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Comparative *in vitro* evaluation of dithiane analogs of tiapamil, Ro 11-2933, Ro 44-5911 and Ro 44-5912 as multidrug resistance modulators

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The analogs of tiapamil are highly active modifiers of P-glycoprotein-mediated multidrug resistance (MDR) *in vitro*. The activity of three analogs of tiapamil, Ro 11-2933, Ro 44-5911 and Ro 44-5912, was compared in K562/DXR and MCF-7/DXR cell lines, using flow cytometry for the determination of intracellular daunorubicin accumulation and MTT assays for the cytotoxic evaluation of the modulators combined or not with daunorubicin. Ro 44-5911 and Ro 44-5912 were not intrinsically more toxic than DL-verapamil and exhibited a significantly higher reversing effect. Ro 44-5912 was shown slightly more efficient than Ro 44-5911 for reversing daunorubicin cytotoxicity. Ro 11-2933 was found to be the most potent in modulating MDR but was not significantly more active than Ro 44-5912. These two compounds were able to achieve a near complete reversion (above 80%) at 5 μmol/l. However, the cytoxicity of Ro 11-2933 was higher with an IC_{50} near 20 μmol/l in both K562 and MCF7 cell lines. Our results indicate that tiapamil derivatives are promising compounds for MDR modulation. Among them Ro 11-2933 and Ro 44-5912 seem to be particularly interesting for *in vivo* evaluations.

Key words: Multidrug resistance, resistance modifiers, tiapamil analogs, verapamil.

Introduction

A major obstacle to the success of cancer chemotherapy is the emergence of resistant tumor cell clones. Multidrug resistance (MDR) is related to the overexpression of 170 kDa P-glycoprotein acting as an ATP-dependent drug efflux pump[1–7] inducing pleiotropic resistance to 'natural' antineoplastic agents such as anthracyclines, vinca alkaloids, epipodophyllotoxins and taxanes.[8–10] In recent years, various compounds have been identified as P-glycoprotein inhibitors, among which are calcium channel blockers (verapamil, diltiazem, nifedipine), cyclosporin and its analogs, calmodulin inhibitors, quinine, dipyridamole, tamoxifen and progestins.[11–16] Unfortunately, their MDR-reversing effect can often be only observed *in vitro* at concentrations found to be toxic in man. Calcium channel inhibitors such as verapamil were often found to generate cardiotoxicity.[17] For these reasons, new calcium channel blockers such as tiapamil analogs have been investigated and it has been found that their vasodilatory activities were lower than that of verapamil in tests measuring relaxation of aortic strip contraction *in vitro*.[18] Among them, Ro 11-2933 appeared as a potent inhibitor of P-glycoprotein-mediated daunorubicin efflux but a weak calcium antagonist. At equimolar doses this molecule was reported to be 10-fold more powerful than verapamil in reversing daunorubicin resistance in murine doxorubicin-resistant P388/ADR tumor cells.[19] In the doxorubicin-resistant A2780/DXR ovarian cell line, a complete restoration of doxorubicin sensitivity was observed.[20] Furthermore, *in vivo* reversal of doxorubicin resistance by Ro 11-2933 was also reported in tumor-bearing mice[21] and in human ovarian xenografts.[22] More recently, dithiane analogs of tiapamil such as Ro 11-5160 and its two enantiomers, Ro 44-5911 and Ro 44-5912, were evaluated *in vitro* and found to be highly active modulators of P-glycoprotein-mediated MDR in KB-8-5 cells selected for vincristine resistance.[23] The efficiency of Ro 44-5912 as a resistance modulator was confirmed in the P388/DXR cell line.[24]

The aim of our investigation was to compare three analogs of the calcium antagonist tiapamil, Ro 11-2933, Ro 44-5911 and Ro 44-5912, by considering their intrinsic toxicity, their effect on daunorubicin intracellular accumulation, and on the restoration of daunorubicin cytotoxicity in the human K562 leukemia and MCF7 breast adenocarcinoma cell lines and their doxorubicin-resistant counterparts (K562/DXR and MCF7/DXR). Verapamil was considered as a reference MDR modulator.

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Materials and methods

Materials and chemicals

Cell culture materials and fetal calf serum were provided by Costar (Brumath, France). Culture media and additives were purchased from Gibco (Cergy-Pontoise, France). Daunorubicin (Rhone Poulenc Rorer Bellon, Neuilly, France), doxorubicin (Pharmacia, St-Quentin-Yvelines, France) and verapamil (Biosedra, Malakoff, France) were diluted in physiological saline. Ro 11-2933, *N*-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(2-naphthyl)-*m*-dithiane-2-propylamine hydrochloride, Ro 44-5911 and Ro 44-5912 (Figure 1) were obtained from Hoffman-La Roche (Basel, Switzerland) and were solubilized in ethanol. All other chemicals were purchased from Sigma (St Quentin Fallavier, France).

Cell lines

K562 human leukemia cell line and its subline K562/DXR, selected for its resistance to doxorubicin, were provided by Professor B Desoize (Institut Jean Godinot, Reims, France). MCF7 human breast adenocarcinoma cell line and its subline MCF7/DXR, selected for its resistance to doxorubicin, were provided by Professor J Robert (Institut Bergonié, Bordeaux, France) with the authorization of Dr K Cowan (NCI, Bethesda, MD). All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine and penicillin in a 37°C, 5% CO_2 atmosphere. The resistant sublines were maintained in doxorubicin containing medium, 1 and 10 μmol/l, respectively, for K562/DXR and MCF7/DXR.

Flow cytometry

Intracellular daunorubicin accumulation analyses were assessed using an Orthocyte flow cytometer (Ortho Diagnostic Systems, Roissy, France) as already described.[25] Cell suspensions (10^6 cells/ml) were exposed to 1 μmol/l daunorubicin for 2 h at 37°C then washed twice with cold RPMI 1640 medium and further exposed to propidium iodide (final concentration 75 μmol/l) for identification of the membrane-altered cells. Cell suspensions were kept in ice before being analyzed. Verapamil was used as a reference MDR modulator.

Modulators (tiapamil analogs or verapamil) were added to daunorubicin (simultaneous exposure) at final concentration ranging between 0.1 and 100 μmol/l. As a control, ethanol (final concentration 3.3% v/v) was added instead of the modulator.

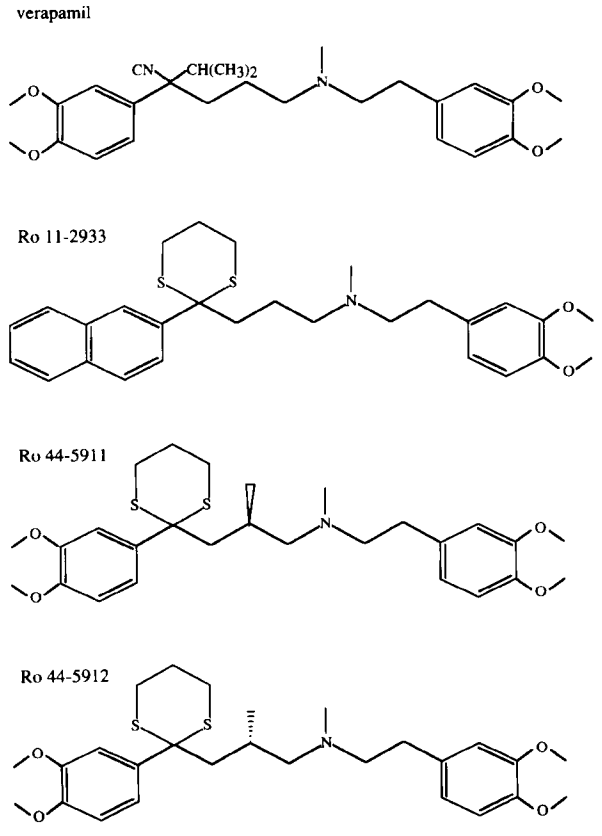

Figure 1. Structural formulas of verapamil and tiapamil analogs Ro 11-2933, Ro 44-5911 and Ro 44-5912.

Logarithmic amplification was used to monitor the two red fluorescence signals of both daunorubicin and propidium iodide. The values obtained from histograms analyses were then converted to linear relative fluorescence intensities using the equation: $F = 10^{(M/N \cdot \Delta C)}$, where F is the relative fluorescence intensity, M is the number of modules of the amplifier (= 3 for the Orthocyte flow cytometer), N is the number of detection channels (= 256 for the Orthocyte flow cytometer) and ΔC is the difference in the median red fluorescence channel numbers between the samples with modulator and the control. Each analysis was performed in triplicate on at least 5000 cells, and the results are expressed as the mean ± SD.

Cytotoxicity assays

Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

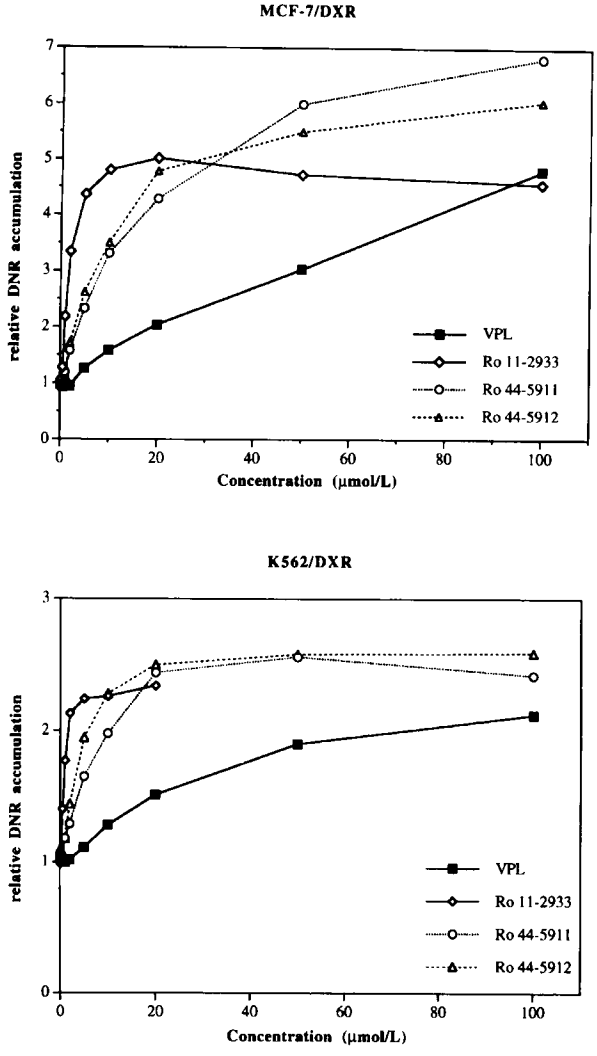

Figure 2. Influence of reversing agents on relative intracellular accumulation of daunorubicin in K562/DXR and MCF7/DXR cell lines. The values obtained for histogram analysis were converted in linear relative fluorescence intensities as described in Materials and methods. Values are the mean of three experiments ($0.03 \leq SD \leq 0.6$).

(MTT) assay. Briefly, cells suspensions containing 5×10^4 viable cells/ml were plated into 96-well dishes and allowed to attach in doxorubicin-free medium for 72 h. The cells were then exposed to the drug(s) (modulators and/or daunorubicin) for 2 h at 37°C and then incubated for 72 h in drug-free medium. MTT was added into each well and the dishes were incubated for 3 h at 37°C to allow its metabolization into crystalline formazan derivatives. The crystals were finally solubilized by addition of sodium dodecylsulfate. The absorbance values were measured at 540 nm on a Multiskan MCC 340 plate reader (Flow Laboratories). The results were compared with the control wells where cells were not exposed to the drug(s) and expressed as percentage of control absorbance values. Drug concentrations inhibiting the cell growth by 50% (IC_{50}) were calculated using median-effect analysis.[26]

The intrinsic cytoxicity of the modulators (verapamil, Ro 11-2933, Ro 44-5911 and Ro 44-5912) was evaluated in both resistant and sensitive cell lines from 0.1 to 100 µmol/l.

MDR modulation was evaluated from daunorubicin cytoxicity assays performed in the presence of the modulators at final concentrations of 0.5, 2 and 5 µmol/l. Daunorubicin final concentration ranges were adapted according to the respective sensitivity of each cell line. As reference, daunorubicin was tested alone in the resistant cell lines within the range 50–500 µmol/l. After this incubation period, the cells were washed twice with PBS and fresh medium was added to allow cells to grow. After 72 h, MTT test was performed as reported before.

Daunorubicin concentrations, with or without modulator, leading to 50% cell growth inhibition were calculated using median-effect analysis.[26] From these data, normalized reversion indexes were deduced: no reversion (reversion index equal to 0.0) corresponding to an IC_{50} equivalent to that obtained in resistant cells without modulator and complete reversion (reversion index equal to 1.0) corresponding to an IC_{50} equivalent to that obtained in sensitive cells.

Statistical analysis

Results were analyzed using Student's unpaired *t*-test with $p < 0.05$ as significance limit.

Results

Flow cytometry

Intracellular daunorubicin accumulation (IDA) in the resistant cells as compared with the sensitive cells was 0.36 (±0.09) and 0.32 (±0.05) (mean ± SD), respectively, for K562/DXR and MCF7/DXR cell lines. In all cases, whatever the cell line, tiapamil analogs (Figure 2) were found to induce an increase in IDA which, in most cases, was significantly higher than that achieved with equimolar verapamil.

MCF7/DXR cell line. For verapamil, the increase of daunorubicin accumulation was linearly related to

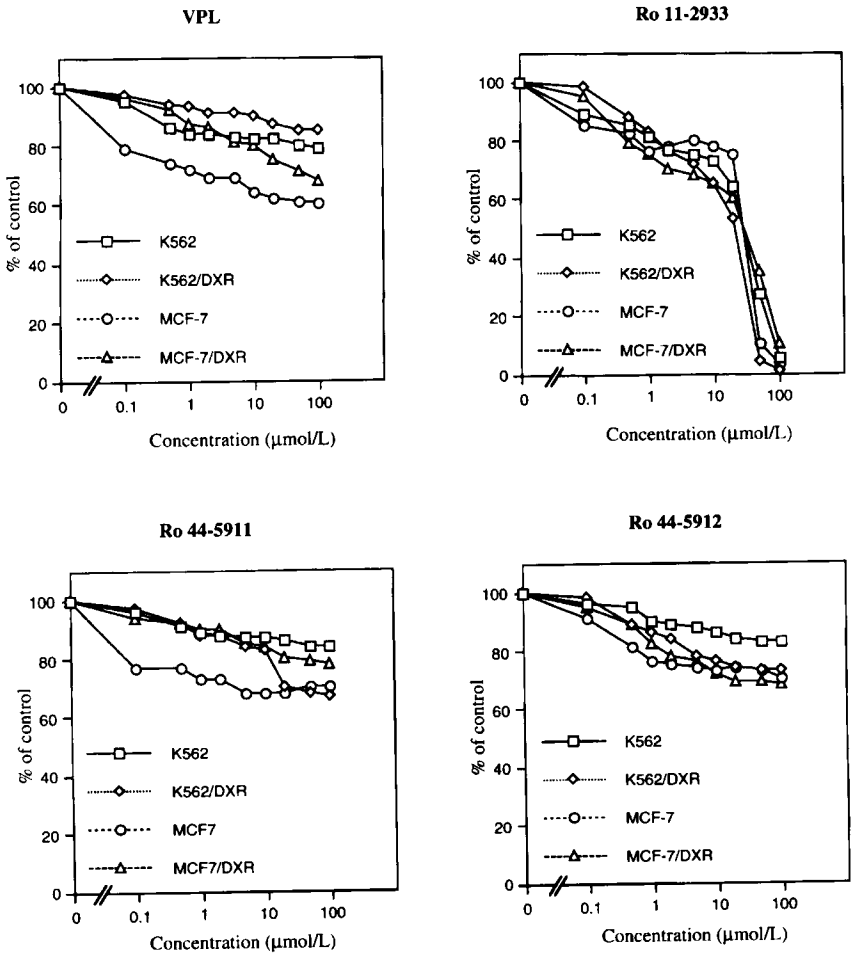

Figure 3. Intrinsic cytotoxicity of modulators, verapamil, Ro 11-2933, Ro 44-5911 and Ro 44-5912 in K562, K562/DXR, MCF7 and MCF7/DXR cell lines determined by MTT assays (mean values of three assays). SD are not presented and were always below 15% of the mean value.

the concentration. At 100 μmol/l (maximal concentration tested) IDA was 4.8-fold higher than in control. For tiapamil analogs, maximal modulation activity was observed at 10 μmol/l for both Ro 44-5911 and Ro 44-5912 and at 5 μmol/l for Ro 11-2933. At these values, these compounds were 2- to 3.5-fold more potent than verapamil. Ro 44-5911 and Ro 44-5912 activities were very close, whereas Ro 11-2933 activity was significantly higher at 1 μmol/l ($p < 0.002$) and 2 μmol/l ($p = 0.03$). From 20 μmol/l, the cytotoxicity of Ro 11-2933 was evidenced by a significant increase in propidium iodide labeled cells (data not shown) and a slight decrease in IDA in the non membrane-altered analyzed cell population. When resistant cells were compared with sensitive cells, IDA was completely restored with 100 μmol/l verapamil, 20 μmol/l Ro 11-2933, and 50 μmol/l Ro 44-5911 and Ro 44-5912.

K562/DXR cell line. As a whole, the results achieved in the K562/DXR cell line were close to those obtained in the MCF7/DXR cell line. Maximal modulation was obtained at 2 μmol/l for Ro 11-2933, and its activity was significantly higher than the other tiapamil analogs between 0.5 and 2 μmol/l ($p < 0.04$) whose maximal activities appeared at 20 μmol/l. Again, Ro 11-2933 proved to be cytotoxic from 20 μmol/l, thus impairing the completion of the IDA analyses at higher concentration. Complete IDA restoration was observed for verapamil at 100 μmol/l, at 2 μmol/l for Ro 11-2933, and at 10 μmol/l for both Ro 44-5911 and Ro 44-5912.

Cytotoxicity assays

Intrinsic cytotoxicity of the three tiapamil analogs (Ro 11-2933, Ro 44-5911 and Ro 44-5912) was eval-

Table 1. IC_{50} values and reversing indexes calculated from MTT assays performed on K562/DXR and MCF7/DXR cell lines exposed to daunorubicin with or without modulators.

Modulators (μmol/l)	K562/DXR		MCF7/DXR	
	IC_{50} (μmol/l)[a]	Reversion indexes[b]	IC_{50} (μmol/l)[a]	Reversion indexes[b]
Control	55.53 (9.03)	0	1902 (887)	0
Verapamil				
0.5	47.00 (8.49)	0.11 (0.15)	185.50 (30.41)	0.86 (0.01)
2	35.05 (14.90)	0.39 (0.28)	159.50 (13.44)	0.88 (0.01)
5	22.90 (10.75)	0.62 (0.20)	70.00 (16.97)	0.92 (0.01)
Ro 11-2933				
0.5	36.70 (11.60)	0.36 (0.22)	50.23 (23.86)	0.93 (0.02)
2	19.80 (13.10)	0.68 (0.25)	28.25 (9.90)	0.94 (0.01)
5	5.65 (1.69)	0.95 (0.03)	15.53 (3.33)	0.94 (0)
Ro 44-5911				
0.5	34.00 (14.00)	0.41 (0.27)	424 (40.80)	0.74 (0.02)
2	25.40 (26.90)	0.57 (0.51)	347 (11.67)	0.78 (0.01)
5	18.58 (12.69)	0.70 (0.24)	32.05 (29.91)	0.94 (0.02)
Ro 44–5912				
0.5	33.23 (3.22)	0.43 (0.06)	90.35 (68.38)	0.90 (0.04)
2	20.90 (4.36)	0.66 (0.09)	89.20 (84.00)	0.91 (0.04)
5	11.10 (3.68)	0.84 (0.07)	23.85 (16.48)	0.94 (0.01)

[a]IC_{50} values are calculated using median effect analysis, results are mean values of triplicated experiments (SD).
[b]Reversion indexes are normalized IC_{50} values according to daunorubicin cytotoxicity in resistant cells without modulator and in sensitive cells. In the latter, IC_{50} values were 3.32 ± 1.24 μmol/l for K562 and 10.35 ± 0.77 μmol/l for MCF7.

uated and compared with that of verapamil, in sensitive and resistant cell lines (Figure 3). No dramatic difference was observed among the four cell lines tested, except for verapamil and Ro 44-5911, which were found to be slightly more cytotoxic in the sensitive MCF7 cell line although at 100 μmol/l no significant difference subsisted. Ro 44-5911 and Ro 44-5912 showed a mild cytotoxicity equivalent to verapamil, inducing approximately 40% growth inhibition at 100 μmol/l. Ro 11-2933 was found to be significantly more cytotoxic than verapamil and the other tiapamil analogs with an IC_{50} near 20 μmol/l in both K562 and MCF7 cell lines.

The modulation of daunorubicin cytotoxicity was evaluated by comparing its IC_{50} values with and without simultaneous exposure of the cells to verapamil and the tiapamil analogs in sensitive and resistant cell lines. No significant modulation of daunorubicin cytotoxicity was observed in the sensitive cell lines in which mean daunorubicin IC_{50} (SD) values were 3.3 (1.2) and 10.4 (0.8) μmol/l, respectively, for K562 and MCF7 cell lines.

K562/DXR were about 17-fold more resistant to daunorubicin than sensitive cells with an IC_{50} of 55.6 (9.0) μmol/l. With all four reversing agents (verapamil, Ro 11-2933, Ro 445911 and Ro 44-5912), modulation of daunorubicin cytotoxicity was related to the concentration (Table 1) with results achieved with 5 μmol/l giving statistically significant higher modulation than 0.5 μmol/l ($0.01 < p < 0.05$), except in the case of Ro 44-5911 because of larger interexperimental variations. All three tiapamil analogs appeared more potent than verapamil, leading to higher reversion indexes and a near complete reversion (> 0.80) was achieved with Ro 11-2933 and Ro 44-5912 at 5 μmol/l, while 5 μmol/l verapamil only reached 0.62.

MCF7/DXR revealed a higher degree of resistance to daunorubicin than K562/DXR (190-fold versus 17-fold) with an IC_{50} of 1902 (88.7) μmol/l. The reversing effect of all modulators appeared from the lowest concentrations, therefore the concentration–effect relationship only appeared for verapamil and Ro 44-5911, with significantly different reversion effects between 2 and 5 μmol/l. Only Ro 11-2933 was significantly more potent than verapamil and Ro 44-5911 at 0.5 and 2 μmol/l ($p < 0.02$).

Discussion

Calcium channel blockers have been largely studied for the modulation of P-glycoprotein-mediated MDR. It has been shown that their reversing activity is independent of their effects on calcium transport[27] and consists in a competitive binding to P-glycoprotein which blocks the efflux of cytotoxic

drugs.[28] Most of them were commercialized drugs and therefore had therapeutic properties that became side effects; when given at high dose for MDR modulation, new molecules were synthesized in the view MDR modulation such as the tiapamil analogs Ro 11-2933 and the two enantiomers Ro 44-5911 and Ro 44-5912.

In this study we showed using flow cytometry that tiapamil analogs could restore daunorubicin uptake in cells more efficiently than verapamil. Both Ro 44-5911 and Ro 44-5912 appeared to be better P-glycoprotein inhibitors than verapamil. In addition, it was recently demonstrated that their resistance modifying activities were not restricted to P-glycoprotein.[23,29]

Through the present study Ro 11-2933 appeared to be more potent than verapamil but was found more toxic than Ro 44-5911 and Ro 44-5912. These results are in agreement with data already reported from *in vitro* and *in vivo* experiments comparing Ro 11-2933 with verapamil.[20–23] In our experiments, at 5 μmol/l, Ro 11-2933 allowed a complete restoration of intracellular daunorubicin accumulation and a near complete recovery of its cytotoxicity (reversion index >0.9). However, this effect might depend on the level of resistance since a 10-fold higher concentration was needed to overcome MDR in K562/DXR (5 μmol/l, reversion index of 0.95) being 10-fold less resistant than MCF7/DXR (0.5 μmol/l, reversion index of 0.93). This paradoxal effect can be compared with the results observed in blast cells from patients in which P-glycoprotein expression is low (10–20%) and correlates with a decrease in daunorubicin accumulation lower than in the K562/DXR cell line.[26] In such cells, even relatively high concentrations (5 μmol/l) of powerful modulators such as verapamil and S9788 were not always able to restore completely the cellular accumulation of daunorubicin. Further extrapolation from this fact would suggest that, even if low levels of resistance would be related to a significant decrease in intracellular accumulation of the drug, they would surely not be only mediated by P-glycoprotein expression and so modulators interacting exclusively with this protein would fail to restore completely the cellular sensitivity. *In vivo*, Ro 11-2933 was found to be less active than Ro 44-5912 in KB-8-5 xenograft tumors treated by vinblastine.[30]

Ro 44-5911 and Ro 44-5912 were very similar for daunorubicin uptake restoration and cytotoxicity in cells, but Ro 44-5912 was shown slightly more efficient than Ro 44-5911 for reversing daunorubicin cytotoxicity. These results are in agreement with those recently published by Eliason *et al.*,[23] who reported that Ro 44-5912 was more active than Ro 44-5911 in KB-8-5 cell line exposed to vincristine. This difference between the restoration of daunorubicin accumulation and the reversing cytotoxicity effect could be related to an enantioselectivity as shown for the two verapamil enantiomers differing in their resistance modifying activities, the racemic mixture having an intermediate activity.[31] Moreover, the ability of each molecule to modify the drug distribution within the cells has been demonstrated for other MDR modulators,[32,33] thus suggesting other intracellular targets.

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