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NOVEL DITHIANE ANALOGUES OF TIAPAMIL WITH HIGH ACTIVITY TO OVERCOME MULTIDRUG RESISTANCE *IN VITRO*

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Abstract—Dithiane analogues of tiapamil are highly active as modifiers of P-glycoprotein mediated multidrug resistance (MDR) *in vitro*. In an assay using the P-glycoprotein over-expressing cell line KB-8-5, the most active analogues for decreasing vincristine resistance were the racemate Ro 11-5160 and its two enantiomers, Ro 44-5911 (R) and Ro 44-5912 (S). In the KB-8-5 assay, the resistance modification index (RMI) of Ro 11-5160 was approximately 12-fold higher than those of the most active reference compounds tested, dipyridamole, cepharanthine, reserpine and cyclosporin A, when compared at concentrations equal to one-tenth of the IC_{50} of each compound ($RMI_{0.1}$). The enantiomers have similar resistance modifying activities, but the (S) enantiomer Ro 44-5912 is somewhat more active, fully reverting the vincristine sensitivity of KB-8-5 cells to the level of the parental KB-3-1 cells at a concentration of 2 μM. The (R) enantiomer attained this level of modification at a concentration of 3.5 μM. These concentrations are both well below their IC_{50} values for KB-8-5 cells (150 μM). The enantiomers appear to interact with P-glycoprotein because they inhibited [^{3}H]azidopine and [^{3}H]-vinblastine binding to plasma membrane fractions prepared from resistant K562/ADR cells. However, in addition to their resistance modifying activities with KB-8-5 cells, these compounds also decreased the IC_{50} values of vincristine and doxorubicin with KB-3-1 cells that do not express detectable levels of P-glycoprotein. Ro 44-5911 overcame doxorubicin and vincristine resistance in three colorectal cancer cell lines (DLD-1, WiDr and COLO 201) that express P-glycoprotein. No effect was seen with the 3 colorectal cell lines on the IC_{50} values of three drugs not related to the MDR phenotype, 5-fluorouracil, 5′-deoxy-5-fluorouridine and *cis*-diaminodichloroplatinum(II). The *in vitro* vasodilatory activity of these dithianes, measured with strips of rat aorta contracted with KCl, was about 5% of that of verapamil. These results suggest that dithianes could be useful agents for MDR modification *in vivo*.

Key words: multiple drug resistance; vincristine; doxorubicin; cell lines; P-glycoprotein; tiapamil analogues

Drug resistance, acquired or intrinsic, is a major impediment to successful treatment of cancer. One form of resistance, called MDR‖ has been the focus of much investigation in recent years, particularly since the gene for P-glycoprotein, a molecule responsible for resistance in many types of cancer, was cloned [1–3]. P-glycoprotein appears to act as an energy-dependent drug efflux pump to prevent high levels of toxic compounds from accumulating in cells [4]. Recently, MDR-associated protein, another protein belonging to the ABC superfamily and capable of conferring drug resistance to cancer cells, has also been cloned [5].

The demonstration that the calcium channel blocker verapamil could reverse the MDR phenotype [6] has led to a wide search for other agents that could overcome drug resistance, yet would have fewer pharmacological side effects. We have developed a sensitive *in vitro* assay using the P-glycoprotein over-expressing cell line KB-8-5 to measure the resistance modifying activity of various agents. We have shown that there are significant differences between the activities of the two enantiomers of verapamil to overcome vincristine resistance *in vitro* [7]. Similar differences were also found between the epimers quinidine and quinine [7].

We have used this assay to examine a large number of compounds from a variety of chemical classes. The most active group of compounds that we have tested consists of dithiane analogues of tiapamil [8], confirming the earlier findings of Kessel and Wilberding [9]. On the basis of our assay results, we have synthesized several new compounds having extremely high resistance modifying activity when tested at doses that had minimal effect on proliferation of KB-8-5 cells. In the course of these

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‖ Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; FCS, foetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulphate; ELISA, enzyme-linked immunosorbent assay; RM, resistance modifier; VCR, vincristine; RMI, resistance modification index; DOX, doxorubicin; CDDP, *cis*-diaminodichloroplatinum(II); 5-fura; 5-fluorouracil; 5′-dfurd, 5′-deoxy-5-fluorouridine; PAGE, polyacrylamide gel electrophoresis.

Table 1. Resistance modifying activities of selected compounds determined with KB-8-5 and KB-3-1 cells

Compound	KB-8-5		Reference*
	$RMI_{0.1\,VCR}$	IC_{50} (μM)	
Dipyridamole	58 ± 7	25 ± 2	16
Cepharanthine	57 ± 4	6.7 ± 4.3	17
Reserpine	57 ± 4	10 ± 3	18
Cyclosporin A	55 ± 2	2.5 ± 0.9	19, 20
Tiapamil	35 ± 1	460 ± 20	9
Verapamil	33 ± 4	76 ± 26	6
Quinidine	30 ± 1	96 ± 21	21
Diazepam	30 ± 1	570 ± 240	This work
Amiodarone	25 ± 1	18 ± 3	22
Gallopamil	23 ± 1	110 ± 81	23
Tamoxifen	23 ± 1	20 ± 1	24
Nicardipine	21 ± 1	17 ± 1	16
Tretoquinol	18 ± 2	80 ± 18	This work
Quinine	18 ± 0.5	26 ± 15	7, 25
Felodipine	16 ± 1	20 ± 1	26
Flupentixol	13 ± 1	18 ± 2	27
Cremophor EL	12 ± 0.2	0.83 ± 0.07†	28
Clonazepam	10 ± 1	26 ± 1	This work
Mefloquine	6.9 ± 0.2	2.3 ± 0.1	This work
Prenylamine	4.9 ± 0.1	19 ± 1	29
Imipramine	4.9 ± 0.1	57 ± 3	30
Phenindamine	4.5 ± 0.1	130 ± 10	This work
Haloperidol	3.0 ± 0.0	27 ± 1	27
Diltiazem	2.9 ± 0.1	63 ± 2	31
Progesterone	2.7 ± 0.0	16 ± 7	32
Doxepin	2.6 ± 0.0	30 ± 3	30
Triton X-100	2.2 ± 0.1	16 ± 1†	33
Trifluoperazine	2.2 ± 0.3	6.4 ± 2.5	29
Propafenone	2.1 ± 0.1	35 ± 4	This work
Staurosporine	1.8 ± 0.1	0.018 ± 0.012	34
Fluphenazine	1.7 ± 0.1	4.3 ± 2.5	27
Nifedipine	1.6 ± 0.1	55 ± 6	16, 35
Propanolol	1.6 ± 0.0	75 ± 20	21
Chloroquine	1.2 ± 0.0	95	16

* Original reference describing resistance modifying activity.
† mg/mL.

studies, we have found that these compounds also significantly enhance the activity of doxorubicin and vincristine against parental KB-3-1 cells, which do not express detectable levels of P-glycoprotein.

MATERIALS AND METHODS

Cells and media. The MDR cell line, KB-8-5, and its parental line, KB-3-1, were obtained from Dr M. M. Gottesman (NCI, Bethesda, MD, U.S.A.). The COLO 201, DLD-1, WiDr colorectal carcinoma cell lines were all obtained from ATCC (Bethesda, MD, U.S.A.).

The cells were cultured in EF medium [11, 12] supplemented with 10^{-5} M α-thioglycerol and 5% FCS. The KB-8-5 cells were routinely grown in the presence of 10 ng/mL of colchicine (Sigma, St. Louis, MO, U.S.A.) and then cultured in its absence for 1 week prior to each assay. The K562/ADM doxorubicin-resistant cell line was cultured in RPMI 1640 medium supplemented with 5% FCS and 100 μg/mL of kanamycin.

Cell proliferation assays. The assay used has been described in detail [7]. Cells were plated in 96-well microtitre plates and were incubated at 37° for 2 days in a fully humidified atmosphere of 5% CO_2 before drugs were added. The volume in each well was 200 μL. The plates were then incubated for a further 5 days, at which time, 50 μL of MTT (Sigma) solution (3 mg/mL) were added. After 4.5 further hr of incubation at 37°, 50 μL of a 25% SDS solution (pH 2.0) were added. The plates were incubated overnight to dissolve the formazan crystals before absorbance was measured at 540 nm using an ELISA reader (Anthos, Salzburg, Austria).

In each assay, controls consisted of several concentrations of cells plated with each concentration of RM. The relationship between log cell number and log absorbance was determined by least squares regression analysis for each concentration of RM and this was used as a standard curve to relate the absorbance measured in the VCR treated groups to fraction survival as has been described [7]. The effect of vincristine was examined at the highest

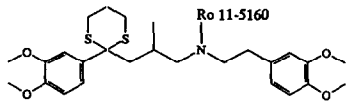
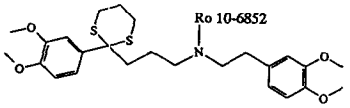
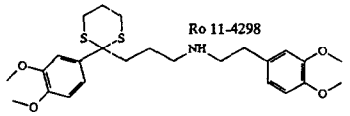
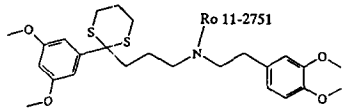
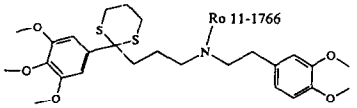
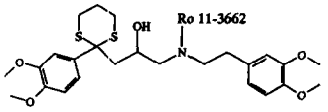
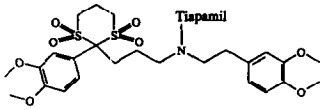
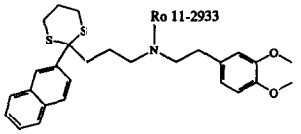
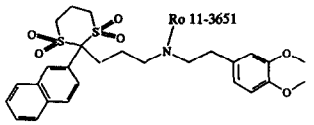
concentration of each cell line, 500 cells/well for KB-8-5 and 300 cells/well for KB-3-1. Regression lines for log percentage survival versus drug concentrations for VCR and RM were used to calculate the doses of compounds resulting in a 50% reduction in cell numbers compared to control cultures (IC_{50}). Because each calculated IC_{50} of VCR is determined using the cell titration curve for that particular concentration of RM, the results are empirically normalized for the inhibitory effects of the RM alone.

The RMI is the IC_{50} of VCR measured in the absence of RM divided by the IC_{50} measured in the presence of each dose of RM. To compare different compounds under similar conditions we further define the $RMI_{0.1}$, which is the RMI at a concentration of RM equal to one-tenth its own IC_{50} [7].

The optimal cell concentrations for the other cell lines were as follows: DLD-1, 300 cells/well; COLO 201 and WiDr, 1000 cells/well. In some experiments, the effects of RM on the sensitivities of cells to other cytotoxic drugs were tested under the same conditions. The drugs used were DOX, CDDP, 5-fura and 5'-dfurd.

Preparation of plasma membranes. Plasma membranes were prepared essentially as described

Table 2. Resistance modifying activities of tiapamil analogues

Compound	KB-8-5		KB-3-1	
	$RMI_{0.1\,VCR}$	IC_{50} (μM)	$RM_{0.1\,VCR}$	IC_{50} (μM)
 Ro 11-5160	720 ± 70	170 ± 120	6.9 ± 1.1	190 ± 55
 Ro 10-6852	190 ± 60	220 ± 20	15 ± 1	260 ± 20
 Ro 11-4298	180 ± 40	53 ± 26	4.3 ± 0.5	26 ± 0
 Ro 11-2751	130 ± 20	35 ± 11	8.8 ± 4.9	54 ± 20
 Ro 11-1766	99 ± 4	200 ± 70	2.4 ± 0.1	420 ± 180
 Ro 11-3662	53 ± 3	200 ± 40	4.6 ± 0.2	390 ± 180
 Tiapamil	35 ± 1	460 ± 20	1.5 ± 0.1	390 ± 180
 Ro 11-2933	24 ± 5	4.6 ± 2.4	1.1 ± 0.0	3.2 ± 1.0
 Ro 11-3651	13 ± 1	19 ± 1	ND*	ND

* ND, not determined.

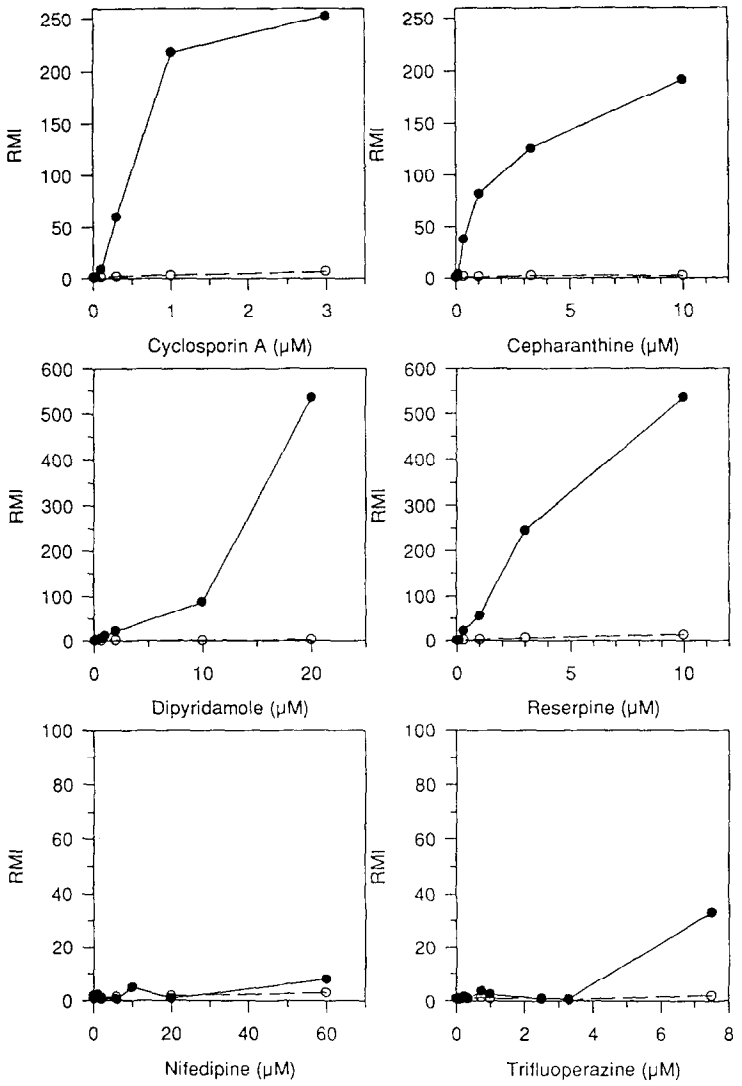


Fig. 1. Relationship between vincristine resistance modifying activity and concentration for cyclosporin A, cepharanthine, dipyridamole, reserpine, nifedipine and trifluoperazine. Closed circles represent results obtained using KB-8-5 cells and open circles represent those obtained using KB-3-1.

[13, 14]. Briefly, cells were washed with PBS, suspended at a concentration of 3×10^7 cells/mL in hypotonic lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.02 mM phenylmethylsulphonyl fluoride, pH 7.4) and incubated for 15 min on ice. The swollen cells were disrupted with 30 strokes using a Dounce homogenizer. The nuclei were removed by centrifugation at 1000 *g* for 10 min. The membrane fraction was pelleted by centrifugation at 100000 *g* for 60 min and resuspended in 10 mM Tris-HCl (pH 7.5) and 0.25 M sucrose. The membrane preparations were stored at −70° prior to use.

Vincristine transport assay. Binding of [^{3}H]VCR was measured by filtration on Millipore MF membranes (pore size 0.22 μM) as described [13, 15]. The membrane preparation was suspended in a total volume of 50 μL containing 125 nCi of [^{3}H]VCR in 10 mM Tris-HCl (pH 7.4), 0.25 mM sucrose and 5 mM $MgCl_2$. The ATP concentration was 3 mM except in control tubes with no ATP. After 10 min of incubation at 25°, the reaction was stopped by adding 4 mL of the same buffer at 4°. The samples were collected on filters that had been pretreated with 3% BSA. After the filters were washed with another 4 mL of buffer, they were dried and radioactivity was determined by scintillation counting.

Photoaffinity labelling with azidopine. Aliquots of 50 μg of plasma membrane preparation were mixed with 20 ng of [^{3}H]azidopine in 25 μL of PBS and incubated for 15 min at room temperature in the dark. Various concentrations of RM were added to determine if they competed with azidopine for binding. The mixtures were illuminated at 365 nm using a 400 W mercury lamp at a distance of 10 cm.

The samples were then analysed by 4–20% gradient SDS-PAGE. The gels were fixed in a mixture of 25% isopropyl alcohol and 10% acetic acid. They were treated with the fluorographic reagent Amplify (Amersham, Bucks, U.K.) for 30 min and labelled bands were visualized by autoradiography.

Relaxation of rat aortic rings. The vasodilatory activity of compounds was kindly determined by Dr M. Clozel (PRPV, Hoffmann-La Roche, Basel, Switzerland). Thoracic aorta, obtained from Wistar-Kyoto rats, were dissected into 5 mm rings. The endothelium was left intact. Each ring was suspended in a 10 mL isolated organ bath filled with Krebs–Henseleit solution (115 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11.1 mM glucose) kept at 37° in an atmosphere of 5% CO_2 and 95% O_2. The rings were connected to force transducers and isometric tension was recorded. The rings were stretched to a resting tension of 3 g. The presence of endothelium was assessed by the relaxation to acetylcholine (10^{-5} M) after precontraction of the ring with noradrenaline (10^{-7} M).

The compounds were dissolved in DMSO at a concentration of 10 mM and further diluted in Krebs–Henseleit solution. Their relaxant effect was tested by applying cumulative doses after the aortic rings had been contracted with 50 mM KCl. Each compound was tested on rings of three different rats. The EC_{50} was the concentration of compound inducing 50% maximal relaxation.

Statistical analyses. All statistical comparisons were made using SigmaStat™. Differences between multiple groups were determined using one-way analysis of variance. Differences between slopes of linear regression curves were calculated using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Our *in vitro* assay with the KB-8-5 cell line has been described in detail [7]. This line overexpresses P-glycoprotein and is approximately 75- to 150-fold resistant to vincristine, 4- to 8-fold resistant to DOX under the conditions we use. In order to correct for the growth inhibitory properties of each RM, we have defined a relative RMI, $RMI_{0.1}$, which is calculated at a dose of RM equal to one-tenth its own IC_{50}. None of the RM we tested have measurable growth inhibition on either KB-8-5 or KB-3-1 cells at this dose. The results for some compounds described in various publications as having resistance modifying activity are shown in Table 1 [16–35].

Only dipyridamole, cepharanthine, reserpine and cyclosporin A have significantly higher resistance modifying activities than verapamil and these are all in the same range, with $RMI_{0.1}$ values between 55 and 58. The results of titration experiments with these compounds are shown in Fig. 1 and clearly show dose–response relationships for RMI with each. As we have demonstrated previously, the relationship is linear at lower doses of RM. However, two distinct patterns are evident, particularly at higher doses of RM. Most compounds we have tested behave like cyclosporin A, cepharanthine and reserpine, having steeper slopes at lower doses and tending to reach plateaus with higher doses, when the IC_{50} values of the cytotoxic drug approach those of the drug sensitive parental line. A few display patterns similar to those of dipyridamole and trifluoperazine where the initial slope is low, but increases sharply at higher doses, usually near the IC_{50} of the compound.

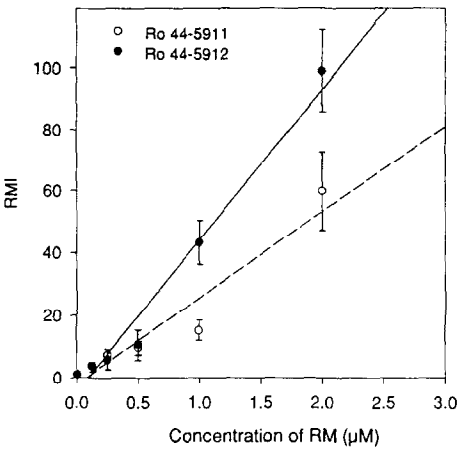


Fig. 2. Comparison of the resistance modifying activities of Ro 44-5911 and Ro 44-5912. Pooled results from five to six experiments for Ro 44-5911 and two to five experiments for Ro 44-5912 are shown. The points represent normalized mean RMI values. In order to compare the results from different experiments, the RMI value at which the IC_{50} of vincristine in the presence of modifier with KB-8-5 cells was equal to the IC_{50} of vincristine alone with KB-3-1 cells in the same experiment was set equal to 100. Also shown are linear least squares regression lines.

We have now tested over 600 compounds in this assay system and some of the most active compounds are dithiane analogues of tiapamil such as several of those shown in Table 2. For comparison, the dithiane tetraoxide, tiapamil, and its dithiane analogue Ro 10-6852 are also given. Two other analogues in Table 2 have also been described as having resistance modifying activity [9], the naphthyl tetraoxide Ro 11-3651 and its dithiane counterpart Ro 11-2933. In each case, the dithiane is more active than the dithiane tetraoxide. Both the dithiane Ro 10-6852 and its desmethyl derivative Ro 11-4298 are highly active with $RMI_{0.1}$ values approximately three times those of the most active reference compounds tested (Table 1). The most active compound, however, is that with a methyl group on the middle carbon between the tertiary amine and the dithiane moiety, Ro 11-5160. Replacing this methyl group with a hydroxyl group greatly reduces the resistance modifying activity (Ro 11-3662).

Because Ro 11-5160 has a chiral centre, the two stereo isomers, Ro 44-5911 and Ro 44-5912, were synthesized and were both highly active in the *in vitro* assay, with $RMI_{0.1}$ values similar to that of the racemate. The dose–response curves reach plateau values at concentrations above 3 μM (data not shown), so detailed titration studies at doses of 2 μM

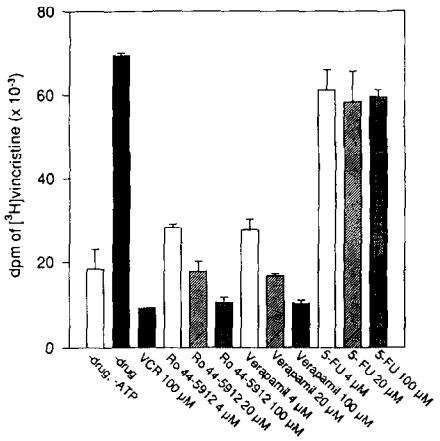


Fig. 3. Inhibition of [^{3}H]vincristine transport by isolated membrane vesicles of P-glycoprotein expressing K562/ADR cells.

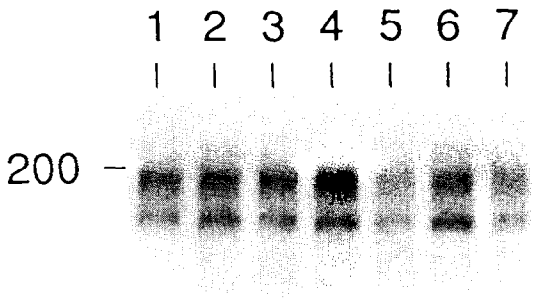


Fig. 4. Competition of RM for [^{3}H]azidopine binding to plasma membrane of K562/ADR cells. Membranes were incubated with [^{3}H]azidopine alone (lane 1), 10 μM (lane 2) or 100 μM (lane 3) verapamil, 10 μM (lane 4) or 100 μM (lane 5) Ro 44-5911 or with 10 μM (lane 6) or 100 μM (lane 7) Ro 44-5912. The position of the 200,000 molecular weight marker is shown at the left.

and below were performed in order to determine more precisely if the two enantiomers differed in their resistance modifying activities. The results shown in Fig. 2 indicate that the two compounds have similar activities, but that the S-enantiomer, Ro 44-5912, is more active than the R-enantiomer, Ro 44-5911. The slopes of the regression lines are 48.8 ± 3.7% per μM for Ro 44-5912 and 27.7 ± 3.3% per μM for Ro 44-5911. The difference between the two slopes is statistically significant ($P < 0.001$). The sensitivity of KB-8-5 cells was restored to that of the parental KB-3-1 cells as measured in control cultures in the same experiments at a concentration of 2 μM Ro 44-5912. The concentration of Ro 44-5911 needed to fully restore sensitivity of KB-8-5 was approximately 3.5 μM.

Two types of experiments were performed to determine if Ro 44-5912 interacts with P-glycoprotein. The first was to measure the ability of this compound to compete with vincristine for transport by P-glycoprotein into isolated membrane vesicles prepared from DOX-resistant K562 human leukaemia cells. The results of this assay are shown in Fig. 3. Transport by P-glycoprotein is dependent upon ATP as can be seen by the low accumulation of radioactivity when ATP is left out of the incubation mixture. Cold VCR (100 μM) competes very well to prevent transport of labelled VCR into the inside-out vesicles. Ro 44-5912 and verapamil were able to prevent transport into the vesicles, whereas 5-fura had no activity in this assay, as expected.

The second type of experiment was to examine the ability of the modifier to prevent photoaffinity labelling by [^{3}H]azidopine of P-glycoprotein from resistant K562 membranes. Compared to verapamil, both Ro 44-5911 and Ro 44-5912 appeared to be better at inhibiting labelling of P-glycoprotein when each compound was added to the mixture at a concentration of 100 μM (Fig. 4).

These dithianes also decreased the IC_{50} values of VCR with the parental KB-3-1 cell line. The degree

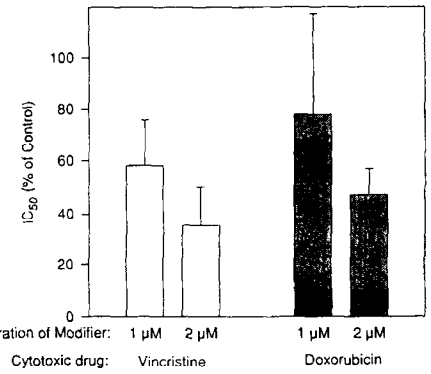


Fig. 5. Effect of dithiane resistance modifier Ro 11-5160 on vincristine and doxorubicin sensitivity of KB-3-1 cells. The bars represent the mean values of the IC_{50} values of vincristine or doxorubicin obtained in the presence of 1 or 2 μM of RM (pooled results from eight to 12 experiments) expressed as a percentage of control IC_{50} ± SEM.

of enhancement was clearly less than that seen with KB-8-5 cells, but was statistically significant at both doses of modifier, as shown for the racemic mixture in Fig. 5. At 1 μM, the IC_{50} of VCR was 60% of control and was about one-third of control at a concentration of 2 μM. Similar effects were also seen with respect to DOX toxicity. The enhancement with KB-3-1 cells was also statistically significant at both doses of modifier. At 1 μM, the IC_{50} of DOX was 78% of control and it was 47% at 2 μM.

In addition, we have examined the resistance modifying activity of Ro 44-5911 using a variety of cell lines of different origins. The results for the combination with doxorubicin and vincristine tested with three colorectal lines, DLD-1, WiDr and COLO 201 are shown in Table 3. We have detected P-glycoprotein mRNA by a polymerase chain reaction assay in these lines (data not shown). The results are compared to those obtained with Ro 44-5911

Table 3. Resistance modifying activity of Ro 44-5911 on three colorectal cell lines with different cytotoxic drugs

Cell line: Ro 44-5911 (μM)	% Growth inhibition by modulator	IC_{50} (RMI) DOX (nM)	VCR (nM)	5-Fura (μM)	5'-dfurd (μM)	CDDP (μg/mL)
DLD-1						
0	—	62	17	3.4	110	1.2
0.1	3	46 (1.3)	14 (1.2)	3.4 (1.0)	110 (1.0)	1.2 (1.0)
1.0	5	23 (2.7)	3.7 (4.6)	3.1 (1.1)	110 (1.0)	1.3 (0.9)
10	17	15 (4.1)	0.76 (22)	2.7 (1.3)	110 (1.0)	1.1 (1.1)
WiDr						
0	—	70	2.3	2.9	98	0.80
0.1	2	55 (1.3)	2.1 (1.1)	3.0 (1.0)	100 (1.0)	0.79 (1.0)
1.0	4	46 (1.5)	1.3 (1.8)	3.0 (1.0)	96 (1.0)	0.83 (1.0)
10	4	23 (3.0)	0.26 (8.8)	3.0 (1.0)	83 (1.2)	0.70 (1.1)
COLO 201						
0	—	290	3.1	9.7	180	2.8
0.1	2	290 (1.0)	2.7 (1.1)	10.3 (0.9)	190 (0.9)	2.4 (1.2)
1.0	2	93 (3.1)	1.7 (1.8)	11.0 (0.9)	220 (0.8)	2.7 (1.0)
10	2	64 (4.5)	0.25 (12)	6.6 (1.5)	150 (1.2)	2.4 (1.2)

plus 5-fura, 5'-dfurd and CDDP, drugs that are not involved in the 'classical' MDR phenotype. Only the cytotoxicities of vincristine and doxorubicin were enhanced by Ro 44-5911 in these experiments. This enhancement was dose dependent. The toxicity of the other three drugs was not influenced by co-incubation with this compound.

The potential cardiovascular activity of these compounds was examined *in vitro* using rat aortic rings contracted with KCl. Verapamil was tested at the same time. The EC_{50} of verapamil was 0.22 ± 0.05 μM. The tiapamil analogues were approximately one-twentieth as active as verapamil. The EC_{50} values for the racemate (Ro 11-5160) and the two enantiomers (Ro 44-5911 and Ro 44-5912) were not significantly different from each other, being 4.9 ± 0.9 μM, 5.2 ± 0.6 μM and 4.1 ± 0.4 μM, respectively. Similar results were obtained in a second experiment with Ro 11-5160.

DISCUSSION

We have tested a large number of different agents for their resistance modifying activities in our *in vitro* assay. Two distinct patterns have been observed for compounds having resistance modifying activity. One, seen with most compounds tested, was a linear relationship between RM concentration and RMI at low RMI values, reaching a plateau at high RMI values. This plateau is indicative that the P-glycoprotein drug pump is becoming saturated at the high doses of RM and the sensitivity of the resistant KB-8-5 line approaches that of the parental KB-3-1 line.

The second pattern, seen with a few compounds such as dipyridamole and trifluoperazine, indicated low to moderate resistance modifying activity at low doses of RM, but rapidly increasing activity as the IC_{50} of the RM was approached. The IC_{50} determined under these conditions represents approximately 1 population doubling over a period of 5 days. Because the doubling time of KB-8-5 cells is approximately 1 day, the same effect could be achieved either by killing 50% of the cells at the initiation of culture or by decreasing the proliferation rate by 20% over the entire 5 day period. Thus, concentrations of RM that significantly inhibit growth of KB-8-5 cells are not necessarily cytotoxic. Our interpretation of this second resistance modification pattern is that these compounds may have non-specific effects on membrane characteristics at concentrations which inhibit cell proliferation and also interfere with membrane permeability, thereby leading to increased accumulation of cytotoxic drugs. Thus, the high RMI values measured under these conditions may be independent of P-glycoprotein mediated effects.

In support of our interpretation is the study by Horio *et al.* [36] comparing the capacity of several agents to inhibit vinblastine transport into isolated membrane vesicles prepared from resistant KB-V1 cells. They demonstrated that verapamil and quinidine had nearly equal activities in this assay, whereas trifluoperazine and nifedipine had very low activities. Diltiazem had intermediate activity in this assay. This order of activity is similar to the order of our $RMI_{0.1}$ values (Table 1). This suggests that by examining the resistance modifying activities of different RMs at one-tenth their respective IC_{50} values, comparisons will reflect specific effects rather than non-specific membrane perturbations. This mode of analysis has also been used to compare resistance modifying activities of various surfactants [37].

Several studies have demonstrated that the activity of calcium channel blockers in modulating drug resistance is independent of their effects on calcium transport [38, 39]. A variety of analogues of different classes of calcium channel blockers, including analogues of verapamil [23] and dihydropyridine

[40, 41], have been examined to find more selective compounds with fewer cardiovascular side-effects. One early report compared several analogues of tiapamil [9] showing that dithiane analogues were more active than dithiane tetraoxides. We have confirmed these findings and further identified several analogues with extremely high $RMI_{0.1}$ values. The most active of these, Ro 11-5160, is approximately 12-fold more active by this measure than the most active reference compounds dipyridamole, cepharanthine, reserpine and cyclosporin A. A comparison with cyclosporin A at equimolar doses in the range of 2–3 μM, suggests similar potency for the two compounds, although cyclosporin A has much greater intrinsic inhibitory activity for cell proliferation at these concentrations.

We have previously demonstrated that the two enantiomers of verapamil exhibit a 4-fold difference in their resistance modifying activities. Therefore, it was of interest to test the pure enantiomers of Ro 11-5160 to determine if they differed in their activities as well. The S-enantiomer Ro 44-4912 was somewhat more active than the R-enantiomer, but the difference was less than two-fold. Both compounds appear to interact directly with P-glycoprotein because they inhibited VCR and azidopine binding to plasma membrane fractions prepared from P-glycoprotein expressing cells. The activity of the dithianes in these two assays relative to verapamil was different, being approximately the same as verapamil in inhibiting VCR transport and much more active as inhibitors of azidopine binding. These differences may reflect differences in the binding sites for the different drugs [42].

One of the most striking features of these compounds is that their resistance modifying activities are not restricted to P-glycoprotein expressing KB-8-5 cells. The IC_{50} values of both vincristine and doxorubicin were also significantly decreased in the KB-3-1 cells. We have been unable to detect significant levels of P-glycoprotein in KB-3-1 cells by polymerase chain reaction analysis (data not shown). This suggests that these compounds may influence other mechanisms of drug resistance in these cells. Work by several groups with the dithiane Ro 11-2933 indicates several possible alternative mechanisms. One is the MDR-associated protein that was cloned from drug resistant small-cell lung cancer cells H69AR [5]. Resistance of H69AR cells is partially overcome by Ro 11-2933 *in vitro* [43]. A second possibility is inhibition of DNA repair in cancer cells following DOX treatment [44–47]. However, we found no evidence of resistance modification by Ro 44-5911 with CDDP in three colorectal carcinoma cell lines suggesting that inhibition of DNA repair *per se* may not play a significant role in resistance of these cells.

Cytotoxicity of another drug not included in the 'classical' MDR phenotype, 5-fura, was also not affected by co-incubation with Ro 44-5911. It has been recently reported that the prodrug of 5-fura, 5'-dfurd, has a cytotoxic activity directly related to P-glycoprotein expression in rat colon carcinoma cells and increases daunorubicin uptake in P-glycoprotein-expressing cells [48]. However, we found no effect of the dithiane analogue Ro 44-5911 on the activity of 5'-dfurd.

In conclusion, we have shown using the well-defined P-glycoprotein expressing KB-8-5 cell assay that certain dithiane analogues have high MDR modulating activity. These compounds also decrease the resistance to VCR and DOX of several other cancer cell lines, originating from intrinsically resistant colorectal tumours and expressing P-glycoprotein. Remarkably, these compounds also enhance the activities of VCR and DOX in KB-3-1 cells that have little or no expression of P-glycoprotein. Because of their possible interaction with other pathways of drug resistance and their low potential vasodilatory activities, these compounds could be useful modulators of drug resistance *in vivo*.

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