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EFFECT OF COMBINATION OF SUBOPTIMAL CONCENTRATIONS OF P-GLYCOPROTEIN BLOCKERS ON THE PROLIFERATION OF *MDR1* GENE EXPRESSING CELLS

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Pharmacologically active *in vivo* doses of P-glycoprotein (Pgp) blockers, specifically verapamil, Cremophor EL and PSC833 cause toxicity in addition to that from the concomitantly used cancer chemotherapeutic drugs. It was shown before that these blockers cause different types of toxicities *in vivo*. We found that these 3 chemically distinct Pgp blockers exert different biophysical effects on the membranes of L1210 MDR cells. They also affect the general metabolism of these cells differently, but all block affinity labeling of Pgp. We could also show that the combination of suboptimal doses of these blockers can restore the uptake of the Pgp substrate rhodamine 123 into L1210MDR, 3T3MDR and KB-V1 cells and can reduce the survival rate of these cells when treated in combination with daunorubicin. Our results suggest that the combination of suboptimal doses of these Pgp blockers may be advantageous in clinical practice.

Resistance to chemotherapeutic agents is a major problem in the treatment of cancer. One of the most common causes of resistance is the overexpression of P-glycoprotein (P-170) (Pgp) by the *MDR1* gene (Pastan *et al.*, 1988). Pgp has the ability to enhance outward transport of chemically unrelated anti-cancer drugs and thereby diminish their effectiveness (Skovsgaard, 1978). Several compounds have been investigated for blocking the function of Pgp and were introduced into the clinic (Raderer and Scheithauer, 1993). While many of them block the function of Pgp, their effective dose also is associated with marked toxicity. For example, verapamil possesses a potential cardiovascular toxicity at a plasma concentration of 1–6 μM, the concentration that is necessary to block Pgp function (Salmon *et al.*, 1991). This dose of verapamil also poses toxicity to other normal and tumor cells (Lampidis *et al.*, 1986). Another agent under clinical development as a Pgp blocker is Cremophor EL (poly oxy castor oil, POCO). This solubilizing agent was found to block Pgp in MDR cells (Woodcock *et al.*, 1990). An effective dose of POCO is 0.1% in blood of dogs and humans (Webster *et al.*, 1993). This dose can cause certain adverse cardiovascular effects: changes in cardiac output, mean blood pressure, hepatic blood flow and renal blood flow (Bowers *et al.*, 1991). One of the most interesting Pgp blockers tried in the clinic is PSC833, a non-immunosuppressive cyclosporin A (CsA) derivative. This drug may cause intrahepatic cholestasis by inhibiting salt-dependent bile flow (Bohme *et al.*, 1994).

To avoid the potential side effects of individually used Pgp blockers, several studies have evaluated the use of combinations of these agents at lower than optimal dose levels. For example, Merlin *et al.* (1995) studied the combination of a synthetic agent, S9788, with either CsA, PSC833 or verapamil. They concluded that the use of lower doses of each Pgp blocker in combination is advantageous because each agent acts differently on the cellular distribution of doxorubicin, the cytotoxic drug used in their study. Another study, by Ross *et al.* (1994), found synergistic effects between CsA and POCO on the accumulation of daunorubicin (D) in resistant cells of AML leukemia patients.

For the above reasons, we have investigated the effectiveness of combinations of suboptimal concentrations of 3 chemically distinct Pgp blockers, verapamil, PSC833 and POCO (Cremophor EL), *in vitro*. We have selected these 3 chemicals not only because their host toxicity profiles are known to be different but also because we could show that they differently influence biophysical parameters of the plasma membrane. Our studies followed that of Callaghan *et al.* (1993), who showed that Pgp function can be modulated by altering the plasma membrane "fluidity". Furthermore, Friche *et al.* (1993) found that with increasing lipophilicity of D analogues, lipophilicity becomes more important than the chemical nature of the rest of the molecule in affecting Pgp function. We speculated then that the combinations of suboptimal concentrations of 2 of the selected 3 Pgp blockers, each with a different effect on biophysical parameters of the plasma membrane, may block additively the function of Pgp. This additiveness could then be exploited to circumvent the different toxicities exerted by them when used individually in clinics at their necessary optimal concentrations.

MATERIAL AND METHODS

Cells and chemicals

The leukemia L1210 cells were stably transfected with recombinant *MDR1* retroviral vector (pHa MDR1/A) as described earlier (Currier *et al.*, 1989; Weaver *et al.*, 1993) and were grown in complete RPMI medium with 10% horse serum. Mouse fibroblast NIH3T3 cells were stably transfected with the *MDR1* gene as described earlier (Currier *et al.*, 1992) and were grown in complete DMEM medium with 10% FCS. The drug resistant human adenocarcinoma KB-V1 cells were obtained by selection in increasing concentrations of vinblastine as described by Currier *et al.* (1989). All MDR cell lines were grown regularly under selection conditions, in the presence of the appropriate colchicine concentration. Before any test with the Pgp blockers, cells were grown without colchicine for 2 generations.

The L1210MDR cells expressed 8×10^3 Pgp per cells, the 3T3 cells 55×10^3 and the KB-V1 cells over 100×10^3, as determined by labeling the cells with FITC conjugate of MRK16 monoclonal antibody (MAb) and by flow cytometry. As standards, beads with defined numbers of MAb binding sites were used (Flow Cytometry Standards, Research Triangle Park, NC).

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Verapamil, rhodamine 123 and Cremophor EL were from Sigma (St. Louis, MO). PSC833 was obtained from Sandoz (Basel, Switzerland) through a standard agreement.

Cell proliferation assay

The L1210MDR cells were seeded at 0.25×10^6 cells/ml in complete RPMI medium in 24 well plates. Different concentrations of Pgp blockers were added in DMSO (<0.1% v/v) followed in 15 min by D (0.01 μg/ml). Cell proliferation was assessed by cell counting on a Coulter Counter (Coulter, Miami Lakes, FL) after 24 hr incubation. Results are expressed according to Ross *et al.* (1994) as percent change in cell survival: 100 × (number of cells remaining following treatment with D plus blocker − number of cells remaining following treatment with D)/number of cells remaining following treatment with D.

The 3T3 cells were also seeded at 0.25×10^6 cells/ml. Treatment of these cells with the different drugs was the same as for the L1210 cells. Cell counts were performed also at 48 hr, and results of these determinations essentially reflected the same results as the 24 hr ones. Therefore, our assay was standardized with the 24 hr timing.

Both MDR cell lines were grown regularly under selection conditions, in the presence of colchicine. Before any test with the Pgp blockers, cells were grown without colchicine for 2 generations.

Fluorescence substrate uptake assay

The fluorescence uptake assay was carried out as described earlier (Weaver *et al.*, 1993). In brief, parental and *MDR1* gene expressing cells were adjusted to 10^6/ml in serum-free RPMI medium. To measure Pgp function in these cells, we used a fluorescent substrate of Pgp, rhodamine 123 (R123), at a concentration of 0.1 μM. This fluorescent substrate was used before for screening of effective P-glycoprotein blockers. Test compounds, the different Pgp blockers, were added to both parental and MDR cell suspensions in combination or singly for 10 min at room temperature. At this point, R123 was added and cell suspensions were incubated for an additional 20 min at room temperature. Cells were then washed in the centrifuge and analyzed for fluorescence intensity in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The time period of 20 min was chosen for incubation because loading of the MDR cells reached a plateau in less than this time and a significant difference in fluorescence could be observed between MDR and parental cells. Fluorescence intensities are recorded in arbitrary units. For the 3T3 and the KB cells the same protocol was used as for the L1210 cells. The effect of combined use of suboptimal doses of Pgp blockers was calculated by the equation:

$$E = (I_{ab} - I) - [(I_a - I) + (I_b - I)]$$

where I is the mean fluorescent intensity of the histogram obtained without any blocker or with blocker *a* or *b*. If $E = 0$, then the effect is additive; if $E > 0$, then the effect is more than additive, *i.e.*, synergistic.

Motional freedom of fatty acid spin probes in the plasma membrane of L1210 cells by electron spin resonance (ESR) spectrometry

To assess the influence of the different Pgp blockers on the motional freedom, the ESR membrane probe 5-doxyl stearic acid (5-dox-SA) (Sigma) was inserted into L1210MDR cell membranes as described earlier (Aszalos *et al.*, 1985). In brief, cells were treated at a concentration of 2×10^6/ml PBS with the Pgp affecting drugs at room temperature for 30 min. Then treated or untreated cells were concentrated by centrifugation to 2×10^6 cells/20 μl PBS. This cell suspension was added to a glass conical test tube containing at the tip 8×10^8 molecules of 5-dox-SA. The test tube was incubated at 37°C for 20 min. This time is needed to get all spin label into the cellular membranes. After incubation, the cell suspension was sucked into a 50 μl micropipet capillary (Clay Adams, Becton Dickinson, Parsippany, NJ) and sealed with Critoseal. The capillary was placed into the cavity of a Varian (Palo Alto, CA) E-9 Century series spectrometer, operated at 9.5 GHz, with 20 mW microwave power, 100 kHz field modulation, 4 gauss modulation amplitude and 100 gauss sweep range. The temperature of the cavity was 22°C. Each experiment was carried out 5 or 6 times with fresh cells, otherwise under identical conditions.

Evaluation of the ESR spectrum

The order parameter (S) and the polarity factor (a'_N) are calculated from the measured ESR experimental components of the motional averaged nitrogen hyperfine tensors, $2T'_{\parallel}$ and $2T'_{\perp}$. The equation used for these calculations and the definitions are from Butterfield *et al.* (1974):

$$S = \frac{(T'_{\parallel} - T'_{\perp})a_{NxL}}{(T_{\parallel} - T_{\perp})_{xL}a'_N}$$

Motional freedom of maleimide nitroxide spin probe labeled proteins in the membrane of L1210MDR cells by ESR spectrometry

Spin labeling of the membrane proteins with maleimide nitroxide (maleimid tempo, Sigma) was done essentially as described previously (Grof and Belagyi, 1983). In brief, 10 μl of ethanolic solution of maleimide nitroxide (2 mg/ml) was taken to dryness in a conical glass centrifuge tube. To this tube the suspension of L1210 cells (3×10^7/ml) in 50 μl PBS was added. After 20–30 min reaction time at 22°C, the suspension was pelleted in 5 sec. The pellet was resuspended and pelleted repetitively until the last supernatant contained no measurable spin label (3 or 4 times). The ESR signal of the final pellet was measured in a 50 μl micropipet capillary as described above for the 5-dox-SA probe.

Electron spin resonance oximetry

Close chamber ESR spin label oximetry was performed essentially as described earlier (Hyde and Subozynski, 1989) and was used here to measure the oxygen consumption of L1210MDR cells in the presence of different Pgp blockers. In brief, L1210MDR cells were treated with POCO (0.1% v/v), PSC833 (10 μM) or verapamil (5 μM) or with the solvent in RPMI for 20 min at room temperature. Then 20 μl of the spin probe, 3-carbamoyl-2,5-dihydro-2,2,5,5-tetramethyl-1h-pyrrol-1 (CTPO) (Sigma), at a concentration of 0.14 mM was added to 5×10^5 cells in 20 μl PBS, and this cell suspension was transferred to a quartz capillary. Spectra were taken by a Varian E-9 Century series spectrometer, periodically. Gas exchange between the sample and the atmosphere was negligible. The ESR spectra were recorded at 22°C. The superhyperfine structure of the center line of the nitroxide was recorded at 1 mW microwave power and 0.05 gauss field modulation. The K parameter, which depends on the superhyperfine structure of the center line and in turn on the concentration of oxygen in the cell suspension, was used to analyze spectra according to Hyde and Subozynski (1989); derivation of the K parameter is shown in Figure 4*b*.

Membrane potential measurements

Membrane potential measurements were carried out essentially as described earlier (Damjanovich *et al.*, 1987). Accordingly, 10^6 L1210MDR cells were washed in the centrifuge and resuspended in phenol red-free RPMI at room temperature. This cell suspension was incubated with the different Pgp blockers or the solvent (DMSO) for 10 min. Then 140 nM bisoxanol (Molecular Probes, Eugene, OR) was added, and the fluorescence intensity of 10^4 cells was measured on the FACScan flow cytometer (Becton Dickinson) at 2 min time. Suspending the cells in 50 mM K^+ buffer (substitution of NaCl

with KCl) depolarized the cells (shifted the fluorescence intensity higher), indicating that the bisoxonal dye measured plasma membrane potential.

Fluorescence recovery after photobleaching (FRAP)

Fluorescence recovery after photobleaching was carried out essentially as described earlier (Szabo *et al.*, 1992). In brief, lateral mobility of MRK-16-FITC, fluorescine isothiocyanate labeled MAb specific for P-glycoprotein, was measured on the plasma membrane of viable L1210MDR cells on the ACAS 570 system (Meridian Instruments, Okemos, MI) using the FRAP data collection and analysis computer routine. The round cell analysis algorithm was used. Step size was 0.5 μM and 20 to 40 cells were analyzed per condition. Cells were treated with POCO (0.1% v/v) or verapamil (5 μM) or the solvent DMSO 10 min before analysis started. Five cells were analyzed in a Cunningham chamber at once, followed by a fresh preparation. Control experiments measured the mobility of NBD-phosphatidyl choline (Avanti Polar Lipids, Birmingham, AL), which probe inserts into the plasma membrane. Results are expressed by the diffusion constant, D (cm^2/sec).

Affinity labeling of P-glycoprotein

One microliter [^{3}H]azidopine (Amersham TRK279, Arlington Heights, IL; 44 Ci/mmol; 1 μCi) was added to 10^6 3T3MDR cells in 100 μl cold PBS, without Ca^{2+} and Mg^{2+}, containing 1% Aprotinin solution (Sigma). Subsequently, 1 μl from: 2 mg/ml or 10 mg/ml D, 0.01 mg/ml or 0.1 mg/ml N-octanoyl-D, DMSO (control), POCO or 1:10 or 1:100 or 1:1000 dilutions of POCO in DMSO were added to individual cell suspensions. All samples were allowed to incubate at room temperature in the dark for 1 hr. The tubes were then placed on ice and exposed to UV (365 nm) light for 45 min. The tubes were spun at 1000 *g* in a microcentrifuge for 3 min, the supernatant was removed and the pellets were frozen on dry ice. The compound N-octanoyl-D was described earlier (Aszalos *et al.*, 1995).

Cells were lysed by resuspending in 50 μl TD buffer (10 mM Tris, pH 8.0, 0.1% Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 10 μg/ml DNase, 1 mM DTT) and subjected to 3 freeze-thaw cycles (dry ice and 37°C). The tubes were further incubated for 10 min at 37°C to complete the DNase digestion. The samples were then spun for 5 min in a microcentrifuge at 4°C. The supernatant was removed and added to 25 μl 3× SDS-PAGE sample buffer (180 mM Tris, pH 6.8, 6% SDS w/v, 36% glycerol v/v, 0.005% bromophenol blue and 2.1 M β-mercaptoethanol). The samples were heated at 42°C for 45 min and analyzed by SDS-PAGE on 7.5% acrylamide w/v, 0.26% N,N-methylenebisacrylamide resolving gels followed either by Western blot analysis using the MAb C219 (1:1500 dilution) or by autoradiography (Enlightning; DuPont-NEN, Wilmington, DE).

RESULTS

Effect of combination of suboptimal concentrations of P-glycoprotein blockers on cell proliferation of MDR1 *gene transfected cells*

Cell proliferation assays were carried out in order to assess the effect of combinations of suboptimal concentrations of 2 chemically distinct Pgp blockers on 2 cell lines transfected with the *MDR1* gene.

For the L1210MDR cells the combination of 2 Pgp blockers at suboptimal concentrations (verapamil: 0.03 μM, POCO: 0.002% and PSC833: 0.03 μM) resulted in antiproliferative effects similar to those maximally achievable with an optimal dose of a singly used blocker in the presence of 0.01 μg/ml D (Table I). This dose of D affected cell proliferation, expressed as % cell survival, to a modest degree only, about 6% (Table I). This fact indicates that a much higher dose of it would be necessary to suppress proliferation to the extent that is achievable when blockers are used simultaneously. Suboptimal concentrations of the 3 blockers were selected on the basis of the fluorescence substrate uptake assay and on preliminary cell culture studies. The best effect in change of cell survival was achieved with the combination of the suboptimal concentrations of PSC833 and POCO: 45%. The combinations acted synergistically as calculated by the method of Ross *et al.* (1994). For example, the decrease in cell survival rate with the suboptimal concentration of PSC833 (0.03 μM) was 9.7 ± 2.5% and that with POCO (0.002% v/v) was 18.0 ± 4.8%. When combined, their effect was 45.0 ± 4.2%. Combination of suboptimal concentrations of these blockers without D affected cell proliferation slightly, *i.e.*, 6.2 to 10.8%. Singly used suboptimal concentration of each blocker in combination with D had modest effects, 9.7 to 18%. Parental L1210 cells had a decrease of 40% with the dose of D (0.01 μg/ml) and this decrease was not affected by Pgp blockers (not shown).

Similar results were obtained with the 3T3MDR cells. D alone, in a concentration of 0.01 μg/ml, resulted in about 5% change in cell survival. Two concentrations of PSC833 (0.06 and 0.08 μM) and 2 concentrations of verapamil (0.4 and 0.5 μM) with D resulted in between 9.5 and 19% dose dependent changes in cell survival (Table I). The dose of 0.04% POCO in combination with D resulted in 26% decrease in cell survival. The different combinations of suboptimal concentrations of Pgp blockers with D affected cell survival additively or nearly additively and close to the extent of the singly used optimal dose blockers with D. Combination of suboptimal concentra-

TABLE I - DECREASE IN CELL SURVIVAL OF L1210MDR AND 3T3MDR CELLS TREATED WITH CLINICALLY USED OPTIMAL AND SUBOPTIMAL DOSES OF VERAPAMIL, POCO AND PSC833 AS P-GLYCOPROTEIN BLOCKERS AND WITH THE CYTOTOXIC DRUG DAUNORUBICIN[1]

Daunorubicin (μg ml)	PSC833 (μM)	Verapamil (μM)	POCO (%)	% Decrease in cell survival (±SD)
L1210MDR				
0.01	—	—	—	6.0 ± 3.1
0.01	0.03	—	—	9.7 ± 2.5
0.01	—	0.03	—	7.0 ± 3.4
0.01	—	—	0.002	18.0 ± 4.8
0.01	0.03	—	0.002	45.0 ± 4.2
0.01	0.03	0.03	—	30.0 ± 5.3
0.01	—	0.03	0.002	28.2 ± 3.7
0.01	5.0	—	—	50.0 ± 5.8
0.01	—	5.0	—	36.5 ± 6.1
0.01	—	—	0.1	30.0 ± 4.7
—	0.03	—	0.002	10.8 ± 2.9
—	0.03	0.03	—	6.2 ± 1.8
—	—	0.03	0.002	9.4 ± 3.1
NIH3t3MDR				
0.01	—	—	—	5.0 ± 3.0
0.01	0.06	—	—	9.5 ± 1.5
0.01	0.08	—	—	16.5 ± 2.2
0.01	—	—	0.04	26.0 ± 4.2
0.01	—	0.4	—	15.2 ± 4.0
0.01	—	0.5	—	19.0 ± 3.3
0.01	0.06	—	0.04	35.0 ± 3.0
0.01	0.08	—	0.04	40.4 ± 7.4
0.01	—	0.4	0.04	42.5 ± 3.1
0.01	—	0.5	0.04	48.0 ± 1.8
0.01	0.06	0.4	—	25.5 ± 7.6
0.01	0.06	0.5	—	28.0 ± 6.8
0.01	0.08	0.5	—	34.0 ± 3.8
0.01	5.0	—	—	26.0 ± 2.2
0.01	—	5.0	—	28.0 ± 3.1
0.01	—	—	0.1	39.0 ± 2.5

[1]Cells, seeded at 0.25 × 10^6/ml, were treated with the blockers for 15 min before D treatment (0.01 μg/ml). Survival was assessed by cell counting at 24 hr incubation time, and percent decrease in cell survival was calculated as given in the Material and Methods section and by Ross *et al.* (1994). Results represent the average of 3 independent assays, each in duplicate.

tions of Pgp blockers and without D had minimal effects on the change of cell survival rate of the 3T3MDR cells, less than that of the L1210MDR cells (not shown). The suboptimal doses of individual blockers used with the 3T3 cells were higher than for the L1210MDR cells, reflecting the higher number of Pgp expressed in the 3T3MDR cells (55×10^3 *vs.* 8×10^3 /cell). These final doses, shown in Table I, were derived by preliminary experiments and from the results of the fluorescence uptake assay. The selected 2 suboptimal doses of verapamil (0.4 and 0.5 μM) and of PSC833 (0.06 and 0.08 μM) show dose dependency in suppression of cell survival when used singly or in combination. Again, the effect of D was not modified by Pgp blockers in the parental 3T3 cells (not shown).

No proliferation assay was performed with the KB-V1 cells.

Effect of combination of suboptimal concentrations of P-glycoprotein blockers on the fluorescent substrate rhodamine 123 uptake by MDR1 *gene transfected cells*

Comparison of R123 uptake was made in the 3 parental and the corresponding *MDR1* gene expressing cell lines treated with optimal and suboptimal concentrations and the combination of the studied 3 Pgp blockers (Fig. 1). Optimal doses of the blockers, when used individually or in combination, restored the R123 uptake to approximately that of the parental in L1210MDR cells (Fig. 1*a*). The optimal doses used in combination restored the R123 uptake to about the same degree as the singly used optimal doses of verapamil (5 μM), POCO (0.1%) or PSC833 (5 μM). However, combinations with PSC833 resulted in somewhat less R123 uptake than with the singly used blockers. These optimal doses were selected based on clinical uses of these blockers (Salmon *et al.*, 1991; Raderer and Scheithauer, 1993; Webster *et al.*, 1993; Bohme *et al.*, 1994) and from preliminary experiments with 3T3MDR cells. Suboptimal doses of the blockers used singly restored R123 uptake only partially, whereas the combinations (POCO plus verapamil, POCO plus PSC833 or verapamil plus PSC833) restored it to or close to the level of the parental cells. Suboptimal doses of the blockers were the same in this assay as in the cell proliferation assay (verapamil: 0.03 μM; POCO: 0.002% v/v; PSC833: 0.03 μM). A high dose of verapamil, 5 μM, did not affect R123 uptake into L1210 parental cells. Combinations of suboptimal doses of the Pgp blockers acted additively, as calculated by the equation given in the Material and Methods section (data not shown).

Qualitatively, the same types of results were achieved with the 3T3MDR cells using the same suboptimal doses of blockers as with the L1210MDR cells, except for PSC833: 0.06 μM (Fig. 1*b*). The extent of blocking Pgp was less than with the L1210MDR cells and was less than with singly used optimal doses of the blockers. The effectiveness of the combination of these suboptimal doses of blockers is less, possibly due to the 7-fold higher number of Pgp expressed by the 3T3MDR cells than by the L1210 cells (55 *vs.* 8×10^3/cell). The singly used optimal doses of blockers restored R123 uptake to the level of that of the parental cells. Combinations of optimal doses with PSC833 resulted in lower R123 uptake than with PSC833 alone. Combination of suboptimal doses of the Pgp blockers acted close to additively, as calculated by the equation given in the Material and Methods section (not shown).

Higher suboptimal concentrations of the blockers were necessary to achieve additive or more than additive effects with the KB-V1 cells (verapamil: 0.9 μM; POCO: 0.01% v/v; PSC833: 0.4 μM) (Fig. 1*c*). The higher concentrations were necessary possibly due to the great number of Pgp expressed by these cells (over 100×10^3/cell). Even the combinations of these higher suboptimal concentrations did not achieve as much R123 uptake as singly used optimal concentration of PSC833 (5 μM).

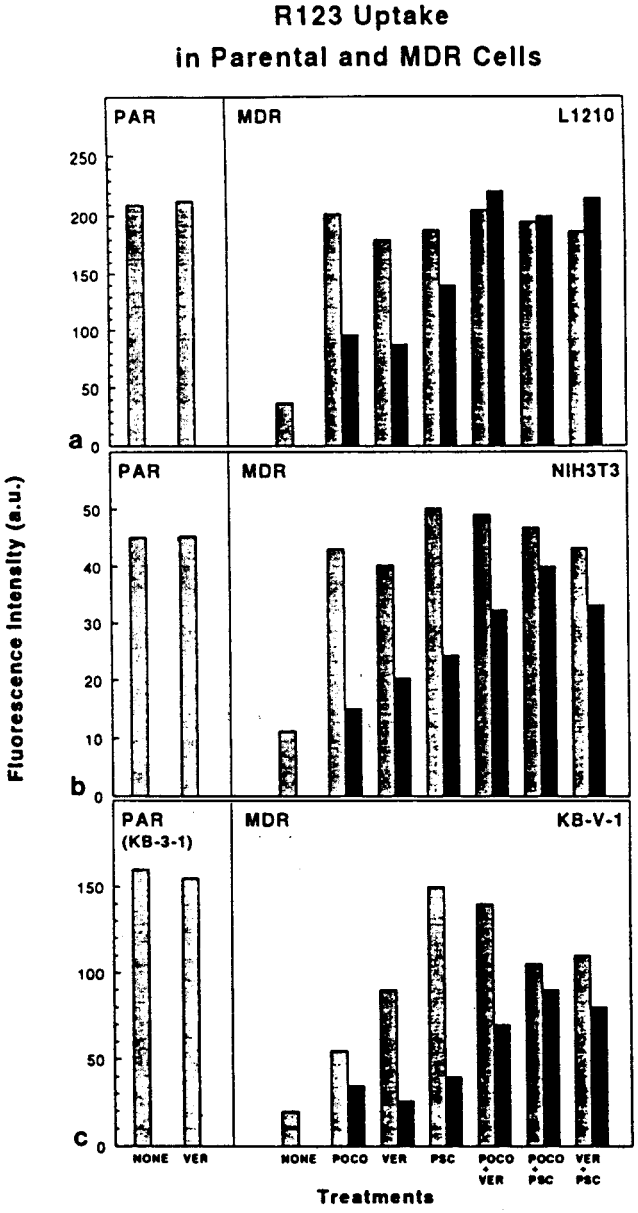


FIGURE 1 – Uptake of the fluorescent substrate rhodamine 123 into parental and *MDR1* gene expressing L1210, 3T3 and KB-V1 cells in the presence and absence of various doses and combinations of P-glycoprotein blockers. Shaded bars represent mean fluorescence intensities of histograms obtained with clinically optimal doses of blockers (verapamil, 5 μM; POCO, 0.1% v/v; PSC833, 5 μM). Solid bars represent mean fluorescence intensities of histograms obtained with suboptimal concentrations of blockers. *(a)* L1210MDR cells: verapamil, 0.3 μM; POCO, 0.002% v/v; PSC833, 0.03 μM. *(b)* 3T3MDR cells: same as for L1210MDR cells except PSC833, 0.06 μM. *(c)* KB-V1 cells: verapamil, 0.9 μM; POCO, 0.01% v/v; PSC833, 0.4 μM. Typical results from 3 or 4 separate experiments. Histograms were obtained with 5×10^3 cells for each cell type.

POCO and PSC833, but not verapamil, change the motional freedom of ESR spin probes in the plasma membranes of L1210MDR cells

ESR spectra were obtained for the L1210 MDR cells treated with the optimal concentrations of verapamil (5 μM), POCO (0.1% v/v) and PSC833 (5 μM). The spin probe, 5-doxyl-SA,

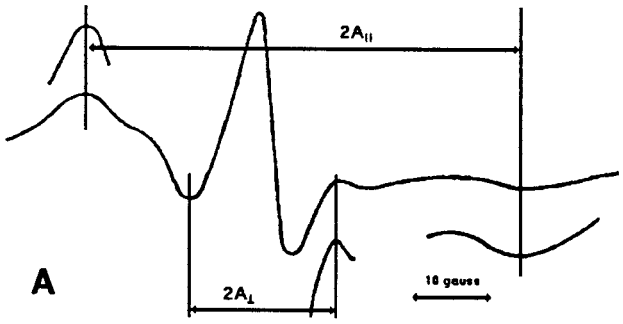
was used at a concentration that produced spectra without interference from contribution of free spin probe signal. Results indicate that significant changes could be obtained in the order parameter (motional freedom of the spin probe) with POCO and PSC833 (Fig. 2*b*). POCO treatment decreased the order parameter significantly from that of the control, 0.558 to 0.501, and PSC833 increased the order parameter to 0.606 in a scale of 1 (solid) to 0 (complete fluidity). Verapamil had no effect on the order parameter of 5-doxyl-SA; $S = 0.571$.

POCO, but not verapamil, changes the motional freedom of maleimide nitroxide labeled membrane bound proteins in L1210 cells

The motional freedom of membrane bound proteins was assessed from parameters of maleimide spin probe reacted with sulfydryl groups of membrane proteins. There are 2 types of sulfhydryl groups that bind this spin label: easily accessible mobile sulfhydryl groups and slow-moving sulfhydryl groups seeded deep in the hydrophilic regions of membranes (Grof and Belagyi, 1983). Therefore, the ESR spectra indicate the motional freedom of both types of sulfhydryl groups connected to membrane bound proteins. The slow motion of the proteins can be characterized by the outer hyperfine coupling tensor, $2T'_{\parallel}$. Larger $2T'_{\parallel}$ could mean less motional freedom or less polar vicinity of the ESR probe. A typical spectrum obtained with L1210MDR cells is shown in Figure 3. The measured $2T'_{\parallel}$ values for untreated cells were 65.1 ± 0.5 gauss ($n = 6$), for POCO (0.1% v/v) treated L1210MDR cells 66.1 ± 0.5 gauss ($n = 5$) and for verapamil (10 μM) treated cells 64.9 ± 0.5 gauss ($n = 3$). These results suggest that POCO, but not verapamil, decreases the motional freedom of spin probes attached to membrane bound proteins, or that POCO shifts the protein bound spin probes to a less polar environment. We prefer the second possibility.

POCO accelerates, PSC833 slows down and verapamil does not alter oxygen consumption of L1210MDR cells

Oxygen consumption of 5×10^5 L1210MDR cells was measured in the presence and absence of optimal concentrations of verapamil (5 μM), PSC833 (5 μM) and POCO (0.1% v/v) with ESR oximetry, as described in the Material and Methods section (Fig. 5). The initial ESR spectrum of the probe is shown in Figure 4*a*. Calculations of K values were done according to Hyde and Subozynski (1989) and as illustrated in Figure 4*b*. Results indicate that clinically relevant doses of POCO accelerate, PSC833 slow and verapamil do not alter the O_2 consumption of the treated cells. Figure 5 shows typical results with one L1210 MDR cell preparation ($n = 4$).



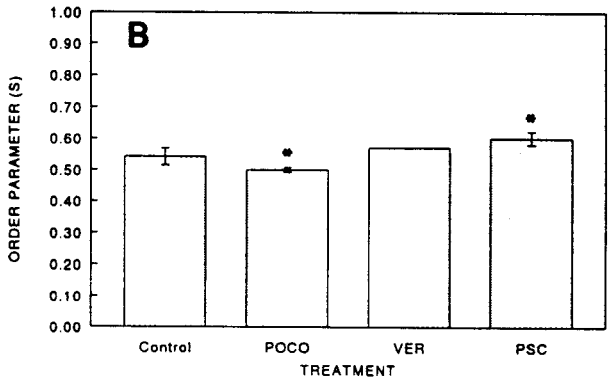


FIGURE 2 – *(a)* Typical electron spin resonance spectrum of 5-dox-SA inserted into L1210MDR cell membrane. *(b)* Order parameters, S, calculated according the equation given in the Material and Methods section and by Butterfield *et al.* (1974). Concentrations of P-glycoprotein blockers were verapamil, 5 μM; POCO, 0.1% v/v; PSC833, 5 μM. Each type of measurement was made 5 or 6 times with fresh cells; SDs are shown on the bars. Significant changes ($p = 0.05$) are marked by *.

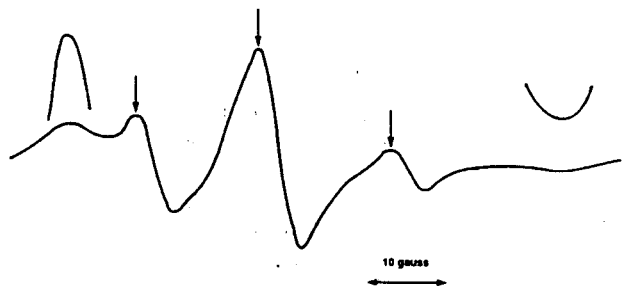


FIGURE 3 – Typical electron spin resonance spectrum of maleimide nitroxide bound to proteins in the membrane of L1210MDR cells. Hyperfine splittings are accentuated by the partial spectrum produced by higher receiver gain setting. The distance between these splittings was used to calculate 2T' tensors as given in the Results section. Arrows indicate spectral contributions from freely moving labeled sulfhydryl groups.

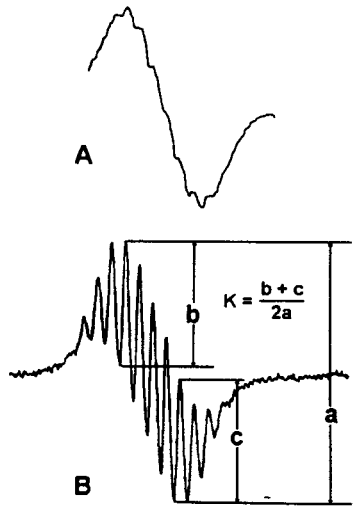


FIGURE 4 – *(a)* Initial electron spin spectrum of CTPO (0.07 μM) in the suspension of L1210MDR cells. *(b)* Final electron spin resonance spectrum of CTPO (no more spectral change). Parameters, *a*, *b* and *c*, used in the calculation of the K value are indicated.

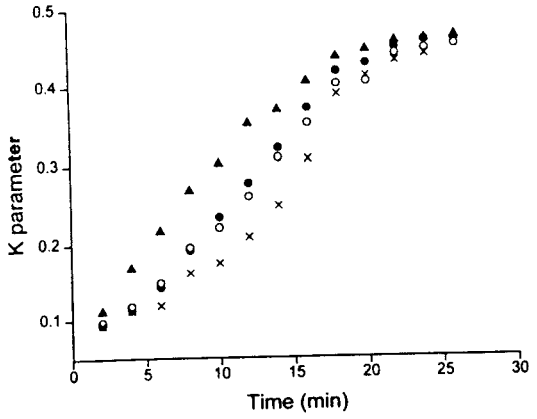


FIGURE 5 – Calculated K values, obtained from equation shown on Figure 4*b*, of spectra of the electron spin resonance oximetry probe CTPO in L1210MDR cell suspension. No drug, ○; verapamil, 5 μM, ●; POCO, 0.1% v/v, ▲; PSC833, 5 μM, ×.

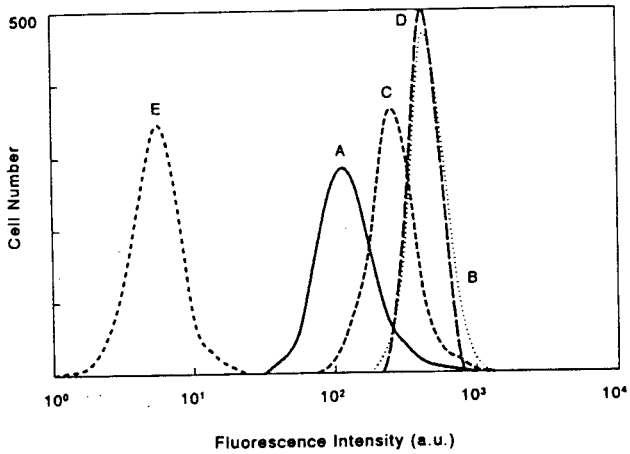


FIGURE 6 – Histograms obtained with fluorescent membrane potential indicator dye bisoxonol (140 nM) in P-glycoprotein treated and untreated L1210 cells. *(a)* Untreated. *(b)* Untreated in 50 mM K^+ buffer. *(c)* PSC833, 5 μM. *(d)* Verapamil, 5 μM. *(e)* POCO, 0.1% v/v. Each histogram was obtained with 5×10^3 cells. One representative result of 3 independent assays.

POCO hyperpolarizes and verapamil and PSC833 depolarizes L1210MDR cells

Shifts in the membrane potential of L1210MDR cells were measured in the presence and absence of the 3 Pgp blockers: verapamil (5 μM), POCO (0.1% v/v) and PSC833 (5 μM). In a typical experiment, 50 mM K^+ buffer treatment of the cells depolarized the cells as expected (Weaver *et al.*, 1993), *i.e.*, shifted the mean fluorescence intensity of the histogram higher, to channel number 456.6 (arbitrary scale), from that of the control cells, channel number 136.3. POCO (0.1% v/v) hyperpolarized the cells (channel 5.96), whereas verapamil (5 μM) and PSC833 (5 μM) depolarized the cells (channels 477.5 and 285.6, respectively). The corresponding histograms are shown in Figure 6. With these treatments the scattergram of the cells did not change significantly (not shown). Similar results were obtained with 2 additional fresh cell preparations.

Verapamil and POCO do not change significantly the lateral diffusion of P-glycoprotein in the membrane of L1210MDR cells

Lateral diffusion of Pgp in the plasma membrane of L1210MDR cells was measured to detect possible differences in this biophysical parameter after treatment of the cells with 2 blockers, verapamil and POCO. These 2 blockers affected plasma membrane "fluidity" (motional freedom of spin probe) differently, as shown above, and differences in the lateral diffusion of membrane bound proteins could be expected. First, we measured the lateral diffusion of the membrane probe NBD-phosphatidylcholine, to standardize our assay. The lateral diffusion of this probe in the plasma membrane of L1210MDR cells was $2.1 \pm 0.6 \times 10^{-8}$ cm^2/sec, in agreement with published values. The diffusion constants of MRK16-FITC MAb bound to Pgp in untreated and in verapamil or POCO treated cells clustered around $5\text{–}6 \times 10^{-8}$ cm^2/sec, without significant differences (Fig. 6). For the untreated cells, $D = 5.7 \pm 0.63 \times 10^{-8}$ cm^2/sec ($n = 20$), and the use of 2 concentrations of verapamil gave values of $D = 6.05 \pm 0.82 \times 10^{-8}$ cm^2/sec ($n = 20$) for 0.02 μM and $D = 4.51 \pm 0.86 \times 10^{-8}$ cm^2/sec ($n = 22$) for 10 μM. The 3 doses of POCO used, 0.01, 0.05 and 0.1% v/v, showed dose-dependent increases (4.04 ± 0.53, 4.15 ± 1.29, $5.16 \pm 1.33 \times 10^{-8}$ cm^2/sec, respectively, $n = > 12$ for each) that were not statistically significant ($p < 0.01$) (Fig. 6).

POCO blocks affinity labeling of P-glycoprotein

POCO dose dependently blocks [^{3}H]azidopine photolabeling of Pgp in 3T3MDR cells (Fig. 7). D, a substrate, and N-octanoyl-D, a non-substrate (Aszalos *et al.*, 1995) of Pgp, also block affinity labeling. The nonsubstrate, N-octanoyl-D, blocks affinity labeling more effectively at a 100-fold lower concentration than the substrate D.

DISCUSSION

Several strategies are being developed to circumvent multidrug resistance to cancer chemotherapeutic agents. One important strategy is to block the function of Pgp, which is one of the causes of multi-drug resistance, without causing additional toxicity along with the concomitantly used cancer chemotherapeutic agents. Because most blockers used individually and at optimal concentrations have some toxicity (Lampidis *et al.*, 1986; Woodcock *et al.*, 1990; Bowers *et al.*, 1991; Webster *et al.*, 1993; Bohme *et al.*, 1994), we have investigated the possibility of using a combination of suboptimal doses of Pgp blockers in *MDR1* gene transfected cells. For our investigation we have chosen 3 chemically distinct Pgp blockers already in development as single agents in the clinic. These 3 blockers differ also in their effect on plasma membranes, as we have shown by several biophysical methods. We postulate that this strategy may circumvent toxicities of individually used blockers in clinical use.

One possible mechanism of action of Pgp, the membrane bound protein responsible for enhanced efflux of drugs from cancer cells, is to function as a proton pump (reviewed by Gottesman *et al.*, 1991). It is also known that changes in the ion distribution across the plasma membrane can affect the membrane potential (Damjanovich *et al.*, 1987). Furthermore, correlations were shown between shifts of membrane potential and blocking of Pgp function before (Vayuvegula *et al.*, 1988). For these reasons we determined the effect of the studied 3 Pgp blockers on the membrane potential of L1210MDR cells. We have found that POCO at the useful clinical dose of 0.1% v/v hyperpolarized these cells (Fig. 5). Contrary to this, clinical doses of verapamil (5 μM) and PSC833 (5 μM) depolarized these cells. These results indicate that there are differences among the modes of action of these Pgp blockers at the plasma membrane level of L1210 MDR cells. They also indicate that shifting the membrane potential higher by a Pgp blocker does not necessarily correlate with blocking Pgp function in all types

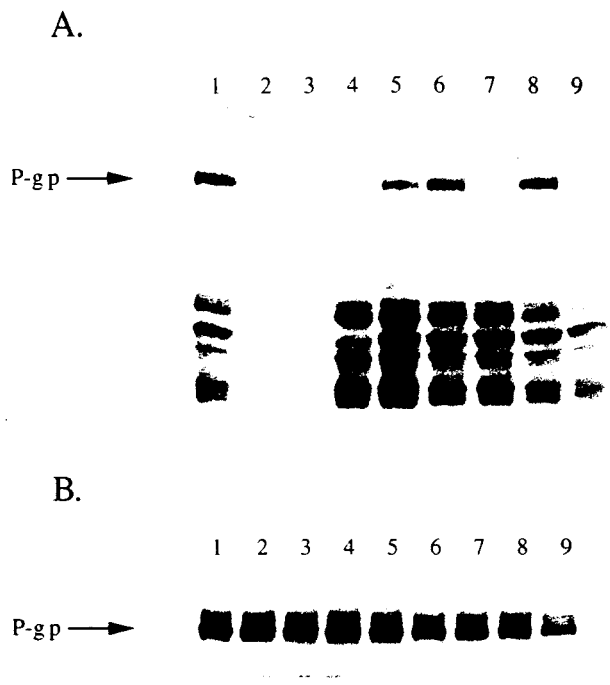


FIGURE 7 – *(a)* [^{3}H]Azidopine photoaffinity labeling of intact 3T3MDR cells. Cells were labeled and samples prepared as described in the Material and Methods section. After SDS-PAGE on 7.5% acrylamide gel, the gel was fixed in 30% methanol/10% acetic acid v/v for 30 min, treated with Enlightning for 30 min and autoradiographed. Lane 1, DMSO control; 2, concentrated POCO; 3, POCO 1:10 dilution; 4, POCO 1:100 dilution; 5, POCO 1:1000 dilution; 6, 0.01 mg/ml N-octanoyl-daunorubicin (D); 7, 0.1 mg/ml N-octanoyl-D; 8, 2 mg/ml D; 9, 10 mg/ml D. *(b)* Western blot analysis of each of the above samples. Samples were run on a 7.5% SDS-PAGE gel and transferred to a 0.45 μm nitrocellulose membrane. The blot was probed with the MAb C219 (1:1500 dilution), and the signal was detected by ECL (Amersham).

of MDR cells, as could be interpreted from previous studies (Vayuvegula *et al.*, 1988). However, one may speculate from our results that the different toxicities of the studied agents may be in part due to their different effect on ion fluxes across plasma membranes.

The 3 studied Pgp blockers cross or intercalate plasma membranes. It was shown before that the modulation of the physical status of the plasma membrane by Pgp blockers can affect the function of this protein (Callaghan *et al.*, 1993; Friche *et al.*, 1993). We determined the influence of these 3 blockers on the "viscosity" of L1210MDR cell membranes by measuring the motional freedom of the spin probe 5-doxyl-SA inserted into the plasma membrane and that of maleimide nitroxide bound to proteins in the plasma membranes. We found that clinical doses of verapamil do not influence either of these biophysical parameters (Fig. 2*b* and Results). Contrary to this, clinical doses of PSC833 and POCO decrease and increase the motional freedom of 5-doxyl-SA, respectively. Our finding with PSC833 is in good agreement with the previous observation that CsA, a close chemical relative of PSC833, decreases the motional freedom of 5-doxyl-SA in the membranes of human lymphocytes (Damjanovich *et al.*, 1987).

Our results indicating that POCO decreases the membrane fluidity are also in agreement with the fluorescence anisotropy measurements of Woodcock *et al.* (1992), who used the resistant leukemic cell line CCRF-CEM/R100 in their studies. POCO, but not verapamil, also shifts the maleimide spin probe bound proteins to a less polar environment (increase in T' value; see Results). We conclude from these experiments that verapamil, PSC833 and POCO used at clinically relevant doses affect the plasma membrane "fluidity" differently.

Measuring oxygen consumption of L1210 MDR cells under our experimental conditions revealed that POCO (0.1% v/v) increases and PSC833 (5 μM) decreases the oxygen consumption of these cells. Verapamil (5 μM) had no effect (Fig. 5). These findings also point in the direction that there are differences among these Pgp blockers in their mode of action on L1210MDR cells. It seems from the above results that there is a parallel between the ability of POCO to increase the motional freedom of the spin probe 5-doxyl-SA and the oxygen consumption and the ability of PSC833 to decrease the value of these 2 biophysical parameters. Verapamil did not affect either of these parameters.

The above oximetry results also indicate that the ATP metabolism of the L1210MDR cells may not be affected in the same fashion by the 3 Pgp blockers (Fig. 5). Combining this with the different effects of these blockers on the motional freedom of the 2 membrane spin probes and the membrane potential, one may argue that these agents affect the lipid environment of P-glycoprotein differently.

It was reported that modulation of membrane composition can effect Pgp function indirectly by altering membrane "fluidity" (Callaghan *et al.*, 1993). This is in agreement with our previous observations (Aszalos *et al.*, 1995) and with our present findings. A similar conclusion was reached by Jaffrezou *et al.*, (1995), who found that cationic amphiphilic drugs, besides blocking Pgp, additionally act on Pgp by altering lipid metabolism and thereby the lipid domain of the membrane. Pgp blockers influence substrate influx in MDR cells (Shalinsky *et al.*, 1993). It remains to be established whether the Pgp blockers used in our study influence, besides the efflux, also the influx.

In the next set of experiments we showed that a combination of suboptimal concentrations of Pgp blockers acts additively or synergistically in the uptake of the fluorescent substrate R123 (Fig. 1). The fluorescent substrate R123 was used to study P-glycoprotein efflux efficiency before (Weaver *et al.*, 1993). In 3T3MDR cells, the combination of these suboptimal concentrations of blockers restored R123 uptake to a lesser degree than in L1210 cells, because of the higher level of Pgp expression in these cells (Fig. 1*a*, *b*). With the KB-V1 cells, higher suboptimal concentrations of blockers were necessary than with the other 2 cell lines to achieve additive or synergistic effects with the combinations (Fig. 1*c*). We attribute this to the much higher number of Pgp expressed by these cells than by the other 2 cells used in this study. These facts also indicate that for total restoration, chemosensitization of any MDR cells, the proper suboptimal concentrations of blockers have to be found.

We have observed in 3T3MDR and KB-V1 cells (less pronounced in L1210MDR cells) that combinations of clinically used optimal concentrations of PSC833 with either POCO or verapamil negatively affect the R123 uptake (Fig. 1). We attribute this to the ability of PSC833 to alter the physical status, decrease the "fluidity" of plasma membranes (Fig. 2), and thereby possibly alter the passive diffusion of the second blocker and R123. This possibility is in agreement with the findings of Shalinsky *et al.* (1993), as mentioned above.

Cell proliferation assays confirmed our fluorescent substrate uptake assay results (Table I). For this assay we used the

cytotoxic Pgp substrate D. We found that combination of suboptimal concentrations of Pgp blockers decreased the cell survival rate to about the same level as singly used blockers at optimal doses in L1210MDR cells. The suboptimal doses of Pgp blockers were the same as in the fluorescent substrate uptake assay. Combination of the blockers alone (without D) had some moderate antiproliferative effects (Table I). Results of cell survival rate calculations according to Ross *et al.* (1994) indicate that with the suboptimal doses used in our studies more than additive effects could be achieved.

Higher suboptimal concentrations of Pgp blockers were needed with the 3T3MDR cell line than with the L1210MDR cell line to achieve maximal decrease of cell survival (Table I). These necessary higher doses reflect the higher number of Pgp expressed in 3T3MDR cells than in the L1210MDR cells (see Material and Methods section). The effect of the suboptimal doses of the blockers was additive. Suboptimal doses of blockers used in these experiments were found experimentally, and these doses are also suboptimal in clinical practice (Salmon *et al.*, 1991; Raderer and Scheithauer, 1993; Webster *et al.*, 1993).

We cannot rule out common action, substrate competition between daunorubicin and the studied 3 Pgp blockers, from our studies. All 3 block affinity labeling of Pgp (Safa, 1988; Friche *et al.*, 1990; Boesh *et al.*, 1991; Fig. 7). However, blocking affinity labeling sites on Pgp does not necessarily indicate substrate behavior, as was shown for several compounds before (Aszalos *et al.*, 1995). On the other hand, we could show that they act differently at the plasma membrane level and on the general metabolism, oxygen consumption, of L1210MDR cells. One can speculate that our findings with the L1210MDR cells may also be generally relevant to other cell lines and to primary cancer cells.

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