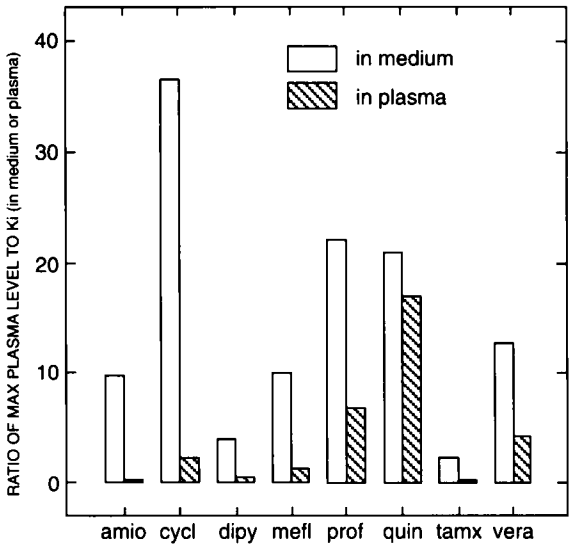

Figure 3. The ratios of the inhibition parameters (K_i) for reversers of P-glycoprotein to the maximal clinical concentrations of these reversers reported in human plasma.[26] The empty bars depict these ratios when the parameter K_i was determined in the conventional RPMI-containing medium (taken from Lan *et al.*[26]), while the hatched bars are for these same parameters measured in undiluted human plasma (Table 1). The reversers are, from left to right: amiodarone, cyclosporin A, dipyridamole, mefloquine, propafenone, quinidine, tamoxifen and verapamil.

We wished to test whether mouse plasma behaved as did human plasma with regard to diminishing the effectiveness of such reversers. To this end, we scaled down our *ex vivo* assay so that it could be used on the small quantities of plasma that we could obtain from mice. Figure 4 depicts data on accumulation of daunomycin into aliquots containing 300 000 *MDR1*-transfected P388 cells in 80 μl of RPMI medium (open circles) or mouse plasma (filled circles) as a function of the concentration of four different reversers. In each case, the plasma has a substantial effect on reducing the effectiveness of the reverser, a stronger effect than in the parallel experiments with human plasma (Figure 1).

We wished to test whether this effect of plasma could account quantitatively for the discrepancy that we had observed[25] between the effects of reversers in RPMI medium and their effects in the whole animal. To this end, we injected groups of mice with cyclosporin A at various doses and, 16 h later, killed the animals and collected their blood. The plasma from these blood samples were then tested in our *ex vivo* assay for their effect on reversing the MDR pump's action on the accumulation of daunomycin. The hatched bars in Figure 5 show the accumulation of daunomycin into 300 000 *MDR1*-transfected P388 cells during 1 h at 37°C in the presence of undiluted plasma from mice injected 16 h earlier with the doses of cyclosporin A depicted on the abscissa. Superimposed on these data are the data points and fitted curve from an experiment in which mice were injected with various doses of cyclosporin A as depicted and the uptake of vinblastine into kidney tissue measured, again 16 h after injection of the reverser and of the cytotoxin. These data are taken from Lyubimov *et al.*[25] and are here computed relative to the control animals that had not been given cyclosporin A. It appears that the effectiveness of cyclosporin A as measured in plasma, using the *ex vivo* assay, is a good measure of its ability to block the MDR pump in the living animal.

To test our *ex vivo* assay on human patients, we obtained blood from two groups of patients who were receiving reversers of the MDR pump in connection with conditions that were unrelated to cancer chemotherapy. Figure 6(a) depicts data obtained in parallel when *MDR1*-transfected P388 cells were added to human plasma prepared from out-dated transfusion blood in the presence of externally added quinidine (solid circles) or to plasma from clinical samples of patients receiving quinidine (open circles). For the clinical samples,

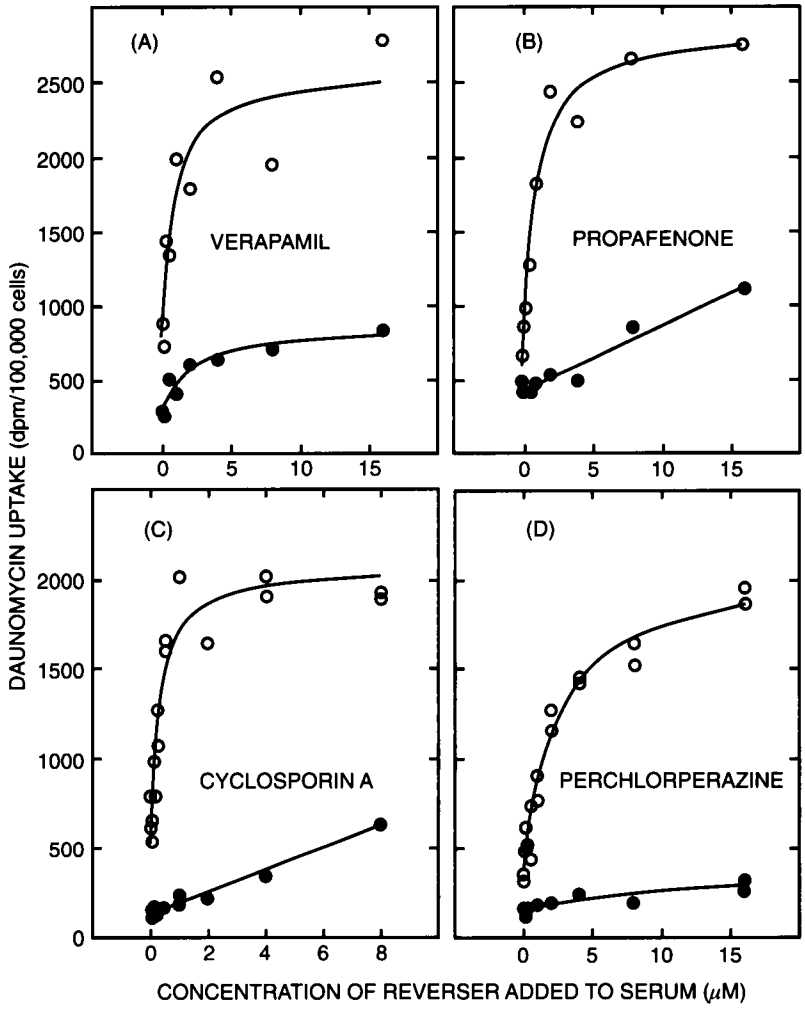

Figure 4. Effect of four reversers on the accumulation of daunomycin into multidrug-resistant P388 lymphoma cells, transfected with the human *MDR1* gene, suspended in a conventional RPMI-based medium (open circles) or in undiluted mouse plasma (filled circles). Cellular accumulation was measured during 60 min incubation at 37°C, from a daunomycin concentration of 2 nM and with a series of dilutions of reverser as indicated. The solid lines are fitted by a form of the Michaelis–Menten equation (see text and Lan *et al.*[26]) which enables extraction of the parameter K_i that measures the ability of the reverser to block P-glycoprotein. The appropriate values are, in μM (in each case, the value extracted for the experiment in RPMI medium is given first): verapamil 0.26 ± 0.25 and 0.66 ± 0.42; cyclosporin A 0.075 ± 0.038 and 2.3 ± 0.21; propafenone 0.19 ± 0.086 and 9.63 ± 6.0; prochlorperazine 0.46 ± 0.13 and 4.9 ± 5.3.

the concentration of quinidine was determined using the Inc-Star Co. analysis kit in routine examinations. The quinidine in the blood from patients receiving this drug is almost as effective, on a molar basis, as quinidine added freshly to human plasma.

Figure 6(b) depicts a comparable experiment in which cyclosporin A, rather than quinidine, was the reversing agent. Here the data are presented as a ratio of the daunomycin uptake at any concentration of cyclosporin A to the uptake when no cyclosporin A is added. The lower and upper interrupted lines represent the amount of accumulation in the absence of any added reverser and in the present of a maximal amount of reverser (verapamil), respectively. Again, the concentrations of reverser in the patients blood (open squares and circles, these being two different collections of blood samples) was determined using a conventional clinical kit. The effectiveness of cyclosporin A as a reverser is low at these levels of cyclosporin A in the blood but is comparable to that obtained at the same levels of cyclosporin A when this is added directly to human

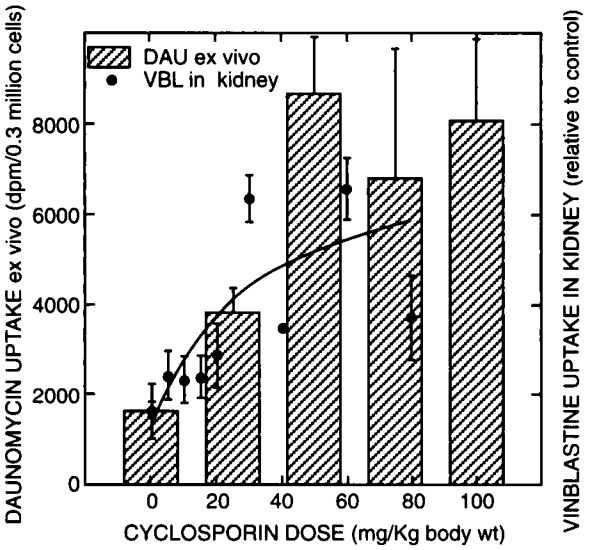

Figure 5. Comparison of the *in vitro* effect of the reverser cyclosporin A on accumulation of vinblastine in the kidney tissue of the mouse (filled circles and solid line) and the *ex vivo* effect on accumulation of daunomycin in P-glycoprotein-transfected P388 lymphoma cells (histogram bars). The data for accumulation in kidney are taken from Lyubimov *et al.*,[25] recalculated as a ratio of the control accumulation value (no cyclosporin A) and then this value being set as equal to the uptake into P388 cells with no cyclosporin A present. In both sets of experiments groups of mice were injected i.p. at time zero with labeled vinblastine and cyclosporin A at the doses listed on the abscissa (see Methods). After 16 h the mice were killed by cervical rupture, kidney tissue was dissected out, homogenized in saline and radioactivity determined by liquid scintillation counting,[25] and blood collected from the femoral vein, into a tube containing EDTA and plasma obtained by centrifugation. *MDR1*-transfected P388 cells and then labeled daunomycin was added and its uptake determined as in Figure 4.

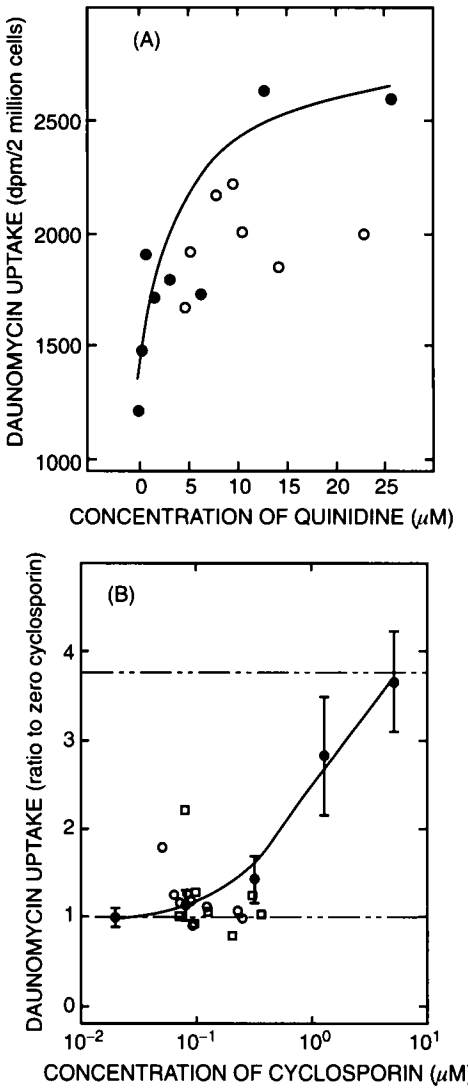

Figure 6. Comparison of the effect of (a) quinidine and (b) cyclosporin A when present in plasma of patients receiving these drugs in routine clinical application (empty circles and empty squares) with their effects when added directly to human plasma prepared from out-dated transfusion blood (filled circles). Plasma concentrations of quinidine and cyclosporin A were measured by routine clinical procedures, while daunomycin accumulation was measured as in Figure 1.

plasma from out-dated transfusion blood (solid circles, with SE of means).

Discussion

The data presented in this paper show quantitatively that both human and mouse blood contains a component or components that compete with P-glycoprotein for reversers of this pump, so that the effectiveness of the reversers is reduced. The binding of cyclosporin A to mouse plasma quantitatively accounts for the discrepancy that we had previously reported as between the effectiveness of cyclosporin A as a reverser when measured in RPMI media containing low concentrations of plasma as opposed to its effects in the living animal. This reduction in reverser effectivenss differs for the different reversers, suggesting that P-glycoprotein and those plasma components that bind these reversers have different binding affinities for the reversers. Quinidine, for example, seems to be the least affected, being apparently poorly bound to plasma, whereas cyclosporin A, tamoxifen and amiodarone are over 10 times less effective in plasma than in the RPMI media usually used for measuring cytotoxin accumu-

lation or cytotoxicity. This effect of plasma is, for mefloquine (Figure 3), largely manifested by its content of plasma albumin. This may be true also for other reversers but for some there may be specific plasma components that bind the reverser in question.

This finding has important clinical consequences since it means that, as has been suspected, the ability of a reverser to block P-glycoprotein as measured in saline medium containing low concentrations of serum (10%) may be only a very poor indication of its effectiveness in the living animal. A consequence of our study is that may be worthwhile to re-investigate[17] the possible effectiveness of quinidine as a reverser of P-glycoprotein in a clinical trial.

The *ex vivo* assay that we have developed will enable a clinician that uses it to monitor the reverser's effectiveness when in the circulating blood of the patient. The clinician can thus measure rapidly the *in vivo* effectiveness of the reverser and should be able bring about an effective reversal of the MDR pump, provided that the patient can tolerate the drug doses that are needed. At the least, the clinician can know, within a short time of administering it, if the reverser dose that is being given is, or is not, sufficient to reverse the MDR pump *in vivo*.

Any newly developed reversers will have to be tested quantitatively by such an *ex vivo* assay to ensure that they are indeed effective in doses that the patient can tolerate. The ability of plasma to bind the various reversers is an additional parameter that needs to be measured *and investigated systematically* when a search is made for new and improved reversers of P-glycoprotein in the endeavor to overcome resistance to cancer chemotherapy.

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