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Modulation of anthracycline accumulation and metabolism in rat hepatocytes in culture by three revertants of multidrug resistance

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Abstract The aim of this study was to compare the action of three multidrug resistance (MDR) modulators, cyclosporine A, S 9788, and verapamil, on the efflux of two anthracyclines, doxorubicin and daunorubicin, and of daunorubicinol, the C-13 alcohol metabolite of daunorubicin. Rat-hepatocyte primary cultures have been used as a model of P-glycoprotein (Pgp) expression. This model allows the study of MDR at different levels of Pgp expression, which increases in parallel with the time in culture; furthermore, the hepatocytes are capable of metabolizing drugs, which enables the determination of the role of Pgp on metabolite efflux. All modulators tested were incubated for 6 h at concentrations of 1, 5, and 15 μ*M* with doxorubicin (0.5 μ*M*) and at 1 and 15 μ*M* with daunorubicin (0.5 μ*M*) on hepatocytes grown for 4 and 48 h in culture. Daunorubicinol (0.5 μ*M*) was tested with modulators at 48 h of culture. In fresh hepatocytes, the three MDR modulators did not induce an increase in the intracellular retention of anthracycline as compared with controls (no MDR modulator). At 48 h of culture, the three test drugs increased doxorubicin intracellular accumulation. In contrast, daunorubicin retention was not modified, but that of its metabolites was increased. Within the concentration range tested, cyclosporine was the most potent modulator without dose-dependent activity. The activity rank order was cyclosporine > S 9788 > verapamil. Cyclosporine and S 9788 were as active in coincubation as in preincubation with anthracyclines. Verapamil had no action when incubated before the addition of anthracyclines. Cyclosporine and S 9788 had an effect on the intracellular retention of daunorubicinol used alone whereas verapamil did not. The action of cyclosporine and S 9788 on the retention of daunorubicinol proves that at least a part of the efflux of C-13 alcohol metabolites of anthracyclines is mediated by Pgp. This study shows that S 9788, cyclosporine, and verapamil are MDR modulators in hepatocytes with high-level Pgp expression. This study also demonstrates that hepatocytes are a potent tool for the study of the action of new MDR modulators on cytostatic drugs as well as on their metabolites.

Key words MDR · Cyclosporine · S 9788 · Verapamil · Anthracyclines · Hepatocytes in vivo

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Introduction

Resistance to anticancer drugs is a major hindrance to the treatment of numerous cancers. One of the best-elucidated mechanisms is the cross-resistance to natural products known as multidrug resistance (MDR). This phenomenon concerns anthracyclines, vinca alkaloids, epipodophyllotoxins, and other intercalators and is due to the overexpression of a membrane high-molecular-weight glycoprotein, P-glycoprotein, which is capable of actively extruding drugs from resistant cells [4, 18]. Several compounds belonging to various pharmacological families can reverse the MDR phenotype in vitro [7]. Among them are calcium channel blockers such as verapamil, immunosuppressants such as cyclosporine A, and calmodulin antagonists such as the phenothiazines. S 9788 is a new triazinoaminopiperidine derivative that originated from Laboratoires Servier (Suresnes, France) and is presently in preclinical and early clinical evaluation [5, 19, 20].

The MDR-reversing activity of S 9788 is characterized by a restoration of anticancer drug accumulation in tumoral cells [10] through P-glycoprotein inhibition. We wanted to know whether this compound was also capable of increasing drug accumulation in normal cells expressing

P-glycoprotein to a high level and whether it was the parent drug and/or the metabolite(s) that accumulated in the cells with high biotransformation potential. We chose a model of rat hepatocytes in primary culture, which overexpress P-glycoprotein [6, 14] and have been extensively studied in our laboratory with respect to anthracycline accumulation, metabolism, and toxicity [13]. Two anthracyclines, doxorubicin and daunorubicin, were studied, as was daunorubicinol, the C-13 alcohol metabolite of daunorubicin. The modulating effect of S 9788 was compared with that of two classic agents, cyclosporine A and verapamil.

Materials and methods

Commercial forms of doxorubicin (from Laboratoire Farmitalia-Carlo Erba) and of daunorubicin (from Laboratoire Roger-Bellon) were used. Daunorubicinol was a gift of Laboratoire Roger-Bellon. S 9788 was freely provided by Laboratoire Servier. Commercial forms of verapamil (from Laboratoire Biosedra) and cyclosporine A (from Laboratoire Sandoz) were used.

Hepatocytes were obtained by enzyme digestion with collagenase from the livers of adult male Sprague-Dawley rats weighing 180–200 g using the technique of Guguen et al. [9]. Briefly, the livers were perfused in situ with Ca^{2+}-free HEPES buffer (pH 7.65) at 37 °C, then with a 0.025% collagenase solution in HEPES buffer. The cell suspension was filtered and washed three times by centrifugation. The cell viability was about 80%–90%. The hepatocytes were seeded at a density of 2.5×10^6 cells/25-cm^2 flask (Nunclon) containing 4 ml culture medium. This medium was a mixture of 75% minimal Eagle's medium and 25% medium 199 and was supplemented with 200 µg bovine serum albumin/ml, 10 µg bovine insulin/ml, and 10% fetal calf serum. At 4 h after seeding, the cells were attached to the plastic dish and the medium was changed. The new medium contained in addition 70 µ*M* hydrocortisone hemisuccinate and was renewed each day.

In a first series of experiments, cells were simultaneously incubated for 6 h with doxorubicin, daunorubicin, and a modulator at either 4 h or 48 h after seeding. The concentration of the anthracycline was set at 0.5 µ*M*. Daunorubicinol (0.5 µ*M*) was tested with modulators at 48 h of culture. Three concentrations of the modulators, 1, 5, and 15 µ*M*, were tested with doxorubicin and daunorubicinol and two were tested with daunorubicin: 1 and 15 µ*M*. In a second series of experiments, we evaluated the schedule dependency of the modulator action; anthracyclines were incubated at a concentration of 0.5 µ*M* for 6 h, and the modulators were incubated at 5 µ*M* either before (for 6 or 1 h) or during (for 6 or 1 h) incubation with the anthracyclines. After incubation, the culture medium was withdrawn and 100 µl was kept for the measurement of lactate dehydrogenase activity; the cell monolayers were washed with saline and the cells were recovered, pelleted, and frozen until anthracycline assay.

The cell toxicity of anthracyclines with and without modulating agents was checked by phase-contrast microscopy at the end of the incubations. The presence of dense, refringent granules and of cell-shape alterations was the reflection of cell toxicity. A quantitative determination was done by the measurement of lactate dehydrogenase (LDH) activity in the cell-culture medium using an LDH-UV kit system purchased from Boehringer-Mannheim.

Anthracyclines were extracted from cells by the technique described by Baurain et al. [1]. Briefly, 1 ml 0.5 *M* phosphate buffer (pH 7) was added to cell pellets; after addition of the internal standard, 100 µl 0.5 *M* sodium tetraborate (pH 9.8) was added for alkalinization, after which 9 ml chloroform/methanol at 4/1 (v/v) was added. The organic layer was recovered, evaporated to dryness, and reconstituted in 50 µl of the solvent used for high-performance liquid chromatography. High-performance liquid chromatography was performed on Lichrocart RP18 cartridges (Merck) combined with a Lichrocart Lichrosorb RP18 microcolumn (Merck). The solvent was a mixture of acetonitrile and 0.05 *M* ammonium formate buffer (pH 4.0) [11] at the following proportions: 5 min isocratic conditions with 20% acetonitrile, 5 min of a gradient leading from 20% to 40% acetonitrile, and 5 min isocratic conditions with 40% acetonitrile. Detection of anthracyclines and metabolites was achieved with a Jasco FP 210 spectrofluorometer, with excitation and emission wavelengths set at 467 and 550 nm, respectively. The results were expressed as the amount (in picomoles) of anthracycline accumulated in 2.5×10^6 cells. All data represent mean values for two experiments performed in duplicate. We also calculated the ratios of the amounts of anthracycline obtained with and without modulator; these ratios are the enhancement factors provided by the modulators.

For Western-blot evaluation of P-glycoprotein, the cells were recovered by gentle scraping in 40 m*M* TRIS buffer (pH 8) and centrifuged (3 min at 400 *g*). The cells originating from several flasks could be pooled so as to obtain a pellet containing 10–12 million cells. The cell pellets were kept at –80 °C. The technique of plasma membrane preparation has been described in detail elsewhere [14]. Proteins of whole-cell lysates were separated by gel electrophoresis on a 6.5% polyacrylamide gel. Protein loading comprised 100 µg in each well. Protein separation was obtained under a constant voltage of 90 V during 16 h at 4 °C with a 25-m*M* TRIS buffer (pH 8.3) containing 192 m*M* glycine and 0.1% sodium dodecyl sulfate (SDS). Proteins were then transfered onto a nitrocellulose membrane under a constant voltage of 150 V during 90 min at 4 °C using a 25-m*M* TRIS buffer (pH 8.3) containing 192 m*M* glycine and 20% methanol. The nitrocellulose membrane was incubated first for 3 h in a suspension of powdered milk to saturate aspecific binding sites, then for 16 h at 4 °C with C219 antibody diluted at 1/100, and then, after five rinses, with a rabbit anti-mouse IgG antibody labeled with alkaline phosphatase (Diagnostics Pasteur) diluted at 1/200 in the saturation solution. Proteins were revealed in a 0.5-mg/ml solution of BCIP (5 bromo-4-chloro-3-indolyl phosphate, Sigma) for 5–15 min. P-glycoprotein appeared on nitrocellulose sheets as a blue spot. A reference P-glycoprotein-containing extract from rat hepatoma tissue culture (HTC) cells was run simultaneously. These cells, rendered resistant to doxorubicin, were a gift of Dr. J. Robert [2].

Results

On Western blotting, one protein band reacted with the C219 antibody at a molecular weight of 150–170 kDa, similar to that of the protein labeled by this antibody in doxorubicin-resistant HTC cells. Figure 1 presents the evolution of P-glycoprotein levels during hepatocyte cul-

Fig. 1 Immunoblot analysis of Pgp expression in hepatocytes in culture as a function of the time in culture. Samples at 100 µg protein/lane were subjected to 6.5% SDS-polyacrylamide gel electrophoresis and immunoblotting with antibody C219 (*H4* Hepatocytes cultured for 4 h, *H48* hepatocytes cultured for 48 h, *T* resistant rat HTC cells, i.e., control)

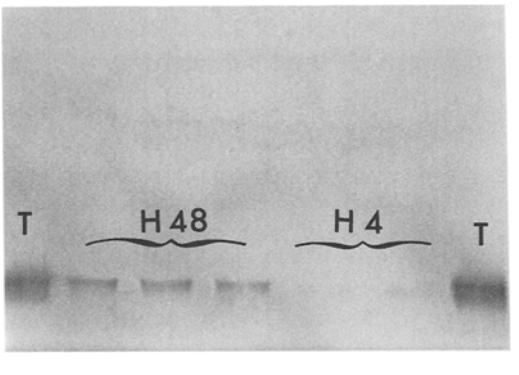

Table 1 Effect of modulators on anthracycline accumulation as evaluated after 6 h of incubation in hepatocytes grown for 4 h. The exposure dose was 0.5 μ*M* for both anthracyclines. Results are expressed as the amount (in pmol) of anthracyclines accumulated in the cell layers (2.5×10^6 cells seeded in 25-cm^2 flasks). Data represent mean values ± SEM for two experiments performed in duplicate. (*e.f.* Enhancement factors provided by the modulators, i.e., the ratio of anthracycline accumulation with modulator to that without modulator, *Doxo* doxorubicin, *Dauno* daunorubicin, *Daunol* daunorubicinol, *Sum* total anthracycline intracellular contents, i.e., daunorubicin + daunorubicinol)

		Doxorubicin		Daunorubicin					
		Doxo	e.f.	Dauno	e.f.	Daunol	e.f.	Sum	e.f.
No modulator		692±58	1.00	434±21	1.00	607±16	1.00	1041±24	1.00
Cyclosporine A	1 μ*M*	707±52	1.02	466±21	1.07	724±34	1.19	1195±52	1.14
	5 μ*M*	712±68	1.02						
	15 μ*M*	678±56	0.97	464±65	1.06	718±40	1.18	1182±102	1.13
S 9788	1 μ*M*	649±33	0.93	384±12	0.89	607±13	1.00	991±24	0.95
	5 μ*M*	701±19	1.02						
	15 μ*M*	746±51	1.08	398±67	0.91	686±13	1.13	1084±78	1.04
Verapamil	1 μ*M*	735±51	1.09	413±15	0.95	647±31	1.07	1060±46	1.02
	5 μ*M*	645±32	0.94						
	15 μ*M*	672±61	0.96	448±18	1.03	696±25	1.15	1144±43	1.10

Table 2 Effect of modulators on anthracycline accumulation as evaluated after 6 h of incubation in hepatocytes grown for 48 h. The exposure dose was 0.5 μ*M* for both anthracyclines. Results are expressed as the amount (in pmol) of anthracyclines accumulated in the cell layers (2.5×10^6 cells seeded in 25-cm^2 flasks). Data represent mean values ± SEM for two experiments performed in duplicate. (*e.f.* Enhancement factors provided by the modulators, *Doxo* doxorubicin, *Dauno* daunorubicin, *Daunol* daunorubicinol, *Sum* total anthracycline intracellular contents, i.e., daunorubicin + daunorubicinol)

		Doxorubicin		Daunorubicin					
		Doxo	e.f.	Dauno	e.f.	Daunol	e.f.	Sum	e.f.
No modulator		236±37	1.00	325±15	1.00	145±8	1.00	470±24	1.00
Cyclosporine A	1 μ*M*	468±54	2.00	363±28	1.12	300±11	2.06	663±38	1.41
	5 μ*M*	606±142	2.50						
	15 μ*M*	568±116	2.40	355±42	1.09	342±27	2.35	697±112	1.47
S 9788	1 μ*M*	309±33	1.34	284±39	0.87	214±38	1.46	498±77	1.05
	5 μ*M*	446±90	1.90						
	15 μ*M*	575±117	2.40	227±27	0.70	433±52	2.97	661±60	1.40
Verapamil	1 μ*M*	309±33	1.34	307±32	0.94	164±12	1.13	471±42	1.00
	5 μ*M*	320±64	1.33						
	15 μ*M*	521±107	2.16	277±23	0.85	279±21	1.92	556±41	1.18

ture. The level of P-glycoprotein was very low at the beginning of hepatocyte culture (H4) and increased during the period ranging from 4 to 48 h postseeding. Incubation of hepatocytes with anthracyclines (0.5 μ*M*) in the presence or absence of modulators (1–15 μ*M*) did not induce cell toxicity at the end of the incubation that was detectable either by visual observation or by the LDH levels in culture media.

There was a 2- to 3-fold lower accumulation of anthracyclines in the cells when they were exposed for 6 h to the drugs after 48 h of culture than when then they were exposed for the same period after 4 h of culture (Tables 1, 2). Whereas no metabolite of doxorubicin was detectable in hepatocytes, daunorubicin was extensively transformed into daunorubicinol, which represented about 60% of the total fluorescence recovered in cells at the end of incubations with hepatocytes seeded for 4 h; this proportion was much lower after incubation with hepatocytes seeded for 48 h (about 31%). In both cases, higher proportions of daunorubicinol were consistently formed in culture medium at the end of the incubations.

The modulators had no effect on anthracycline accumulation as evaluated at 4 h after seeding, and the transformation of daunorubicin into daunorubicinol was not modified by the presence of the modulators in these cells (Table 1). In contrast, the three modulators had a definite effect on anthracycline accumulation when incubations were performed after 48 h of hepatocyte culture (Table 2). Cyclosporine A was the most effective agent; at a concentration of 1 μ*M*, this agent was capable of increasing doxorubicin accumulation by a factor of 2, whereas verapamil and S 9788 produced only a 1.3-fold enhancement of doxorubicin accumulation. At a concentration of 5 μ*M*, S 9788 was significantly superior to verapamil in enhancing doxorubicin accumulation, and all three modulators showed equivalent activity at 15 μ*M*, giving the same enhancement

Table 3 Effect of modulators on daunorubicinol accumulation as evaluated after 6 h of incubation in hepatocytes grown for 48 h. The exposure dose of daunorubicinol used alone was 0.5 μ*M*. Results are expressed as the amount (in pmol) of daunorubicinol accumulated in the cell layers (2.5×10^6 cells seeded in 25-cm^2 flasks). Data represent mean values ± SEM for two experiments performed in duplicate. (*e.f.* Enhancement factors provided by the modulators, *Daunol* daunorubicinol)

		Daunol	e.f.
No modulator		165±5	1.00
Cyclosporine A	1 μ*M*	312±6	1.87
	5 μ*M*	317±10	1.89
	15 μ*M*	332±21	1.99
S 9788	1 μ*M*	191±6	1.14
	5 μ*M*	264±9	1.58
	15 μ*M*	300±14	1.79
Verapamil	1 μ*M*	153±17	0.90
	5 μ*M*	159±14	0.94
	15 μ*M*	183±28	1.08

factor. It should be emphasized that cyclosporine A was as potent at the low concentration as at the high concentration. At 48 h of culture, the doxorubicin accumulation more or less reached the level found at day 0 under the effect of cyclosporine at 5 and 15 μ*M* and S 9788 at 15 μ*M* (Tables 1, 2).

Similar effects were obtained with daunorubicin with the original finding that the proportion of daunorubicinol was increased in the presence of the modulators and represented most of the accumulated species in these conditions (Table 2). Accumulation of daunorubicin itself was only slightly increased, particularly under the action of cyclosporine (Table 2). With any of the three modulators tested, the total intracellular daunorubicin content obtained at day 0 was not obtained at day 2. Cyclosporine was active in enhancing the retention of daunorubicinol used alone, which increased by a factor of 2 in the concentration range tested. S 9788 had a dose-dependent effect, as it also did for doxorubicin. Verapamil had no effect (Table 3).

In modifying the schedule of administration of the modulator, we observed that cyclosporine A and S 9788 retained their efficiency when they were added to the culture medium 6 or 1 h before the anthracycline and withdrawn during the incubation with the anthracyclines; in contrast, verapamil had an effect only when used in coincubation with anthracyclines (Table 4). It is also remarkable that all three modulators were as efficient when they were added to the culture medium during the last hour as when they were added during the 6 h of anthracycline incubation.

Discussion

The present study was designed to evaluate the enhancement of anthracycline accumulation in hepatocytes induced by the presence of MDR modulators. These compounds have presently entered clinical trials with the aim of reversing the resistance phenotype presented by numerous tumors. However, the effects that these compounds may have on normal tissues are difficult to evaluate, and there is a need for continuous preclinical evaluation of these side effects. The model of rat hepatocytes in tissue culture may constitute a means of accomplishing this evaluation, since it is known that normal hepatocytes in situ express P-glycoprotein to an appreciable level and can therefore accumulate higher amounts of anticancer drugs in the presence of MDR modulators.

In hepatocytes seeded for 4 h, a greater amount of anthracyclines could be accumulated than in hepatocytes seeded for 48 h, and no difference in anthracycline accumulation was induced by the modulators tested. This

Table 4 Effect of the schedule of incubation with the modulators on the enhancement of anthracycline accumulation. Results are presented as enhancement factors, i.e., the ratio of anthracycline accumulation with modulator to that without modulator. Data represent mean values ± SEM for two experiments performed in duplicate. The modulator dose was 5 μ*M* and the anthracycline exposure dose was 0.5 μ*M*. (*Cyclo A* Cyclosporine A, *Dauno* daunorubicin, *Daunol* daunorubicinol, *Sum* total anthracycline intracellular contents, i.e., daunorubicin + daunorubicinol)

			Daunorubicin		
		Doxorubicin	Dauno	Daunol	Sum
No modulator		1.00	1.00	1.00	1.00
Modulators maintained for 6 h before anthracycline incubation	Cyclo A	1.68±0.10	1.46±0.07	3.35±0.25	1.87±0.07
	S 9788	1.26±0.10	1.12±0.06	1.90±0.12	1.27±0.04
	Verapamil	0.98±0.07	0.98±0.09	1.12±0.17	1.02±0.11
Modulators maintained for 1 h before anthracycline incubation	Cyclo A	1.60±0.08	1.34±0.08	3.12±0.07	1.72±0.09
	S 9788	1.67±0.07	1.00±0.07	1.92±0.34	1.22±0.16
	Verapamil	1.01±0.11	0.86±0.02	0.97±0.18	0.90±0.05
Modulators maintained during the 6 h of anthracycline incubation	Cyclo A	1.68±0.11	1.26±0.07	3.56±0.32	1.75±0.05
	S 9788	1.56±0.07	1.15±0.03	2.24±0.16	1.38±0.04
	Verapamil	1.26±0.14	1.06±0.08	1.53±0.10	1.16±0.08
Modulators maintained during the last hour of anthracycline incubation	Cyclo A	1.29±0.16	1.44±0.11	2.44±0.33	1.64±0.14
	S 9788	1.36±0.14	1.25±0.03	2.04±0.16	1.41±0.04
	Verapamil	1.20±0.07	1.26±0.11	2.00±0.42	1.40±0.12

observation can be attributed to the very low expression of P-glycoprotein observed at the beginning of hepatocyte cultures [6, 14]; without their P-glycoprotein target, the modulators cannot influence anthracycline accumulation. After 48 h of culture, however, there is a high level of P-glycoprotein expression accompanied by an important reduction in anthracycline accumulation that can be restored by MDR modulators. It has been shown in tumor cells by Spoelstra et al. [24] that anthracycline efflux is the sum of a passive diffusion and an active efflux. In cells expressing low levels of P-glycoprotein (SW116, K38, KB 8.5), the passive diffusion accounts for about 75% of the total efflux, whereas in cell expressing high levels of P-glycoprotein (Colo 320, 2780AD), the proportion of the passive diffusion becomes negligible and accounts for less than 10% of the total efflux of drug from the cells. Although they are different in nature and origin, it is likely that rat hepatocytes in culture behave similarly, the passive diffusion being predominant at 4 h after seeding and the P-glycoprotein-mediated efflux becoming more important after 48 h of culture and coexisting with passive diffusion.

Doxorubicin was not metabolized by rat hepatocytes, whereas daunorubicin was extensively metabolized into its 13-dihydro derivative, daunorubicinol. This finding is in agreement with results previously reported elsewhere [13]. All anthracyclines can be reduced by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytosolic aldoketoreductase, but this enzymatic system presents species and substrate specificity: it is more active in human tissues than in rat tissues, and anthracyclines having a methyl group in the C14 position (daunorubicin, idarubicin) are more extensively metabolized than those having a hydroxymethyl group in this position (doxorubicin, epirubicin) [13, 21]. It is remarkable that most of the effect of MDR modulators on daunorubicin accumulation actually involved its metabolite; the concentration of daunorubicin itself was only slightly increased in the presence of any of the three modulators, whereas the concentration of daunorubicinol was substantially increased, thus providing the main contribution to the global effect observed. This phenomenon is probably due to the better availability of daunorubicin to the aldoketoreductase system allowed by inhibition of its efflux. If leukemic cells behave similarly to hepatocytes in this respect, this could protect the cells from the expected increase in cytotoxicity and prevent any efficacy of the P-glycoprotein inhibition provided by MDR modulators, because daunorubicinol is much less active than daunorubicin [12, 22] and because the aldoketoreductase system is ubiquitous and present in myeloid leukemic cells [25]. In other studies concerning the inhibition of daunorubicin efflux by MDR modulators, no attempt was made to distinguish between the unchanged compound and its 13-dihydroderivative [3, 8].

The action of cyclosporine and S 9788 on the retention of daunorubicinol proves that at least a part of the efflux of the C-13 alcohol metabolites of anthracyclines from the cells is mediated by P-glycoprotein as also observed for the parent drugs. Cyclosporine A was the most active agent at a concentration of 1 μM; its effect did not increase further when its concentration was raised to 5 and then 15 μM. S 9788 had the same effect as cyclosporine A at 5 μM and was more effective than verapamil at this concentration. Finally, all three compounds had the same potency at 15 μM. Cyclosporine A has been recognized as being more potent than verapamil in tumor cells [10, 16, 17, 23]. It has been shown that cyclosporine A has a higher affinity for P-glycoprotein than does verapamil [15]. The commercial form of cyclosporine used contains the excipient cremophor, which has MDR-revertant properties [8, 26]. The effect of the latter may increase the potency of cyclosporine. It should be emphasized that cyclosporine A has a prolonged action on anthracycline accumulation, since preincubation with it has the same effect as coincubation. In contrast, verapamil presents no such property and must be present with the anthracycline to modulate its intracellular accumulation. This phenomenon could be related to a higher degree of binding of this drug with its target on P-glycoprotein; it could also be due to the existence of other targets, possibly intracellular, from which cyclosporine A cannot be simply removed by washing the monolayer. The existence of such intracellular targets for some modulators has been postulated in a recent study [10]; it must be added that S 9788 behaved mostly like cyclosporine A in our study as well as in the study of Huet et al. [10].

The present study shows that cyclosporine, S 9788, and verapamil are MDR modulators in hepatocytes expressing P-glycoprotein at a high level. The tested concentrations of cyclosporine A and verapamil are supratherapeutic. S 9788 is now in early clinical development and the peak concentration is about 2 μM. This study also demonstrates that rat hepatocyte culture is an interesting model of P-glycoprotein expression by normal cells and allows the effect of modulators on both cytotoxic drugs and their metabolites to be studied.

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