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Reversion of the P-glycoprotein-mediated multidrug resistance of cancer cells by FK-506 derivatives

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FK-506 is a resistance-modulating agent (RMA) for tumor cells whose multidrug resistance (MDR) involves a P-glycoprotein (Pgp)-mediated anti-cancer drug efflux. The family of FK-506 relatives and derivatives includes analogs which display a whole range of chemosensitizing strengths, from no detectable RMA activity to a complete reversion of the MDR phenotype. Similarly, FK-506 analogs display a whole range of immunosuppressive activities, including inactive ones. FK-506 was compared for RMA activity with 11 FK-506 analogs which were at least 20-fold less active than FK-506 for the inhibition of the bi-directional mixed lymphocyte reaction displayed the whole range of RMA activity. One such strong RMA derivative of FK-506 (SDZ 280-629) was further shown able to restore completely daunomycin retention by highly resistant MDR P388 tumor cells.

Key words: Anticancer drugs, multidrug resistance, P-glycoprotein, resistance-modulating agents.

Introduction

There is now general consensus that one of the major mechanisms by which tumor cells may become resistant to several of the most efficient anti-cancer drugs (ACDs) available today is a decreased intracellular ACD bioavailability. Such a multidrug resistance (MDR) phenotype of tumor cells is mediated by the overexpression of P-glycoprotein (Pgp) molecules, i.e. transmembrane glycoproteins which belong to the class of ABC (ATP-binding cassette) superfamily of active transporters. Pgp pumps drugs out of the cells by an ATP-dependent process.[1–3]

Various ways have been undertaken to restore the chemosensitivity of the MDR tumor cells. Besides attempts to discover ACDs which would not be Pgp pump substrates though maintaining a sufficient activity towards their molecular target for cell cycle arrest, the other pharmacological approach consisted in trying to inhibit the Pgp pump function enough to restore sensitivity to the classical ACDs within the range of doses compatible with their *in vivo* therapeutic window.[2]

The latter type of approach resulted in a variety of chemosensitizers, shown either to inhibit MDR and/or to interfere with ACD binding to Pgp. The structures of such resistance-modulating agents (RMA) are as widely different as the structures of the ACDs which are Pgp substrates.[2] The earliest identified RMAs were quinidine and verapamil, both of which have been used in clinical attempts of MDR reversion. Nevertheless, the most active RMAs were recently shown to belong to two structurally and phylogenically unrelated classes of highly *N*-methylated cyclic 4-butenyl-4-methylthreonine (Bmt)-containing peptides and pipecolic acid (Pec)-containing peptolides, such as, respectively, the cyclosporin derivative SDZ PSC 833[4] and the novel semi-synthetic cyclopeptolide SDZ 280-446.[5]

Though several Bmt-containing cyclosporins and Pec-containing cyclopeptolides were strong RMAs, other natural compounds with new structures should not be disregarded as potential RMAs since they might be more suitable in specific circumstances. The FK-506 macrolide antibiotic was such a potential lead structure and its RMA property was shown by various groups.[6–9] In our own study,[6] though higher dosages of FK-506 than cyclosporin A were required to achieve the same level of chemosensitization, a broader range of concentrations was active and direct cytotoxic effects were weaker with FK-506 than with cyclosporin A. However, the high immunosuppressive activity of FK-506 should exclude its clinical use as an RMA.

In the case of the cyclosporin family, there was no correlation between immunosuppression and

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resistance modulation.[10] Therefore FK-506 derivatives lacking significant immunosuppressive activity were screened for their capacity to neutralize Pgp-mediated MDR. Using an MDR-subline of Chinese hamster ovary (CHO) cells, a better restoration of their sensitivity to colchicine (COL) and doxorubicin (DOX) could be obtained by several such FK-506 derivatives, in comparison with the parent compound FK-506. Using a subline of MDR cells from the murine monocytic leukemia P388, one of these compounds (SDZ 280-629) was further shown to be capable of completely restoring their retention of daunomycin (DAU).

Materials and methods

Drugs

For *in vitro* experiments, COL (Sandoz, Basel, Switzerland), vincristine (VCR, vincristine sulfate, Serva, Heidelberg, Germany), DAU (Sigma, St Louis, MO) and DOX (Sigma) were prepared as stock solutions in culture medium. Cyclosporin and FK-506 derivatives (Sandoz) were prepared as stock solutions in absolute ethanol. All FK-506 derivatives used in this paper were tested in a two-way mixed lymphocyte reaction in concentrations up to 0.1 μg/ml (roughly 0.1 μM). The FK-506 IC_{50} in this test ranges from 0.02 to 0.2 ng/ml; none of the other compounds showed activity at the highest concentration (0.1 μg/ml) tested. Other assays of immunosuppressive activity showed the derivatives to be at least 20-fold less active than FK-506.

Tumor cell lines

The cell lines belonged to two species and two cell classes: the murine monocytic leukemia P388N (Par) and P388Dox^R (MDR) and the CHO fibroblastoid carcinoma AUXB1 subclone AB1SII (Par) and CH^{R}C5 subclone C5S3.2 (MDR).

The MDR cell lines were continuously grown in the presence of the drug used for their selection; 8–24 h before each experiment the culture medium of the MDR cell lines was removed and the cells were grown in drug-free medium. The origins and detailed conditions for *in vitro* growth and analyses of these cell lines were as published earlier.[4,10]

Previous studies have shown that the Par P388 cells were not at all chemosensitizable even by large concentrations of our stronger RMAs.[4,5] The MDR P388 cells, which had been selected to MDR by growth in the presence of DOX, expressed a high MDR level which was nevertheless completely reversible by adequate RMA treatment. At variance, the Par CHO subline displayed a low degree of Pgp expression conferring a low and easily reversible resistance to drugs such as COL and DOX, whereas the MDR CHO subline, which was selected by growth in the presence of COL, showed high MDR level corresponding to high Pgp expression. While maximal gains of sensitivity of only about 10- to 20-fold (depending on the ACD and on the status of the Par CHO cell culture) can be obtained for the Par CHO cells even using the strongest chemosensitizers, gains of 300- to 400-fold are commonly reached with the MDR CHO cells. Conversely, weak chemosensitizers which did not display enough activity to neutralize the strong Pgp function of MDR CHO cells could be sufficient, at high concentrations, to knock out the mild resistance of the Par CHO cells.[10,11]

In vitro cytotoxicity studies

Tumor cell growth and its drug-mediated inhibition were measured as described previously.[4,10,11] The growth levels obtained without RMA and ACD but with their solvents were taken as representing 100% growth.

The ACD IC_{50}s were calculated from the dose–response curves obtained by plotting the measured growth versus the ACD concentration. Cultures performed in the absence of ACD (but in the presence of its solvent) with the whole range of RMA concentrations allowed the construction of RMA dose–cell growth response curves and the determination of the RMA IC_{50}s.[4,11]

In chemosensitization assays, only RMA concentrations giving less than 10–20% growth inhibition of the particular cell line were considered to give significant results. A complete ACD dose–cell growth response curve was constructed at each RMA concentration. A whole range of 'IC_{50+}' values were thus obtained in the *presence* of the different RMA concentrations, the 'IC_{50-}' values being obtained in the *absence* of RMA (but in the presence of its solvent).

The increases of ACD sensitivity or 'gains' in sensitivity of the RMA-treated cells were given by the ratio IC_{50-}/IC_{50+}, a gain being calculated for each RMA concentration.

Intracellular fluorescence studies for DAU retention

The method was adapted for 96-well microplates from our former procedure.[12]

Briefly, samples of 5×10^5 cells were incubated in a 7% CO_2 humidified atmosphere at 37°C for 30 min in 0.2 ml medium containing 20 μM DAU in the absence or presence of RMA. The DAU excess not taken up or not retained by the cells was removed by three washes (2 min centrifugation at 200 *g* and 4°C) and reincubated for 15 min at 37°C in drug-free medium (lacking both DAU and RMA). After two further washes by centrifugation in DAU- and RMA-free medium, the cells were fixed in PBS -3.7% formaldehyde and analyzed for intracellular DAU fluorescence with a FACScan cell analyzer (Becton-Dickinson, Mountainview, CA) equipped with an argon laser (15 mW) tuned at 488 nm. Dead cells and debris were excluded by setting a gate on the basis of their decreased forward light scatter.

In the fluorescence histograms, the *X*-axis was a logarithmic scale for the fluorescence level and the *Y*-axis was an arithmetic scale for the number of cells recorded in each channel. In order to facilitate the comparison of the Par P388 and MDR P388 cells, both histograms were then overlayed on a single diagram.

In order to simplify the comparisons, some data are presented as the percentage of the MDR P388 cell geometric mean fluorescence to the Par P388 cell geometric mean fluorescence.

Results

Chemosensitizing activity of FK-506 for Par and MDR CHO cells

Preliminary studies with the two sub-lines of CHO cells which have been extensively used for the characterization and comparison of various RMAs[11] confirmed the data obtained with the MDR P388 cell line:[6] *in vitro* FK-506 could sensitize Par CHO and MDR CHO cells to COL and VCR (Table 1). With the Par CHO cells, a near 10- to 20-fold plateau of sensitization to COL or VCR was commonly observed with several RMAs.[10,11] Such a maximal effect was reached here by 3.0 μg ml (3.7 μM) of FK-506; this RMA concentration was also able to sensitize significantly the highly resistant MDR CHO cell line. Although higher gains could be obtained with 10 μg/ml (12.4 μM) of FK-506, the emergence of direct inhibitory effects of this RMA for CHO cell growth restricted the significance of these preliminary data to the lower RMA dosages. This level of MDR-reversing activity of FK-506 was roughly similar to the ones of verapamil and amiodarone.[11]

Table 1. FK-506 dose-dependent chemosensitization of Par and MDR CHO cells to COL and VCR: gains of sensitivity

FK-506 dose[a] (μg/ml)	Par CHO		MDR CHO	
	COL gain	VCR gain	COL gain	VCR gain
0.1	1.1	1.0	ND	ND
1.0	2.1	3.3	1.1	2.0
3.0	9.5	9.7	11	5.6
5.0	ND	14	29	22
10	9.4	31	91[b]	185[b]
20	9.3[b]	40[b]	toxic[c]	toxic[c]
≥30	toxic[c]	toxic[c]	toxic[c]	toxic[c]

[a] 1 μg/ml = 1.245 μM.
[b] There was ±20% inhibition of cell growth brought by FK-506 alone, making the significance of its chemosensitizing activity questionable.
[c] FK-506 alone gave a strong inhibition of cell growth.

Because a slight chemosensitization to VCR was sometimes observed with very poor Pgp-directed RMAs or with non-Pgp expressing cell lines, our RMA screening for FK-506 derivatives was performed with COL and DOX as ACDs.

Comparisons of the RMA activity of FK-506 derivatives on CHO cells

The screening of a variety of FK-506 derivatives and related macrolides indicated that the property of being a RMA was not equally shared by all of the FK-506 family members as shown in a study of chemosensitizing activities of FK-506 related compounds, including FK-506 and 11 derivatives lacking significant immunosuppressive activity (Table 2).

All compounds gave IC_{50}s higher than 10 μM, though half of them directly inhibited CHO cell growth at that concentration. Nevertheless, at the 5 μM concentration, less than 10% direct cell growth inhibition (GI) was mediated by these RMAs, with the exceptions of SDZ 281-151 and SDZ 281-112.

That 5 μM concentration of almost all compounds, but one (SDZ 280-956) which was totally

Table 2. Comparison of 12 members of the FK-506 family for their direct antiproliferative effects (GI class), and for their chemosensitizing properties for COL gain and DOX gain

RMA	Read-out[a]	*In vitro* responses of Par CHO and MDR CHO tumor cells to the RMA at 1, 5 and 10 μM					
		Par CHO			MDR CHO		
		1	5	10	1	5	10
FK-506	GI	A	A	B	A	A	C
	COL	4.2	7.5	8.2	1.3	22	51
	DOX	3.3	6.6	9.8	1.9	38	117
SDZ 281–112	GI	A	A	D	A	C	D
	COL	8.4	8.5	8.7	5.9	108	94
	DOX	6.9	15	10	7.3	220	223
SDZ 281–116	GI	A	A	A	A	A	A
	COL	6.8	13	10	1.2	4.7	38
	DOX	4.7	11	14	1.2	5.8	43
SDZ 281–151	GI	A	A	D	A	B	D
	COL	7.3	7.8	7.3	1.3	37	61
	DOX	6.5	12	8.8	1.9	106	140
SDZ 281–155	GI	A	A	A	A	A	A
	COL	3.1	8.3	9.0	1.3	25	56
	DOX	2.6	7.0	8.8	1.9	27	87
SDZ 280–536	GI	A	A	D	A	A	D
	COL	5.4	7.4	7.5	1.1	9.1	29
	DOX	3.9	6.8	8.3	1.4	9.9	76
SDZ 280–538	GI	A	A	B	A	A	A
	COL	3.4	7.9	8.3	1.4	18	67
	DOX	3.2	7.5	10	2.1	30	101
SDZ 280–629	GI	A	A	C	A	A	C
	COl	7.6	9.0	8.3	8.9	140	314
	DOX	7.7	12	11	9.0	364	401
SDZ 280–956	GI	A	A	B	A	A	A
	COL	0.9	1.0	1.3	1.1	1.1	1.2
	DOX	0.9	1.0	1.1	1.0	1.0	1.0
SDZ 280–958	GT	A	A	B	A	A	A
	COl	2.3	6.9	7.4	1.1	1.6	6.7
	DOX	2.1	7.2	9.5	1.1	2.3	10
SDZ 280–964	GI	A	A	A	A	A	A
	COL	2.6	8.5	9.2	1.0	1.3	4.7
	DOX	1.8	6.7	12	1.1	1.5	5.4
SDZ 280–966	GI	A	A	B	A	A	A
	COL	2.8	8.8	8.9	1.4	31	74
	DOX	2.4	6.9	8.3	1.8	40	117

[a] Read-out: GI = direct cell growth inhibition *in vitro* by the RMA alone was labeled as class A: for less than 10%; B: for 10–20%; C: for 20–30%; D: for 30–40%. IC_{50}s for these 12 compounds were higher than 10 μM. COL, gain of sensitivity for COL, DOX, gain of sensitivity for DOX.

inactive, brought more than 7-fold sensitization of the Par CHO cells to COL and to DOX. The same concentrations of these RMAs, however, gave widely variable degrees of MDR CHO cell sensitization to COL or DOX: from as much as 364-fold for the DOX sensitization by SDZ 280-629 to virtually no significant ACD sensitization by SDZ 280-958 and SDZ 280-964 (gains smaller than 2), SDZ 280-956 being again fuly inactive. Interestingly, all compounds which were active gave higher gains of sensitivity for DOX than for COL.

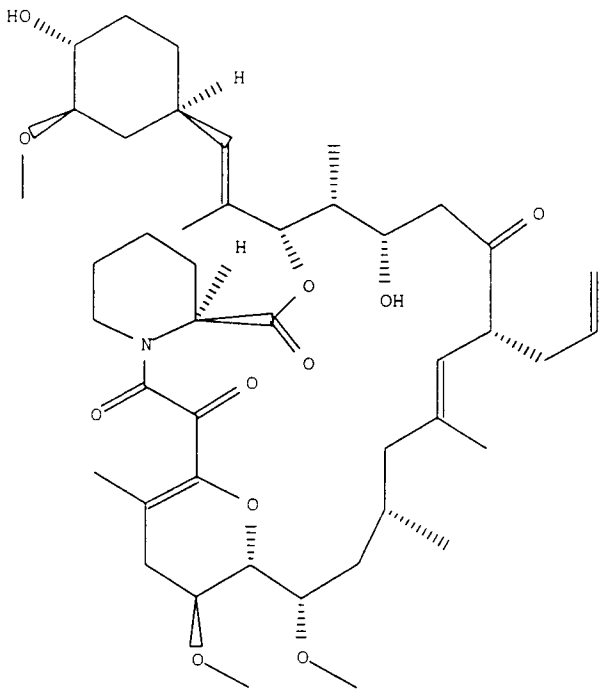

Figure 1. Structure of SDZ 280-629. SDZ 280-629 (empirical formula: $C_{44}H_{67}NO_{11}$; molecular weight: 786.02) is 9(*R*)-dehydro-10-desoxy-$\Delta^{10,11}$-FK-506 (according to the atom numbering in European Patent 184162).

The best RMA in this series was thus SDZ 280-629 (Figure 1), whose activity was further studied with another pair of Par and MDR tumor cell lines, by another assay for Pgp inhibition.

DAU retention restoration in MDR P388 cells by FK-506 and SDZ 280-629

These assays were performed in comparison with SDZ PSC 833 and cyclosporin A as reference compounds. The intracellular DAU retention was measured by the degree of anthracycline fluorescence of the P388 cells following *in vitro* exposure to DAU. These flow cytometry analyses of DAU retention were performed with both Par and MDR cells as a function of the RMA concentration.

In the absence of RMA treatment, the MDR P388 cells displayed low fluorescence levels corresponding to 3.5% of the Par P388 cell fluorescence levels. An RMA presence during the exposure of the Par P388 cells to DAU had no detectable effect on DAU retention, as measured by unchanged fluorescence levels of the Par P388 cells. As observed earlier,[12] low to very low concentrations of the reference RMAs (cyclosporin A and SDZ PSC 833) allowed definite shifts of the fluorescence profiles obtained for the RMA-treated MDR P388 cell populations (not shown).

Only the highest tested FK-506 concentration (30 μM) could induce a marked shift of the MDR cell profile towards higher fluorescence, though remaining far from the Par cell fluorescence level. SDZ 280-629 was much less active than cyclosporin A at 3 μM; however, at 10 μM it caused a net increase of MDR cell fluorescence, and at 30 μM it was almost as active as cyclosporin A, restoring for the MDR cells most of the DAU retention shown by Par P388 cells, as shown in a typical experiment comparing cyclosporin A, FK-506 and SDZ 280-629 (Figure 2).

Thus, FK-506 could not restore the normal 'Par P388' fluorescence level in the MDR P388 cells. For the other three RMAs, there were wide differences of strength (Table 3): SDZ PSC 833 nearly restored the DAU retention of MDR P388 cells to the Par P388 cell level ($\pm$90%) at a concentration of 0.1–0.3 μM only (depending on the experiment), whereas the same approximate level of restoration required much larger concentrations of the other tested RMAs. Cyclosporin A at 10 and 30 μM induced, respectively, 80 and 90% of the DAU retention restoration, while the same RMA concentrations allowed 40 and 80% restoration in the case of SDZ 280-629, and only 15 and 20% in the case of FK-506.

Table 3. Restoration of DAU retention in MDR P388 cells by FK-506 and SDZ 280–629, in comparison with cyclosporin A and SDZ PSC 833

RMA	Dose (μM)	MDR P388/Par P388 fluorescence ratio[a]
Cyclosporin A		
	3	51.8 $\pm$ 8.0
	10	77.8 $\pm$ 12.0
	30	90.2 $\pm$ 8.9
SDZ PSC 833	0.1	69.3 $\pm$ 34.9
	0.3	93.2 $\pm$ 20.2
	1.0	111.5 $\pm$ 10.5
FK-506	3	9.6 $\pm$ 2.4
	10	14.9 $\pm$ 3.3
	30	19.4 $\pm$ 3.6
SDZ 280–629	3	15.0 $\pm$ 4.4
	10	40.5 $\pm$ 9.9
	30	82.8 $\pm$ 18.0
Ethanol solvent		3.5 $\pm$ 0.6

[a] Means $\pm$ SD from four independent experiments.

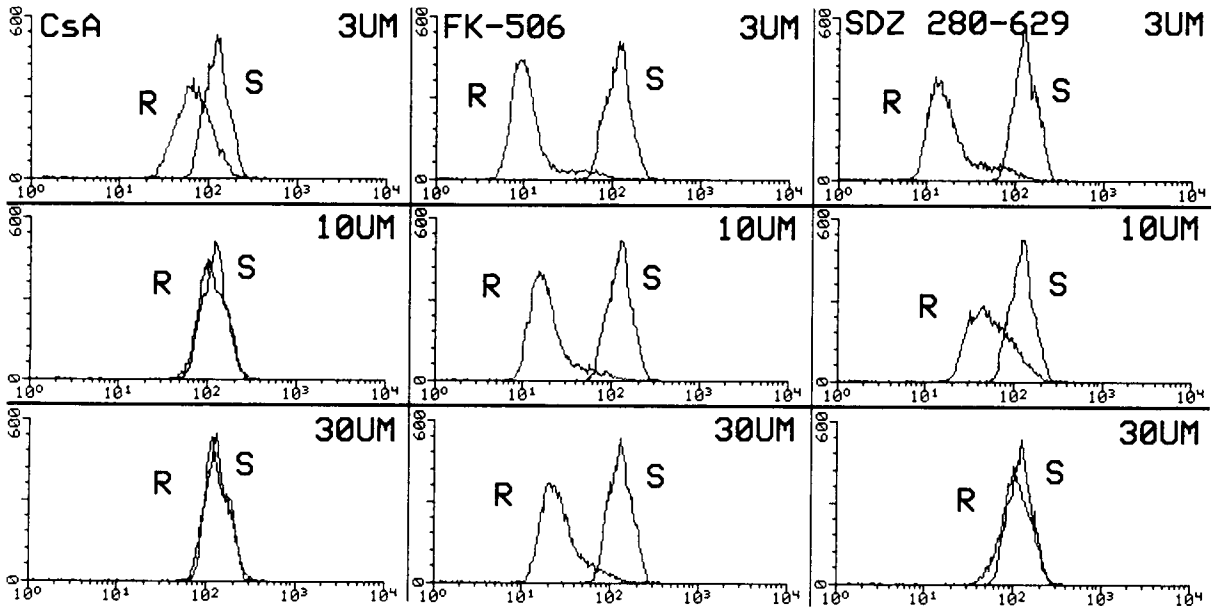

Figure 2. DAU retention by MDR P388 cells. Comparison of the effects of FK-506, SDZ 280-629 and cyclosporin A at 3, 10 and 30 μM on the cell population profile revealed by DAU retention. Each histogram shows the Par P388 cell population profile (labeled 'S') and the MDR P388 cell population profile (labeled 'R'). This example shows that similar profiles for the MDR P388 cells (profiles R) are obtained with 10 μM SDZ 280-629 or 3 μM cyclosporin A, as well as 30 μM SDZ 280-629 or 10 μM cyclosporin A; FK-506 is definitely weaker. The fluorescence, measured by flow cytometry, is recorded on the logarithmic scale of the *X*-axes and the cell numbers are expressed on the *Y*-axes.

Therefore, the FK-506 derivative SDZ 280-629 could restore the DAU retention in MDR P388 cells with the same range of activity as cyclosporin A, whereas the parent FK-506 compound only displayed a very low level of activity. Nevertheless, SDZ 280-629 remained less active than the cyclosporin SDZ PSC 833[4] or the cyclopeptolide SDZ 280-446.[5]

Discussion

A series of derivatives of the natural macrolide antibiotic FK-506 were shown to display a range of chemosensitizing activities for weakly resistant Par CHO and highly resistant MDR CHO cells.

For the chemosensitization to ACD-mediated inhibition of CHO cell growth, the most active FK-506 derivative described here, SDZ 280-629, displayed a level of activity comparable to that displayed by cyclosporin A, while the natural macrolide FK-506 could be considered as being about 3–5 times weaker than cyclosporin A. Similarly, with MDR P388 cells, cyclosporin A was found to be 3–5 times more active than FK-506 for the restoration of DOX sensitivity.[6] Nevertheless, for the restoration of COL- or DOX-mediated inhibition of MDR cell growth, SDZ 280-629 actually remained an order of magnitude weaker than our previously described cyclosporin derivative SDZ PSC 833[4] and semi-synthetic cyclopeptolide derivative SDZ 280-446.[5]

For the restoration of DAU retention by MDR P388 cells, the natural FK-506 macrolide was poorly active in comparison with cyclosporin A. Only at the highest tested concentration (30 μM) was SDZ 280-629 as active as cyclosporin A: its capacity to restore DAU retention was more concentration dependent since SDZ 280-629 was about 3-fold less active than cyclosporin A when these RMAs were compared at 3 μM.

Interestingly, all compounds which were MDR active gave higher gains of sensitivity for DOX than for COL. It is unknown whether this is related to the different 'pharmacophores' that the Pgp molecules would specifically use for effluxing COL and DOX, and whether this would suggest that these FK-506 derivatives were more efficient at inhibiting the DOX efflux than the COL efflux. Such preference was not observed in earlier studies with the cyclosporin and cyclopeptolide families. Point mutations on the Pgp molecules being sufficient to produce rather different profiles of cross-resistance, different pharmacophores might be

involved for the Pgp-mediated processing of drugs such as COL and DOX.[13] This was also suggested by the results of studies on the drug and RMA binding to Pgp.[14-16] Therefore, it might be that FK derivatives were RMAs displaying preferential binding to the hypothetical DOX pharmacophore.

Nevertheless, all these FK derivatives were definitely weaker, at equimolar concentrations, than our best cyclosporins and cyclopeptolides,[5] although with one of them (SDZ 280-629), a complete restoration of sensitivity of the highly resistant MDR CHO cells could be achieved, a rather unusual finding with RMAs which do not belong to the cyclosporin or cyclopeptolide families.

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