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SDZ PSC 833 and SDZ 280-446 are the most active of various resistance-modifying agents in restoring rhodamine-123 retention within multidrug resistant P388 cells

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Multidrug resistance (MDR) of tumor cells may result from overexpression of P-glycoprotein (Pgp) but may be down-modulated by resistance-modifying agents (RMAs). The cyclosporin SDZ PSC 833 and the cyclopeptolide SDZ 280-446 were found to be the strongest RMAs known to date for restoring the sensitivity of MDR cells to anticancer drugs, as well as for restoring their retention of daunomycin, a fluorescent anthracycline. Using rhodamine-123 (Rhod-123), another fluorescent probe of Pgp function which also differentiates sensitive and MDR cells, several RMAs were compared for their capacity to inhibit Pgp function. At variance with the data obtained with the daunomycin probe, a series of RMAs did not detectably restore Rhod-123 retention by the MDR cells. With the remaining RMAs, achieving the same levels of Rhod-123 retention required 3 times lower RMA concentrations when the RMA was added to the MDR cells for both the initial uptake and the efflux of Rhod-123 rather than for its uptake only. Nevertheless, the data emphasized the large superiority of SDZ PSC 833 and SDZ 280-446 over all other RMAs.

Key words: Flow cytometry, fluorescence microscopy, multidrug resistance, P388 tumor cells, resistance-modifying agent, rhodamine-123.

Introduction

Multidrug resistance (MDR) is one of the most frequent causes of failure of combined chemotherapies. Tumors often display such character through a common mechanism by which they overexpress a particular transmembrane protein called P-glycoprotein (Pgp).[1] Pgp molecules have been shown to rapidly expel, in an ATP-dependent way, various structurally unrelated anticancer drugs (ACDs) out of the cells as soon as they passively enter them.

This Pgp pump-escaping mechanism leads to the decrease of intracellular concentrations of ACDs below their active thresholds. Pgp could either act as a transmembrane energy-consuming pump or be organized as a track for ACDs in a flippase model.[2] As one of the obvious therapeutic goals was to block the effluxing function of the pump, various molecules were identified as restoring ACD sensitivity or at least as decreasing the Pgp-mediated resistance of MDR cells.[1,3,4] Such chemosensitizers or resistance-modifying (RM) agents (RMAs) were most often unrelated to each other or to ACDs, though they shared lipid solubility at physiological pH, cationic charge and molar refractivity.[5] Early reports showed that calcium channel blockers (e.g. verapamil), antimalarial drugs (e.g. quinacrine), anti-arrhythmic compounds (amiodarone and quinidine) and immunosuppressive (cyclosporin A and FK-506) agents were able to sensitize tumor cell lines whose MDR was Pgp-dependent.[6–8]

Recently, SDZ PSC 833, a non-immunosuppressive cyclosporin analog, and SDZ 280-446, a semisynthetic cyclopeptolide, were selected for their large RM activity. They were shown to be, both *in vitro* and *in vivo*, the most potent RMAs known to date.[9–11] By fluorescence methods, their ability to restore intracellular drug retention was clearly shown using daunomycin, a natural fluorescent anthracycline which shows preferential nuclear retention.[11,12] With MDR murine monocytic leukemia

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P388 cells (MDR-P388) and their parental Pgp-devoid cells (Par-P388), we have compared 14 potential RMA molecules for their ability to restore the retention of rhodamine-123 (Rhod-123). This fluorescent dye has another intracellular retention target as it specifically labels mitochondria[13,14] while also being a Pgp substrate.[15,16]

Materials and methods

Cell lines

Par-P388 and MDR-P388 murine monocytic leukemia cells were obtained from Dr M Grandi (Farmitalia, C Erba Research Center, Milano, Italy). While Par-P388 cells were fully sensitive to all tested ACDs, cells of the MDR-P388 subline[17] showed a relative resistance of about 200-fold to a variety of ACDs (doxorubicin, etoposide, daunomycin, vincristine and colchicine) in comparison with Par-P388 cells.

Both cell lines were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM pyruvate, 100 IU/ml streptomycin–penicillin, 5×10^{-5} M β-mercaptoethanol, 1% of non-essential amino acid solution 100X, 10 mM HEPES (all from Gibco) and finally complemented with 10% heat-inactivated fetal calf serum (J BIO SA, Les Ulis, France).

The medium for MDR-P388 cells was further supplemented with 300 ng/ml doxorubicin in order to keep their MDR properties stable. This drug was removed just before experimental use.

Rhod-123

Rhod-123 (Eastman Kodak Co, Rochester, NY, USA) was prepared as a stock solution at 1 mg/ml in sterile distilled water. This lipophilic cationic fluorochrome specifically stains mitochondria of living cells.[13,14] It was shown to be a good probe for MDR studies as it specifically stains Par cells while it is effluxed from MDR cells as a Pgp substrate.[15,16] Preliminary fluorescence microscopy and flow cytometry tests of ranges of Rhod-123 concentrations on both P388 cell lines led to the use of 5 μg/ml Rhod-123 as it gave bright staining of mitochondria of Par-P388 cells and no detectable fluorescence of MDR-P388 cells. At variance with similar research performed with daunomycin as a Pgp probe and with fixed cells for analysis (see ref. 12, and Boesch and Loor, manuscript in preparation), the fluorescence analyses were done on living cells in the present assays. Indeed, Rhod-123 stains mitochondria of living cells by a typical transmembrane potential-related retention,[18] so that fixing the cells would abolish the specific retention of the probe in the mitochondrial compartment.

RMAs

β-Lumicolchicine, verapamil, valinomycin, gramicidin S (Sigma Chemical Co., St Louis, MO, USA) as well as cyclosporin A, SDZ PSC 833, SDZ 280-446, SDZ 33-242 (all from Sandoz AG, Basel, Switzerland) and amiodarone (Labaz, France), were prepared at 100 μM as stock solutions in absolute ethanol. Quinidine (Siegfried, Zofingen, Switzerland), quinacrine (Serva, Heidelberg, Germany) and procaine (Merck, Darmstadt, Germany) were diluted at 100, 100 and 1000 μM in culture medium, respectively. Stock solutions of catharanthine and vindoline (Pr Petit, Institut des Substances Naturelles, Gif-sur-Yvette, France) were prepared at 1000 μM in 90% ethanol in 0.1N HCl and at 500 μM in 0.9% NaCl in 0.1N HCl, respectively, and diluted in culture medium.

Fluorescence microscopy

Rhod-123 retention within both Par-P388 and MDR-P388 cell lines was studied by fluorescence microscopy and flow cytometry analyses. Two conditions were tested: 'co-treatment', i.e. in the presence of the RMA only during the uptake phase of the dye, and 'post-treatment', i.e. in the continuous presence of the RMA during both uptake and efflux phases of the dye from cells, since the latter conditions might reveal higher activity for rather weakly active RM molecules.

In 'co-treatment' conditions, samples of 5×10^5 cells of the Par-P388 and MDR-P388 lines were incubated for 15 min at 37°C (uptake phase) in a 7.5% CO_2 humidified incubator (Heraeus) in 1 ml medium containing 5 μg/ml Rhod-123, in the presence or the absence of various concentrations of the RMAs. The dye and RMA excess neither retained nor taken up by cells was removed by centrifugation (200 g at 4°C). The capacity of the cells to efflux the dye was evaluated after 15 min at 37°C in 1 ml of RMA- and dye-free medium (efflux phase). After two further washes, the cell suspension was observed with a Leitz Ortholux II microscope equipped with a fluorescence vertical illuminator and a filter block I2 (510 nm dichroic mirror,

for blue light excitation). In the absence of Rhod-123, the intrinsic fluorescence of quinacrine was not detected in our conditions (i.e. $\lambda_{em} = 515$ nm, $\lambda_{exc} = 488$ nm).

In 'post-treatment' conditions, the cells were treated and studied as described for co-treatment assays, except that the RMA was present throughout the whole procedure (only Rhod-123 was removed for the efflux phase).

Only cells showing typical mitochondrial fluorescence were taken into account ($n \geq 300$). Results are expressed as an index of fluorescence from '0' to '4' evaluated as follows: '0', no fluorescence as for unstained cells; '1', marginal fluorescence; '2', clear fluorescence; '3', bright fluorescence but fainter than for Par-P388; '4', bright fluorescence identical to that of Par-P388 stained without RMA.

Flow cytometry

Samples of 10^6 cells were exposed to the dye and RMA in both co-treatment and post-treatment conditions. Following the last centrifugation after the efflux phase, the cell pellet was resuspended in 1 ml culture medium (4°C). The cells were then washed 3 times in cold PBS–Dulbecco (Seromed, Berlin, Germany) just before analysis.

Flow analyses were done with a FACS analyzer ATC 3000 (Brucker, Spectrospin, Wissembourg, France) by using 400 mW of 488 nm light from an argon ion laser. Sort windows were used on forward and side scatter to eliminate debris. Size and fluorescence ($\lambda_{em} = 515$ nm) intensity were recorded for 5000 cells at a rate of about 200 cells/s.

Results are represented as 'relative retention', i.e. Rhod-123 retention by MDR cells with regard to Rhod-123 retention by Par cells at identical RMA concentrations. Dose–response curves were constructed with the relative retention on the y-axis versus RMA concentration on the x-axis.

Results

Fluorescence microscopy

'Co-treatment' conditions. In RMA-free samples, Par-P388 cells displayed a bright green mitochondrial fluorescence looking like a wool ball following Rhod-123 staining, whereas MDR-P388 cells did not show any staining. Both cell lines presented no intrinsic fluorescence in the absence of Rhod-123 exposure. None of the potential RMAs tested changed the type of fluorescence of Par-P388, except for two cyclic peptides, gramicidin S and valinomycin (not shown). Valinomycin-treated cells showed a diffuse staining which was not restricted to mitochondria, suggesting that its K^+-ionophore property could disrupt the negative inside potential of the mitochondrial membrane leading to that phenomenon. Gramicidin S concentrations higher than 30 μM induced a marked decrease in Par-P388 cells suggesting that high gramicidin S doses were toxic or allowed Rhod-123 to leave the cell by lipoproteic membrane structure perturbations.

When tested on MDR-P388 cells (Table 1), SDZ 33-242, verapamil, quinidine, quinacrine, valinomycin, catharanthine, vindoline, procaine and β-lumicolchicine could at best restore a marginal retention of Rhod-123 even at very high concentrations.

Table 1. Effects of various RMA molecules on the Rhod-123 retention restoration in MDR-P388 cells in co-treatment conditions[a]

RMA (μM)	0.3	0.8	1	2.5	3	5	8	10	25	30	50	100	500	1000
Cyclosporin A		0		0.5			2.5		3					
SDZ PSC 833		2.5		3.5			3.5		4					
SDZ 280-446		1		1.5			3.5		4					
SDZ 33-242		0		0			0		0					
Verapamil	0		0		0			0		0		1		
Quinidine	0		0		0			0		0		0		
Quinacrine	0		0		0			0		0		0		
Amiodarone	0		0		0			0		2		3		
Gramicidine S	0		0		0			3		0.5		0.5		
Valinomycine								0	0		0	0.5		
Catharanthine						0		0	0		0	0	0	0
Vindoline													1	
Procaine														0
β-Lumicolchicine								0	0		0	0		

[a] Results are expressed as index of restoration of fluorescence correlated to the '4' maximum for Par-P388 fluorescence.

Although 10 μM gramicidin S restored most of the normal dye retention by MDR-P388 (graded '3' in comparison with '4' for Par-P388), this activity occurred within a rather narrow range of concentrations, suggesting that higher concentrations were toxic for the cells.

Amiodarone clearly restored Rhod-123 retention at 30 μM and was even more active at 100 μM, while not reaching the fluorescence levels expressed by the Par-P388 cells. With cyclosporin A, a similar suboptimal restoration of Rhod-123 retention in the MDR-P388 cells was obtained by only 8 μM, while the same concentration of SDZ 280-446 was sufficient to completely restore Rhod-123 retention in the MDR-P388 cells. SDZ PSC 833 was shown to be the most active compound, since it totally restored the dye retention at 2.5 μM, with a high activity already at 0.8 μM. By cross-comparisons, SDZ PSC 833 could then be tentatively set as 10-, 4- and 100-fold more active than cyclosporin A, SDZ 280-446 and amiodarone, respectively.

'Post-treatment' conditions. Each experiment in the 'post-treatment' condition was done in parallel with a co-treatment assay, the maximum score of fluorescence ('4') being the one shown by Par-P388 cells in the respective conditions.

The maintenance of SDZ 33-242 or β-lumicolchicine throughout the whole MDR cell treatment did not change their uptake of Rhod-123, but differences of staining were recorded with the other RMAs.

The first clear signs of Rhod-123 retention by MDR-P388 cells required 30 μM amiodarone, 10 μM cyclosporin A, 10 μM SDZ 280-446 or 1 μM SDZ PSC 833 in co-treatment conditions, while 10 μM amiodarone, 3–10 μM cyclosporin A, 1 μM SDZ 280-446 and 0.3 μM SDZ PSC 833 were sufficient in post-treatment conditions (Table 2). Although these comparisons might be tempered by the subjective (non-quantitative) aspects of fluorescence intensity evaluations performed by microscopy, it appeared that a 3-fold factor could be estimated between the RMA concentrations giving a similar effect in the co- and post-treatment procedures.

While fluorescence microscopy studies had the merit of allowing us to keep the mitochondrial specificity of the fluorescence under control, there was a definite need for more objective measurements, i.e. flow cytometry.

Flow cytometry

'Co-treatment' conditions. Several molecules which were structurally unrelated but shared hydrophobicity were first tested in co-treatment conditions in order to set their potential activity in restoring the dye accumulation in MDR-P388 cells. In the absence of RMA, MDR-P388 cells did not significantly retain Rhod-123, their relative retention being recorded as 0.2–2.75% (i.e. as a percentage of the fluorescence displayed by the Par-P388 cells). Except for gramicidin S, which led to a dramatic fall of Rhod-123 retention at 30 and 100 μM, none of the tested RMAs had any significant effect on Par-P388 cells (not shown).

Five out of the 14 RMAs tested showed activity (Figure 1) in the restoration of the Rhod-123 retention in MDR-P388 cells (the stronger the RMA was, the sooner the relative retention reached 100%, i.e.

Table 2. Compared effects of various RMA molecules on the Rhod-123 retention restoration in MDR-P388, in co- and post-treatment conditions[a]

RMA (μM/treatment)	Cyclosporin A		SDZ PSC 833		SDZ 280-446		SDZ 33-242		Amiodarone		β-Lumicolchicine	
	co-	post-	co-	post-	co-	post-	co-	post-	co-	post-	co-	post-
0.1	0	0	0	0.5	0	0	0	0				
0.3	0	0	0	1	0	0.5	0	0	0	0		
1	0.5	0.5	1.5	2.5	0.5	1	0	0	0	0		
3	0.5	0.5	2.5	3.5	0.5	2	0	0	0	0		
10	1.5	2.5	3.5	4	1.5	3	0	0	0	1.5	0	0
25											0	0
30	2.5	3.5	4	4	3.5	4	0	0	1.5	3		
50											0	0
100									2.5	4	0	0

[a] Results are expressed as index of restoration of fluorescence correlated to the '4' maximum for Par-P388 fluorescence.

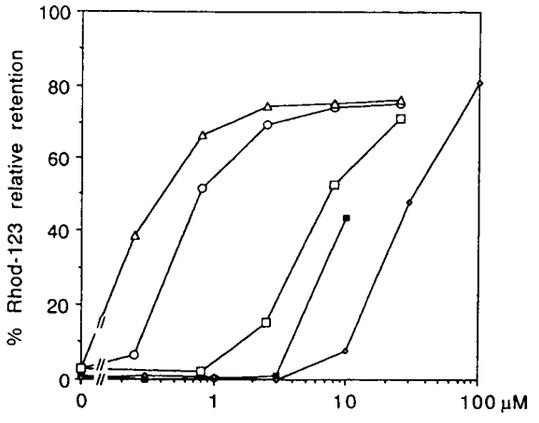

Figure 1. Restoration of Rhod-123 retention by MDR-P388 cells by micromolar concentrations of: cyclosporin A (□), amiodarone (◇), SDZ PSC 833 (△), SDZ 280-446 (○) and gramicidin S (⊞) in co-treatment conditions. Flow cytometry results are expressed as Rhod-123 relative retention by MDR-P388 related to Par-P388 cells under same conditions ($n = 5000$).

a restoration of dye retention by MDR-P388 cells equal to the one by Par-P388 cells). At 10 μM, gramicidin S induced good Rhod-123 retention in MDR-P388 cells (43.7% relative retention); however, this molecule worked within a quite narrow dose range since, as for Par-P388 cells, Rhod-123 fluorescence of MDR-P388 cells completely disappeared at 30 and 100 μM. Amiodarone, cyclosporin A, SDZ 280-446 and SDZ PSC 833 at 100, 30, 8 and 2.5 μM, respectively, led to almost complete restorations of the Rhod-123 retention by MDR-P388 cells.

While 0.25 μM SDZ PSC 833 already induced 38.5% relative retention, only 6.4% relative retention was achieved by the same concentration of SDZ 280-446. The first significant effect for cyclosporin A (15.3% retention) and amiodarone (7.5% retention) required 2.5 and 10 μM, respectively. The detectable shifts of Rhod-123 fluorescence observed with MDR-P388 (Figure 1) indicated the pecking order of activity of the five more potent RMAs. SDZ PSC 833 was about 2.5- and 10-fold more active than SDZ 280-446 and cyclosporin A, respectively, and from 30- to 100-fold more active than weaker RMAs such as gramicidin S and amiodarone.

None of the other nine molecules displayed significant activity on the restoration of the accumulation of Rhod-123 in MDR-P388 cells since the relative retention never exceeded 3% within the tested range of concentrations (not shown).

'Post-treatment' conditions. Post-treatment assays were performed in parallel to co-treatment assays in order to get a fair correlation of the data. The same pecking order of activity of the RMAs as set for co-treatment assays was preserved.

The presence of each of the four interesting RMAs (amiodarone, cyclosporin A, SDZ 280-446 and SDZ PSC 833) during the efflux phase of Rhod-123 regularly induced a shift of the curves, indicating that they could achieve their blocking function on Pgps at weaker doses than when present only during the Rhod-123 uptake phase (Figure 2).

Post-treatment conditions with 10 μM cyclosporin A induced 61.1% relative retention of Rhod-123, while in co-treatment conditions the dye retention was very low. Amiodarone gave 23.7 and 89% relative retention at 10 and 100 μM, respectively, in post-treatment conditions, while co-treatment conditions allowed lower restoration levels with 12.3% relative retention at 30 μM and 57.3% at 100 μM. While complete restoration of Rhod-123 retention was obtained at 10 μM SDZ PSC 833 and 30 μM SDZ 280-446 in co-treatment conditions, 3-fold lower concentrations (i.e. 3 and 10 μM, respectively) were required in post-treatment conditions to achieve the same effect. Thus, for a similar Rhod-123 retention, the effective RMA doses had to be 3 times higher when used only during the Rhod-123 uptake phase than when also continuously present during the efflux phase.

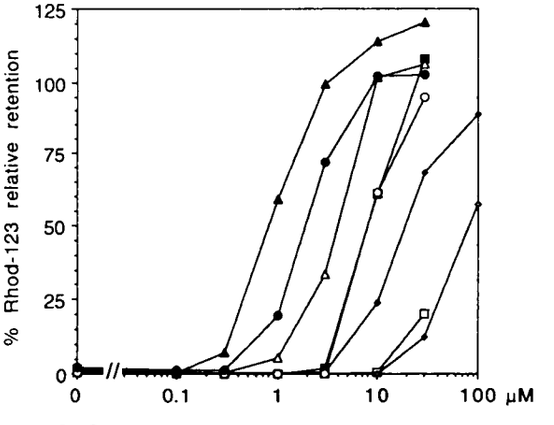

Figure 2. Compared effects of cyclosporin A (□, ■), amiodarone (◇, ◆), SDZ PSC 833 (△, ▲) and SDZ 280-446 (○, ●) on the restoration of the retention of Rhod-123 by MDR-P388 cells in co- (open symbols) and post- (full symbols) treatment conditions. Flow cytometry results are expressed as Rhod-123 relative retention by MDR-P388 related to Par-P388 cells under same conditions ($n = 5000$).

Discussion

SDZ PSC 833 and SDZ 280-446 were previously studied by proliferative *in vitro* assays for their ability to overcome the Pgp-mediated MDR phenomenon in various cell lines.[9–11] SDZ PSC 833 and SDZ 280-446 were also shown to restore the cellular accumulation of daunomycin, an anthracycline which displays natural fluorescence targeted on the nucleus.[11,12] Rhod-123, a molecular probe for Pgp-mediated MDR studies,[15] was used here to compare several RMAs for their capacity to inhibit the Pgp effluxing function. The cationic hydrophobic Rhod-123 specifically stains mitochondria by transmembrane potential-related retention.[18] Therefore, daunomycin and Rhod-123 are structurally different Pgp substrates or probes of Pgp function, while having different intracellular targets. Comparisons of the effects of several Pgp-blocking RMAs on these two probes might show marked or subtle differences, which should help our understanding of how Pgp molecules process their widely different substrates.

Fluorescence microscopy and flow cytometry data correlated well, and indicated that SDZ PSC 833 was the most potent out of the five active RMAs tested for the restoration of the accumulation of both Pgp substrates, daunomycin[11,12] and Rhod-123 (this study), within MDR-P388 cells. Thus, it induced the restoration of the retention of Rhod-123 at lower doses than cyclosporin A, amiodarone and gramicidin S, while nine other molecules were totally inactive. SDZ 280-446 was also a very potent RMA with the same order of RM activity as SDZ PSC 833.

In co-treatment assays, either in fluorescence microscopy or flow cytometry, a major restoration of Rhod-123 retention required 2.5 μM SDZ PSC 833, 8 μM SDZ 280-446, 25–30 μM cyclosporin A or 100 μM amiodarone.

Other Pgp substrates tested could not interfere with Rhod-123 retention. Among these were ACD-related compounds which were definitely able to interfere with the interaction of their parent compounds with Pgp. This was the case for vindoline and catharanthine, which are constituent alkaloids of the vinblastine molecule competing with vinca alkaloids for Pgp binding,[5] and for β-lumicolchicine, which is a UV light-induced inactive form of colchicine competing at high doses with colchicine at the Pgp level.[7]

Other well known Pgp-targeted RMAs such as verapamil, quinidine and quinacrine could induce high daunomycin retention in the same MDR-P388 cell subline at 100 μM,[12] but were not detectably active in our conditions on Rhod-123 retention.

Nevertheless, other RMAs (SDZ PSC 833, SDZ 280-446, cyclosporin A and amiodarone) displayed the same range of activities on Rhod-123 retention as when tested for daunomycin retention. It is unknown whether the apparent lack of activity of the other tested RMAs merely results from quantitative differences in the extent of Pgp blockade or from qualitative differences in the mechanism of Pgp inactivation. The latter (qualitative) hypothesis might explain why RMAs known to restore daunomycin retention did not impair Rhod-123 efflux by the Pgp even when used at high dosages.

Recent work suggested that verapamil and cyclosporin A might interfere by distinct mechanisms with doxorubicin (14-hydrodaunomycin) efflux in Pgp-expressing cells,[19] and a flippase mechanism was proposed for substrate-processing by Pgp.[2] The present study showed that some RMAs (e.g. cyclosporins) could interfere with a wider range of Pgp substrates than others (e.g. verapamil). Processing of Pgp substrates could occur by either a pore or a flippase, or both, or other mechanisms depending on the type of substrate. Various RMAs might belong to diverse classes depending on their capacity to interfere with either all or a single particular Pgp-mediated mechanism.

The same pecking order of activity for the different RMAs was recorded in both co- and post-treatment assay conditions. Nevertheless, obvious shifts were recorded between the incidence of the RMAs in co- and post-treatment conditions: the continuous presence of the RMAs during the efflux phase allowed us to achieve the same Rhod-123 retention restoration levels at 3 times lower concentrations than for co-treatment.

The involvement of two different RMA-binding sites in the MDR cell membrane is one of several possible explanations: a high affinity RMA-binding site would be easily saturated in both types of experimental conditions while the other, lower affinity RMA-binding site would need the continuous presence of RMA to contribute to the full inhibition of the Pgp pump itself or of any other Pgp-mediated MDR mechanisms. As a working hypothesis, one may speculate that the high affinity binding sites were located directly on the Pgp molecules, but that the lower affinity sites need not to be on the Pgp molecule itself, though they presumably sit within the plasma membrane.

In this study, the use of Rhod-123 as Pgp substrate was thus shown to be a helpful tool for the evaluation of the activities of various molecules

known or supposed to be RMAs. Furthermore, the comparison of data obtained with other fluorochromes or with widely different Pgp substrates might help to define whether various substrate-binding sites might be present on the pump and the type of mechanism involved in the effluxing function of Pgp.

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