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Extent and persistence of P-glycoprotein inhibition in multidrug-resistant P388 cells after exposure to resistance-modifying agents

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The low daunomycin (DAU) retention in P388 cells displaying P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) can be increased by the presence of various resistance-modifying agents (RMAs). Taking the DAU retention restoration as an indicator of Pgp function inhibition and using a few RMAs, including SDZ PSC 833, SDZ 280-446, cyclosporin A (CsA) and verapamil, we compared different conditions of MDR cell exposure to the RMA. The 'co+post-RMA' treatments (RMA present during both DAU uptake and efflux phases) generally led to higher DAU retention levels than the 'co-RMA' treatments (RMA present during the DAU uptake phase only). The magnitude and persistence of Pgp function inhibition induced by the RMA was further examined by only pulsing the cells with the RMA and growing them in RMA-free medium before the DAU retention assay ('pre-RMA' treatment). While recovery of Pgp function was nearly complete within minutes after a pulse exposure to verapamil, this took increasing times with CsA, SDZ 280-446 and SDZ PSC 833, the latter RMA leaving traces of inhibition of Pgp function even 2 days after the pulse exposure of the MDR-P388 cells. The persistence of Pgp inhibition conferred by some RMAs being much longer than by others, this feature should be taken into account when designing chemotherapy protocols in the clinic.

Key words: Cyclosporin A, drug retention, multidrug resistance, P-glycoprotein, SDZ 280-446, SDZ PSC 833.

Introduction

The overexpression of a particular transmembrane glycoprotein, called P-glycoprotein (Pgp), is one of the causes of the 'multidrug-resistant' (MDR) phenotype, leading to tumor cell resistance to a broad spectrum of structurally unrelated anti-cancer drugs (ACD).[1,2] These Pgp molecules seem to work by pumping the ACD out of the cells by an ATP-dependent mechanism, therefore decreasing the intracellular ACD concentration below its active threshold. Two models for Pgp function were proposed: either Pgp would be organized as a hydrophilic pore through which the drug is effluxed or the Pgp would be acting as a flippase exporting ACD which would first interact with the membrane lipid bilayer before interacting with Pgp.[3]

Several resistance-modifying agents (RMAs), such as cyclosporin A (CsA), verapamil, amiodarone, quinidine and quinacrine, have been shown to sensitize MDR tumor cells by enhancing the intracellular ACD concentration and, hence, inhibit the Pgp function.[4–11] Using a photoaffinity labeling reagent, CsA was shown to bind to both cyclophilin and Pgp.[12] Since the immunosuppressive activity of CsA was known to be mediated through binding to cyclophilin and structure–activity details for this activity were known,[13] via appropriate chemical derivation, it was possible to find derivatives which were non-immunosuppressive but were very active in reversing MDR. This reversion presumably occurred by interacting with Pgp since the cell lines used had been characterized as ones in which only this form of resistance was present.

By *in vitro* ACD-mediated inhibition of cell growth, the non-immunosuppressive cyclosporin analog SDZ PSC 833 and the semi-synthetic cyclopeptolide SDZ 280-446 were shown to reverse the resistance of different MDR-tumor cell lines at much lower concentrations than other tested RMAs (CsA and verapamil).[14–16] On the MDR-P388 cells, SDZ PSC 833 and SDZ 280-446 completely restored the sensitivity to six ACD: colchicine, vincristine, doxorubicin, daunomycin (DAU), etoposide and taxol.[14–17] Part of this work leading to the identification of improved derivatives has been published,[14] SDZ PSC 833 being [(3′-deoxy-3′-oxo-MeBmt)-1]-CsD[15] and currently evaluated in the clinic. Similarly, several potent RMAs could be de-

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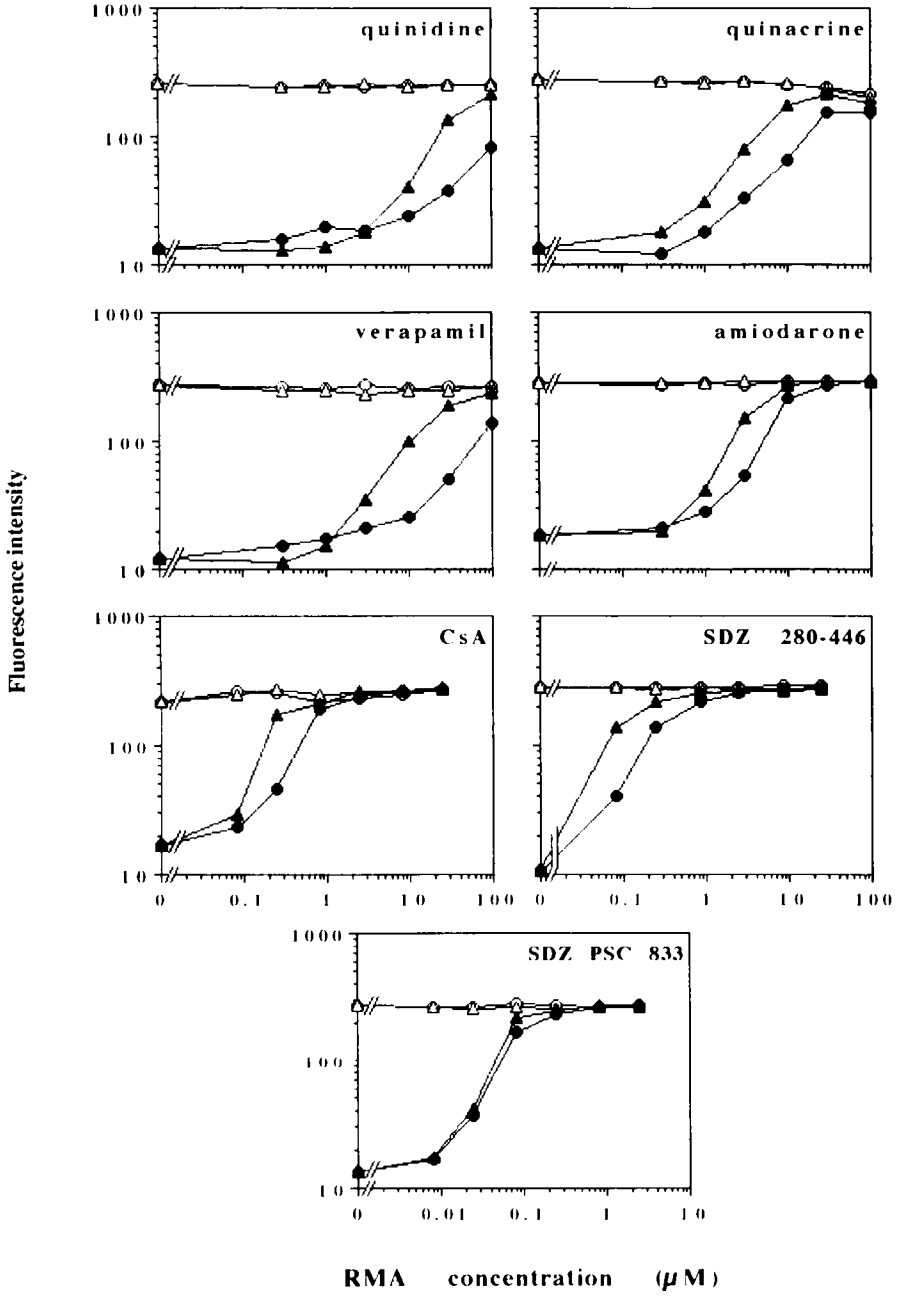

Figure 1. Effect of 'co-RMA' and 'co + post-RMA' treatment conditions on the DAU retention in Par-P388 (open symbols) and MDR-P388 (closed symbols) cells. The fluorescence levels of the cell suspension were measured by flow cytometry and shown on a logarithmic scale (*y*-axes) versus the RMA concentrations (*x*-axes). The fluorescence intensity measured in 'co-RMA' and 'co + post-RMA' treatment conditions was represented by circles and triangles, respectively. The methodology of measurements was identical to ref. 14.

cells were only pulsed with the RMA ('pre-RMA' treatment) for various times, washed in RMA-free medium and then assayed for their capacity to accumulate (and to retain) DAU in the absence of any further RMA addition.

The 'pre-RMA' treatment conditions did not change the DAU accumulation and retention in Par cells (data not shown). In the case of the RMA-pulsed MDR cells, the DAU retention levels were, for each RMA, very similar whether the duration of the 'pre-RMA' treatment was 30 or 1 min only (Table 2). Indeed, such pulse exposures of the MDR cells to 10 μg/ml SDZ PSC 833 (8.2 μM), SDZ 280-446 (8.5 μM) or CsA (8.3 μM) led to levels of DAU retention in MDR cells which were about, respectively, 95, 75 or 25% of the DAU retention levels shown by untreated Par cells. In contrast, pulse exposures of the MDR cells to 100 μM verapamil, for up to 30 min, hardly changed their initial DAU retention capacity, and even 300 μM verapamil could only lead to 20% of the Par cell DAU retention whatever the duration of the pretreatment (data not shown). In view of the very high dosages of verapamil required to obtain marginal effect in

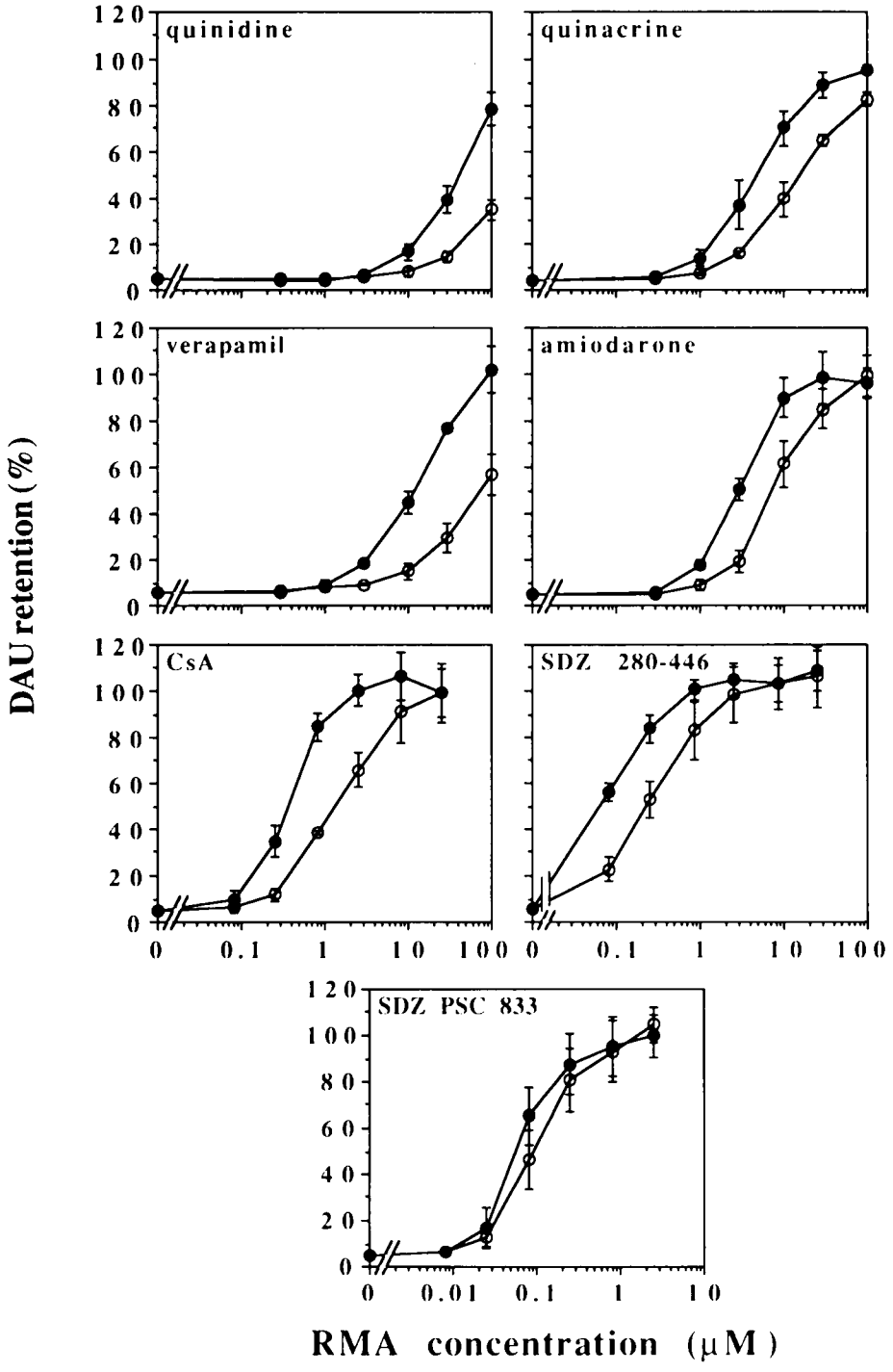

Figure 2. Effect of RMA on the DAU retention in MDR cells under 'co-RMA' (open symbols) and 'co + post-RMA' (closed symbols) treatment conditions. The fluorescence, measured by flow cytometry, was shown as the ratio (%) of the mean fluorescence channel of the MDR cells to that of the Par cells (*y*-axis) versus the RMA concentrations (*x*-axis). The data are expressed as the means (± SD) of three to four independent experiments.

'pre-RMA' conditions, no further experiments were performed with that RMA.

Duration of the inhibition of Pgp function after RMA pulse treatment

Since a 'pre-RMA' treatment could substantially restore the MDR cell capacity to accumulate DAU and to retain it, the duration of that effect was further evaluated. Its persistence was studied after a single 30 min pulse of RMA at a few concentrations: 3, 6 and 10 μg/ml (2.5, 5 and 8.2 μM) for SDZ PSC 833; 10 and 30 μg/ml (8.5 and 25 μM) for SDZ 280-446; and 30 μg/ml (25 μM) for CsA. After such pretreatment, the growth of both types of P388 tumor cells was not significantly affected for the next few days in culture, except for some inhibition of Par cell

Table 1. RMA EC_{50}[a] in 'co-RMA' and 'co + post-RMA' treatment conditions

RMA/treatment	RMA EC_{50} (μM; mean ± SD)	
	'co-RMA'	'co + post-RMA'
Quinidine	>100	42 ± 6
Verapamil	79 ± 20	12 ± 2
Quinacrine	15 ± 3	4.9 ± 1.6
Amiodarone	7.6 ± 2.1	3.0 ± 0.5
CsA	1.4 ± 0.2	0.36 ± 0.05
SDZ 280-446	0.24 ± 0.08	<0.08
SDZ PSC 833	0.079 ± 0.013	0.050 ± 0.007

[a] Concentration of RMA such that the MDR cells recovered 50% of the DAU retention shown by the Par cells. The results are expressed as means ± SD from three independent experiments.

growth after CsA pretreatment. In these pretreatment experiments, the mean DAU retention of MDR cells obtained in the control samples (i.e. in the absence of RMA) reached about 4.5% of the Par cell DAU retention. 'Pre-RMA' treatment conditions did not alter the DAU retention capacity of the Par cells over the next few days in culture (data not shown).

Immediately after their 30 min RMA pulse and their transfer to RMA-free medium, i.e. at the zero time of this assay of Pgp function recovery, the MDR cells displayed a substantial DAU retention level as it reached about 80% after pretreatment with SDZ PSC 833 (2.5–8.2 μM), 60 and 80% after pretreatment with, respectively, 8.5 and 25 μM SDZ 280-446, and 50% after pretreatment with 25 μM CsA (Figure 3).

Table 2. Effect of the duration of a single-pulse 'pre-RMA' treatment on the restoration of the capacity of MDR-P388 cells to accumulate and retain DAU

RMA (10 μg/ml)	Pretreatment duration (min)	DAU retention (%)[a] [mean ± SD (*n*)[b]]
0	—	4.91 ± 1.43 (8)
SDZ PSC 833	1	93.1 ± 18.5 (5)
	3	94.8 ± 13.6 (5)
	5	94.0 ± 12.1 (7)
	30	96.9 ± 12.1 (7)
SDZ 280-446	1	76.0 ± 22.1 (4)
	3	74.6 ± 23.7 (4)
	5	77.1 ± 19.2 (5)
	30	78.1 ± 17.4 (5)
CsA	1	24.7 ± 6.8 (3)
	3	24.5 ± 4.6 (3)
	5	24.4 ± 4.0 (3)
	30	28.5 ± 6.8 (3)

[a]The results are expressed as means ± SD of the DAU retention of the MDR-P388 cells in percent of that of Par-388 cells.
[b]*n*, number of experiments.
10 μg/ml = 8.2 μM for SDZ PSC 833, 8.5 μM for SDZ 280-446 and 8.3 μM for CsA.

With SDZ PSC 833 (2.5–8.2 μM), a 25% recovery of the Pgp function was found after 0.5–6 h in culture, since the MDR cells showed a 60% DAU retention instead of 80% at the zero time. The DAU retention capacity (15–20%), recorded 15–24 h after MDR cell pretreatment, was still substantially higher than the baseline of DAU retention observed with untreated MDR cells. Later on, a Pgp function inhibition could still be detected since, after some 40 h culture in RMA-free medium, the DAU retention remained slightly higher for the RMA pretreated cells than for untreated cells.

With SDZ 280-446, 0.5–6 h culture in RMA-free medium was sufficient to restore about 40% of the Pgp function: the DAU retention levels of the MDR cells decreased from 60 to 35% (at 8.5 μM RMA) and from 80 to 45% (at 25 μM RMA). Some further 40–45% Pgp function could be recovered by the cells cultured for 15–24 h. At that time, the MDR cells pretreated with 25 μM SDZ 280-446 still showed a 10% DAU retention level, i.e. twice as high as the baseline DAU retention level of untreated cells. However, the latter level was reached during the next day of culture in RMA-free medium.

With 25 μM CsA, the level of DAU retention of the MDR cells at zero time only reached 48% of the Par cell level. Within 1 h of culture in CsA-free medium, the DAU retention dropped to 20% of the Par cell level, suggesting a fast and strong (about 60%) recovery of the Pgp function. From 15 h following the CsA pretreatment, no trace of inhibition of the Pgp function of the MDR cells could be detected.

Discussion

MDR cell lines were often characterized by a decreased ACD accumulation;[1,2] a variety of RMA could sensitize them and reverse the MDR phenotype by increasing the intracellular ACD concentration.[2,4–11] Essentially, two types of assays were performed for measuring Pgp function and its inhibition: restoration of ACD sensitivity of cell growth in culture and restoration of the retention of Pgp probes (DAU or others, radiolabeled or fluorescent) in MDR cells.

Assays of the first kind measured the consequences of inhibiting Pgp function for cell growth. They required RMA concentrations which were not inhibitory or toxic by themselves. This was the basis of screening assays which led to the discovery of

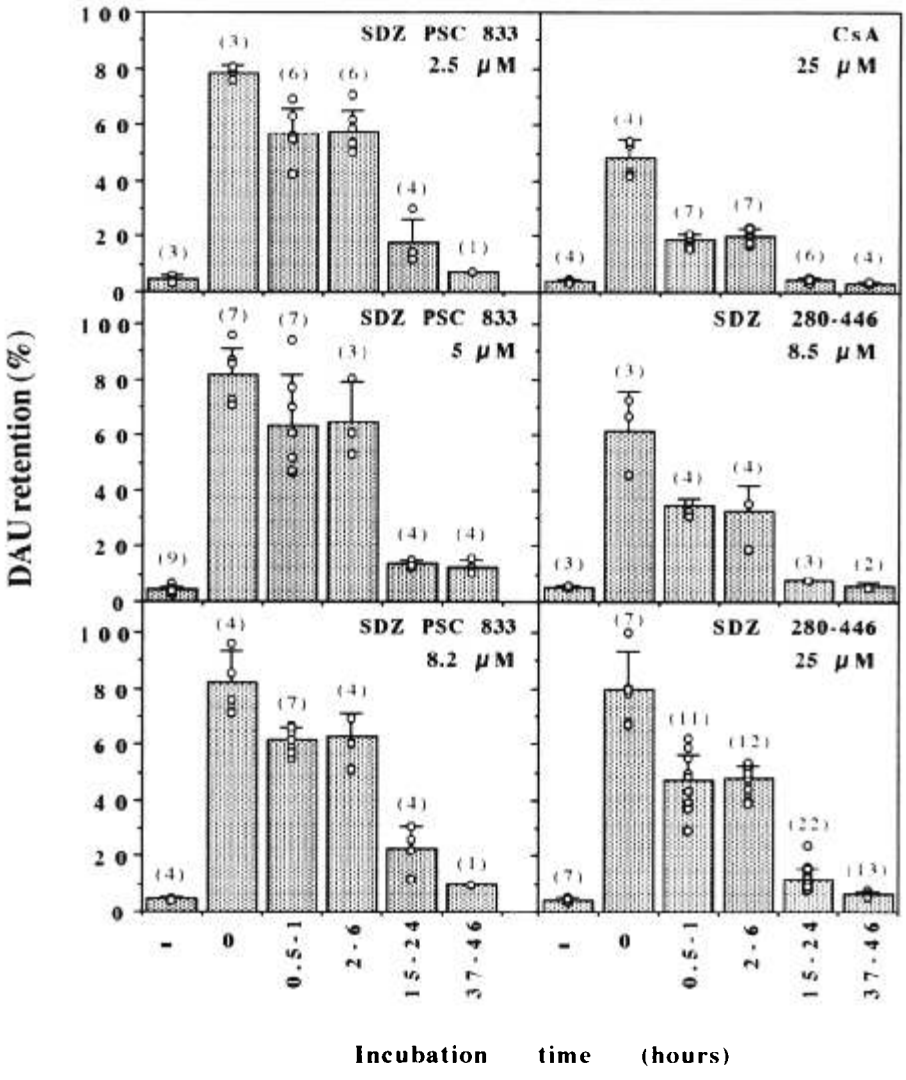

Figure 3. Persistence of the effect of a 'pre-RMA' treatment on the DAU retention in the MDR cells: comparison of SDZ PSC 833, SDZ 280-446 and CsA. The DAU retention levels were measured as mean fluorescence of MDR cells and shown as the ratio (%) of the mean fluorescence channel of the MDR cells to that of the Par cells (*y*-axis) against the duration of the RMA pretreated cell culture in the RMA-free medium (*x*-axis). These data represent the results of several independent experiments, each symbol representing an individual measurement on cells taken after various times in culture. (*n*), number of experiments.

preclinically interesting RMAs. Among these, the non-immunosuppressive cyclosporin derivative SDZ PSC 833 and the semi-synthetic cyclopeptolide SDZ 280-446 restored the sensitivity of various MDR tumor cell lines to ACD-mediated growth inhibition.[15,16] The doses of SDZ PSC 833 and SDZ 280-446 for which a total reversion of the resistance was obtained were *per se* devoid of toxicity on the MDR cell line. To obtain a 10-fold sensitization of MDR cells to DAU, much lower concentrations were required for SDZ PSC 833 (0.045 μM) and SDZ 280-446 (0.068 μM) than for CsA (0.3 μM) or verapamil (3.3 μM). A complete reversion of the DAU resistance could be obtained with 0.8 μM SDZ PSC 833 or 2.5 μM SDZ 280-446 within the range of RMA dosages which did not *per se* cause a substantial inhibition of cell growth, whereas it could not with CsA or verapamil.[15,16] In a 3 day growth inhibition assay on the MDR-P388 cells, the RMA IC_{50}s were the following: 1.1 μM for CsA, 4.9 μM for SDZ PSC 833, 6.1 μM for SDZ 280-446, > 5 μM for amiodarone, > 5 μM for quinacrine, > 30 μM for verapamil and > 90 μM for quinidine.[16,18]

Assays of the second kind essentially measured the pumping/effluxing capacity of the Pgp molecules. Such short-term assays allowed us to probe the nature of the biological mechanism involved in resistance on live, metabolically active cells, regardless of whether the inhibitory compounds were toxic or not for cell growth in long-term culture. Low concentrations of SDZ PSC 833 and SDZ 280-446 were thus shown to increase the DAU retention in MDR-P388 cells, the cyclosporin being about three times more active than the cyclopeptolide in that particular cell/assay combination.[16,18] The IC_{50} data obtained on growing cell cultures could not be simply compared with the EC_{50} values calculated from the cellular tests of DAU retention, since the former assays took a few days whereas the latter were completed within 2 h.

In our previous experiments,[16,18] the MDR cells had been treated with the RMA only during the DAU-loading phase, in order to inhibit the Pgp function while the cells were exposed to DAU. That protocol, now termed the 'co-RMA' treatment condition, was milder than the 'co + post-RMA' treatment protocol also performed in the present studies. All tested RMAs apart from SDZ PSC 833 caused a substantially higher DAU retention when present from the beginning of the DAU uptake phase to the end of the DAU efflux phase, than when present only during the DAU uptake phase. Thus, some RMAs such as verapamil might need to be kept at a high concentration on the MDR cells in order to bring a substantial inhibition of their Pgp function. These results were in agreement with other studies showing that the removal of verapamil led to a rapid loss of ACD from MDR tumor cells.[20,21] In contrast, SDZ PSC 833 gave more similar results when used in 'co-RMA' or 'co + post-RMA' treatments, which indicated that the DAU efflux mechanism was not so readily recovered once inhibited by SDZ PSC 833.

In order to evaluate whether cell-associated RMA would bring a substantial level of inhibition of the Pgp function, the MDR cells were pulse-treated with the RMA ('pre-RMA' treatment) for various times, washed in RMA-free medium, and only then assayed for their capacity to accumulate and retain DAU. A pretreatment with 10 μg/ml SDZ PSC 833 (8.2 μM) or SDZ 280-446 (8.5 μM) could bring substantial degrees of Pgp function inhibition (95 and 76%, respectively) measured right after the MDR cell transfer to RMA-free medium. At variance, 100 μM verapamil for up to 30 min left little inhibition of the Pgp function as soon as the MDR cells were put in RMA-free medium; 300 μM verapamil or 10 μg/ml (8.3 μM) CsA was required to leave a 20–25% DAU retention capacity right after the RMA pulse. In spite of the high concentration of verapamil required to achieve a substantial restoration of DAU retention, the cells were found to stay alive (and be capable of active metabolism, since this is required for DAU efflux). How fast they recovered their full capacity to efflux DAU was not further studied, their initial level of Pgp function inhibition being very low and the rapid reversibility of verapamil-mediated Pgp inhibition being well documented already.[20,21]

For each RMA tested, SDZ PSC 833, SDZ 280-446, CsA or verapamil, similar DAU retention levels in the MDR cells were restored whether the pretreatment period was 1 or 30 min. Exposing the cells to SDZ PSC 833 or SDZ 280-446 for as little as 1 min thus allowed sufficient RMA uptake by the cells, so that their Pgp function was substantially inhibited. Presumably, a longer cell exposure to the RMA did not result in a higher cellular uptake of the RMA, since the inhibition of the Pgp function was not higher. This might indicate that the partition equilibriums between the cell-associated RMA and the medium-soluble RMA were quickly reached when exposing the cells to the RMA. Although presumably RMA concentration-dependent, this equilibrium was definitely more dependent on the nature of the RMA than on the duration of the exposure.

Such a fast establishment of a partition equilibrium between cell-absorbed RMA and medium-soluble RMA was a feature to be considered when trying to interpret the higher inhibition of Pgp function achieved in 'co + post-RMA' conditions compared with 'co-RMA' conditions. This higher level of inhibition should not be due to a cumulative uptake of RMA within cells which are permanently exposed to a same concentration of RMA; rather, it should be due to some loss of RMA by the cells during their incubation in RMA-free medium. Assuming that each RMA has its specific partition coefficient between the cell and the extracellular medium, the more or less rapid loss of RMAs by cells incubated in RMA-free medium would lead to a more or less rapidly established equilibrium between the cell-associated RMAs and the medium-soluble RMAs. RMAs which would have low binding avidity for the cells would quickly re-equilibrate with the medium, as soon as the cells are put in RMA-free medium. Thus, the lower DAU retention, in 'co-RMA' treatment compared with 'co + post-RMA' conditions, would be due to the decrease of the effective (i.e. cell-associated) concentration of RMA during the DAU efflux phase, thus allowing a higher DAU efflux. Therefore, compounds such as verapamil and quinidine probably quickly left the MDR cells or rapidly dissociated from the Pgp molecules so that Pgp function was quickly regained. This interpretation looks compatible with the rapid restoration of DAU or rhodamine-123 efflux functions following verapamil treatment performed in other conditions.[19–21] In the case of CsA, and possibly other RMAs, their higher cellular retention and/or Pgp binding avidity would be sufficient for keeping, after their removal, a substantial level of Pgp inhibition in living cells (see below).

In order to evaluate how long CsA, SDZ PSC 833, and SDZ 280-446 were able to inhibit the Pgp function, the MDR cells were exposed to a pulse of RMA, cultured for different durations in RMA-free medium and only then tested for their ability to retain DAU

('pre-RMA' treatment). With CsA, the MDR cells recovered their Pgp function early after the RMA pre-treatment and a complete recovery was observed within a day. With SDZ 280-446, a rapid decrease of the DAU retention by MDR cells was observed at 0.5–1 h after RMA pre-treatment, and a slow but total recovery of the Pgp function was obtained after 24 h of cell culture. The Pgp inhibition also decreased after SDZ PSC 833 pretreatment, though not so fast as with SDZ 280-446 and CsA. Even after 2 days in culture in SDZ PSC 833-free medium, the baseline DAU retention typical of the MDR cells was not yet completely recovered. This prolonged effect on drug retention was thus consistent with the data obtained in the 'co-RMA' and 'co + post-RMA' treatment assays indicating that, among the tested RMAs, SDZ PSC 833 might inhibit Pgp function in a less reversible fashion. However, a few cycles of cell proliferation occurred meanwhile, so that the kinetics of late Pgp function recovery might not be directly correlated to a dissociation of the RMA from the Pgp.

A whole panel of hypotheses may be proposed to account for the differences of persistence of the Pgp inhibition after exposure to a simple pulse of RMA (pre-RMA treatment). Since photolabeling of Pgp by substrate analogs can be inhibited by several RMAs, such as verapamil,[23] quinidine[24] and CsA,[12] as well as SDZ PSC 833 and SDZ 280-446 (B. Ryffel, personal communication), the simplest hypothesis would be that SDZ PSC 833 displayed a particularly high affinity for Pgp, e.g. with a low K_d so that the pump inhibition might be poorly reversible. However, this is only one of several alternative hypotheses.

In general terms, one may assume that different RMAs may show qualitative and/or quantitative differences in their binding to Pgp molecules (different binding sites on the Pgp molecules and/or different affinities). Some RMAs might bind to the same sites as ACDs which are transported by the Pgp; if they were too slow pump substrates, such RMAs might thus give a competitive inhibition. Both CsA and FK-506 might belong to this first category, as they were shown to be transported by Pgp.[22] With time after pulsing the MDR cells with CsA, its effective concentration within the cell plasma membrane would decrease, releasing the cells from RMA-mediated Pgp inhibition. By extension, it might be suggested that SDZ PSC 833 and SDZ 280-446 would quit the plasma membrane much more slowly, those RMAs being very slow or sticky Pgp substrates and clogging the pump. However, a different hypothesis would be that the latter two RMAs might give another form of non-competitive inhibition, e.g. by destroying the functional organization of Pgp as a pump. Such highly hydrophobic RMAs might indeed work *within* the plasma membrane, causing actual denaturation of individual Pgp molecules and/or disrupting their organization as functional pumping units. Recent data on the mapping of Pgp epitopes expressed on the surface of cells would suggest this is a viable hypothesis (B. Jachez *et al.*, in preparation).

Recently, a SDZ PSC 833-resistant mechanism was found to co-exist with Pgp, for DAU efflux in a particular MDR-CEM cell subline,[25] leaving open the possibility that MDR-P388 cell exposure to a pulse of SDZ PSC 833 might lead to a transient emergence of such a mechanism of DAU efflux. Whether it were mediated by a non-Pgp molecule or by a metabolically altered Pgp molecule are different alternatives. One should indeed consider that the different levels of resistance-modifying activity shown by the different RMAs studied here might reflect different modifications of cell metabolism, causing a variety of different alterations of the Pgp molecules, such as their phosphorylated status, membrane recycling, turn-over, biosynthesis rate, etc. Fully understanding the mechanism of action of RMAs would really represent a whole panel of hypotheses to be evaluated for each RMA. Unfortunately, even RMA binding to Pgp in a plasma membrane extract would only suggest, not prove, it would also occur within intact cells; even if the latter were shown, it would still not be a sufficient demonstration that this is the mechanism by which that RMA inhibits Pgp function. Past experience with CsA generated so many different receptors and mechanisms of action for its immunosuppressive properties, that we feel the time is not yet ripe for similar speculations with SDZ PSC 833 about its resistance-modifying mechanisms.

Conclusion

The cyclosporin D derivative SDZ PSC 833 and the semi-synthetic cyclopeptolide SDZ 280-446 could thus induce a stronger and more stable inhibition of Pgp function of our MDR-P388 cells than a few other tested RMAs, such as verapamil, whose continuous presence seems to be required for maintaining a substantial inhibition of Pgp function. Such marked differences of persistence of the inhibition of Pgp function must be taken into account when designing preclinical *in vivo* animal and clinical drug schedules for the chemotherapy of MDR tumors.

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