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Interaction of the P-Glycoprotein Multidrug Transporter (MDR1) with High Affinity Peptide Chemosensitizers in Isolated Membranes, Reconstituted Systems, and Intact Cells

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ABSTRACT. P-Glycoprotein-mediated multidrug resistance can be reversed by the action of a group of compounds known as chemosensitizers. The interactions with P-glycoprotein of two novel hydrophobic peptide chemosensitizers (reversins 121 and 205) have been studied in model systems *in vitro*, and in a variety of MDR1-expressing intact tumor cells. The reversins bound to purified P-glycoprotein with high affinity (77–154 nM), as assessed by a quenching assay using fluorescently labeled purified protein. The peptides modulated P-glycoprotein ATPase activity in Sf9 insect cell membranes expressing human MDR1, plasma membrane vesicles from multidrug-resistant cells, and reconstituted proteoliposomes. Both peptides induced a large stimulation of ATPase activity; however, higher concentrations, especially of reversin 205, led to inhibition. This pattern was different from that of simple linear peptides, and resembled that of chemosensitizers such as verapamil. In both membrane vesicles and reconstituted proteoliposomes, 1–2 μM reversins were more effective than cyclosporin A at blocking colchicine transport. Reversin 121 and reversin 205 restored the uptake of [^{3}H]daunorubicin and rhodamine 123 in MDR1-expressing cells to the level observed in the drug-sensitive parent cell lines, and also effectively inhibited the extrusion of calcein acetoxymethyl ester from intact cells. In cytotoxicity assays, reversin 121 and reversin 205 eliminated the resistance of MDR1-expressing tumor cells against MDR1-substrate anticancer drugs, and they had no toxic effects in MDR1-negative control cells. We suggest that peptides of the reversin type interact with the MDR1 protein with high affinity and specificity, and thus they may be good candidates for the development of MDR1-modulating agents to sensitize drug resistance in cancer. BIOCHEM PHARMACOL **58**;4:571–586, 1999.

KEY WORDS. peptide derivatives; azidopine photolabeling; ATPase activity; drug binding; drug transport; proteoliposomes; fluorescent dye transport; daunorubicin transport

In the current chemotherapeutic treatment of human cancer, the existence or development of multiple resistance against a wide range of anticancer drugs is a significant problem. Cytotoxic chemotherapy has proven to be an effective treatment for several human cancerous diseases, but in the case of so-called intrinsic or acquired multidrug resistance, antineoplastic compounds that are usually highly effective, e.g. vincristine, vinblastine, daunorubicin, or doxorubicin, fail to produce cures. One of the major causes of such multidrug resistance is the appearance of special integral membrane proteins in the tumor cells. The Pgp** multidrug transporter, or MDR1, catalyzes the ATP-

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** *Abbreviations*: AM, acetoxymethyl ester; α-MEM, α-minimal essential medium; BOC, *tert*-butyloxy-carbonyl; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfonate; CHO, Chinese hamster ovary; DINIB, 4,9-dihydro-3-isobutyl-2-methyl-1-(*p*-nitrophenacyl)-4–9-dioxo-1*H*-naphthyl[2,3-*d*]imidazolium bromide; DPPE, dipalmitoylphosphatidylethanolamine; FBS, fetal bovine serum; MDR, multidrug resistant; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OBzl, O-benzyl; OtBu, O-*tert*-butyl; PC, phosphatidylcholine; PCR, polymerase chain reaction; Pgp, P-glycoprotein; R121, reversin 121; R205, reversin 205; RCSF, Relative Curve Shift Factor(s); and Z, benzyloxycarbonyl.

dependent extrusion of various hydrophobic cytotoxic drugs, e.g. *Vinca* alkaloids and anthracyclines, as well as other natural antibiotics, and maintains their cellular level at a subtoxic concentration. Pgp belongs to the ABC superfamily of membrane transporters, whose members carry out ATP-dependent transport of substrates across membranes in prokaryotes and eukaryotes [1]. Studies using reconstituted purified Pgp *in vitro* have indicated clearly that the protein operates as a promiscuous efflux pump for hydrophobic compounds, powered by ATP hydrolysis [2–5]. Purified Pgp also displays a high level of constitutive ATPase activity, which can be modulated by various substrates [6–8]. The biochemistry and biology of Pgp have been the subject of several recent reviews [9–13].

Pgp is currently one of the best available targets for improvement of cancer chemotherapy. One approach is to modify the cytotoxic drugs to avoid their interaction with the multidrug transporter; another is to search for relatively non-toxic compounds that interfere with drug extrusion. A large number of compounds have been identified (referred to as chemosensitizers, MDR modulators, or reversers) that are able to restore the cytotoxicity of chemotherapeutic drugs to MDR cells *in vitro,* and in experimental drug-resistant tumors *in vivo* [14–16]. Chemosensitizers appear to compete, although in a complex and poorly understood way, for the substrate binding site(s) on Pgp, but there is still no clear understanding of their mechanism of action at the molecular level. At least two chemosensitizers, verapamil and cyclosporin A [17–19], themselves are transported by Pgp. There has been much interest in the use of chemosensitizing agents in the treatment of drug-resistant tumors, and many clinical trials have been carried out, or are ongoing, with chemosensitizing agents [15, 16, 20]. So-called "first generation" chemosensitizers, such as verapamil, suffered from the problems of inadequate effectiveness and high toxicity. Appreciation of these difficulties led to the development of improved "second generation" chemosensitizers, e.g. cyclosporin A, with 10-fold higher potency. However, toxicity still remains a serious problem [16, 21, 22], and the quest for better chemosensitizers continues.

MDR cells are also resistant to killing by hydrophobic peptides [23, 24], and several research groups have explored the interaction of peptides with Pgp. Some hydrophobic peptides are Pgp substrates, whereas others are not [24, 25]. Those peptides that interact with the transporter have been reported to stimulate ATPase activity and block drug transport in membrane vesicles and intact MDR cells [25, 26]. We have shown that a simple hydrophobic tripeptide is transported by Pgp in both native plasma membrane vesicles from MDR cells and proteoliposomes containing purified reconstituted Pgp [5]. More recently, we have demonstrated that both linear and cyclic peptides bind directly to purified Pgp, using a fluorescence quenching assay [27]. Short linear hydrophobic peptides, therefore, may represent a new class of compounds for consideration as potential chemosensitizers of lower toxicity than those in current use.

In the present study, we examined the interaction with Pgp of two novel hydrophobic peptide chemosensitizers (R121 and R205). The reversins are a family of di- and tripeptide derivatives consisting of naturally occurring L-amino acids with bulky aromatic or alkyl groups. The hydrophobic side chains of these peptides are expected to increase greatly the affinity of their interaction with Pgp. The increased size of the molecules is also expected to enhance their interaction with the transporter, which has large natural products among its preferred substrates. The relatively simple structure of these peptides allows their large-scale synthesis by classical peptide synthetic methods at relatively low cost.

In this work, we present some basic experimental data on the interactions of R121 and R205 with Pgp, using purified protein, simple membrane systems, and intact MDR1-expressing cells. Binding of the peptides to the transporter has been measured, using a fluorescence quenching technique, and the effects of the peptides on Pgp ATPase activity have been characterized. Low concentrations of the two reversins were able to block Pgp-mediated drug transport in both plasma membrane vesicles and proteoliposomes containing highly purified Pgp.

In the present study, we also have used relatively simple *in vitro* test systems to specifically assess the interactions of the reversins with the human multidrug transporter in intact cells. The first type of assay is based on the measurement of radiolabeled drug or fluorescent dye accumulation in intact cells. MDR1-expressing tumor cells actively extrude drugs (e.g. daunorubicin) and several fluorescent indicators. This MDR1-specific drug and dye extrusion is blocked by competing substrates and various inhibitors of the MDR1 transporter, whereas these agents have no effect on drug or dye accumulation in MDR1-negative cells [26, 28–30]. The other key assay used was the direct measurement of the reversal of drug resistance in cultured tumor cells. In the present study, we employed a test system developed in the National Cancer Institute to evaluate compounds potentially modulating multidrug resistance in cancer cells. This assay uses human tumor cell lines with variable expression levels of MDR1, and of various tissue origins, and applies a calculation method providing a quantitative measure of MDR1 modulation [31, 32]. All the results presented here strongly support the notion that reversins are, indeed, effective in restoring drug sensitivity of MDR tumor cells, and, thus, this class of compounds may be good candidates for the development of highly effective and specific agents for circumventing drug resistance in cancer.

MATERIALS AND METHODS

Materials

The synthesis of R121 [BOC-Asp(OBzl)-Lys(Z)-OtBu] and R205 {[BOC-Glu(OBzl)]$_2$-Lys-OMe} has been published in the European Patent Bulletin, Section 02.05.97 (European publication number: 0770091; International publication number: WO 95/31474; publication date: November 23,

1995). The molecular mass of R121 is 641.5 Da, and that of R205 is 798 Da. The reversins are practically insoluble in water, and freely soluble in DMSO or ethanol. Peptides were added to binding, transport, and ATPase assays as stock solutions in DMSO, and controls contained the appropriate level of solvent, which never exceeded 0.2% (v/v). This DMSO concentration had no inhibitory effects on ATPase activity or colchicine transport.

Cell Lines and Plasma Membrane Preparation

The MDR CHO cell lines CH^RB30 [33] and CH^RC5 [34] and the drug-sensitive parent cell line AuxB1 [34] were cultured as described previously [24, 35]. These cells express predominantly (> 95%) the hamster homolog of the human MDR1 gene product [8]. Plasma membrane vesicles from the CH^RC5 and CH^RB30 cell lines were isolated as reported earlier [36]. Recombinant baculovirus carrying the human *MDR1* gene was generated, and Sf9 (*Spodoptera frugiperda*) cells were infected and cultured according to the procedures described previously [37–39]. Virus-infected Sf9 cells were harvested, and their membranes were isolated and stored as reported by Sarkadi *et al.* [38]. *MDR1*-transfected mouse NIH 3T3 cells (NIH MDR1 G185) were prepared and characterized for their drug resistance as described previously [40, 41]. KBV1 (MDR1) and KB3 human tumor cells were cultured in Dulbecco's modified Eagle's medium; K562 human tumor cells were grown in RPMI medium, supplemented with 10% FBS [41].

ATPase Activity

The ATPase activity of isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation. In brief, a membrane suspension (~10 μg of membrane protein) was incubated at 37° in 100 μL of buffer containing 50 mM Tris-MES (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCl, and 5 mM NaN_3, and the ATPase reaction was started by the addition of 5 mM MgATP. The reaction was stopped by the addition of 100 μL of 5% (w/v) SDS solution, and the amount of P_i was determined immediately. ATPase activity was estimated by the difference in P_i levels obtained by a sensitive colorimetric assay [38, 39] between 0-min (reaction stopped immediately with SDS) and 20-min incubation periods. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μM) were determined. Isolated membranes of uninfected or β-galactosidase-infected Sf9 cells had no drug- or reversin-stimulated ATPase activity. Mg^{2+}-ATPase activity of Pgp in CHO cell plasma membrane vesicles and reconstituted proteoliposomes was determined by measuring the release of P_i from ATP, in the presence of 5 mM Mg^{2+}, as reported earlier [2, 25, 42].

[^{3}H]Azidopine Photoaffinity Labeling

Photoaffinity labeling of Pgp in CH^RC5 plasma membrane vesicles with [^{3}H]azidopine (200 nM, 52 Ci/mmol; Amersham) was carried out as described [24, 35], in the presence of increasing concentrations of reversins. Membrane proteins were analyzed by SDS–PAGE on a 7.5% gel, followed by autoradiography. The Pgp band intensities on the autoradiograms were quantitated by scanning densitometry, followed by analysis using the SigmaGel program (SPSS Inc.). The value of IC_{50}, the concentration producing 50% inhibition of azidopine photolabeling, was estimated for each compound from a plot of Pgp band intensity versus concentration.

Pgp Purification and Reconstitution

Pgp was purified to 95% purity by a procedure involving a differential two-step extraction of CH^RB30 plasma membrane with the zwitterionic detergent CHAPS, followed by removal of contaminant glycoproteins on concanavalin A-Sepharose [43]. Highly purified Pgp was reconstituted into lipid bilayer vesicles of 1:1 (w/w) egg PC:DPPE as previously described for partially purified protein [2]. The final proteoliposome lipid:protein ratio was ~45:1 (w/w). Protein was quantitated by the method of Bradford [44] for plasma membrane, and by the method of Peterson [45] for purified Pgp, using bovine serum albumin (crystallized and lyophilized, from the Sigma Chemical Co.) as a standard.

Binding of Reversins to Pgp

The affinity of binding of peptides to purified Pgp labeled with MIANS was determined using a fluorescence quenching procedure, as previously described for drugs, chemosensitizers, and peptides [27, 43]. Binding studies were carried out in the presence of 100-nm extruded vesicles of asolectin (soybean phospholipids). Fluorescence intensities were corrected for dilution, scattering, and the inner filter effect. Quenching data were fitted using nonlinear regression (SigmaPlot for Windows, SPSS Inc.) to an equation for interaction with a single type of binding site, and K_d values were estimated, as previously described [27, 43].

Colchicine Transport by Pgp in Vesicle Systems

Steady-state uptake of [^{3}H]colchicine into CH^RB30 plasma membrane vesicles or reconstituted proteoliposomes containing Pgp was determined using protocols previously developed in our laboratory [2, 5, 25, 46]. Briefly, membrane vesicles (30 μg protein) or proteoliposomes (0.8 to 1.6 μg protein) were mixed in a 100-μL final volume of transport buffer with 1 μM [^{3}H]colchicine, 5 mM Mg^{2+}, and 1 mM ATP, together with an ATP-regenerating system (creatine phosphate/creatine kinase). After various times at 23°, vesicles/proteoliposomes were harvested by rapid filtration on Whatman GF/F filters, and immediately washed

with 5 mL of ice-cold buffer. Filters were dried, and radioactivity was quantitated by liquid scintillation counting. Drug binding to filters and nonspecific uptake into vesicles/proteoliposomes were determined in the absence of membrane vesicles/proteoliposomes, and in the absence of ATP and a regenerating system, respectively. Transport inhibition data were analyzed using the median effect equation [47] as previously reported [25, 27, 48].

[^{3}H]Daunorubicin Accumulation in Intact MDR Cells

CH^RC5 or AuxB1 cells were plated at 10^5 cells/mL in 24-well plates (Nunc) and cultured for 3 days. Prior to the start of the assay, wells were rinsed twice with 1 mL of serum-free α-MEM. Serial dilutions of the reversins then were added in α-MEM, in a volume of 150 μL/well. Following a 20-min incubation at 37°, 150 μL of 0.25 μM [^{3}H]daunorubicin (DuPont-NEN; 1.9 Ci/mmol) was added, at a concentration of 0.1 μCi/mL. The plate was incubated for a further 30 min at 37°. Drug uptake was terminated by rapidly rinsing each well of the plate three times with 1 mL of ice-cold PBS. Cells were lysed with 0.2% (w/v) SDS in 20 mM Tris–HCl, pH 7.7. Radioactivity in the cell lysates was determined by scintillation counting. Daunorubicin uptake determinations were carried out in triplicate. The protein content of each well was measured using the Pierce bicinchoninic acid assay system (Pierce).

Rhodamine 123 Accumulation in Intact MDR Cells

CH^RC5 or AuxB1 cells were plated at 2×10^4 cells/well in 96-well plates and cultured for 2 days. Wells were rinsed twice with 200 μL of serum-free α-MEM, and reversins (100 μL/well) were added in α-MEM buffer at the required concentration. Plates were incubated at 37° for 20 min prior to the addition of 100 μL/well of 25 μM rhodamine 123 (Sigma) in α-MEM buffer. After further incubation of the plates for 30 min at 37°, uptake was terminated by rinsing the plate three times with 200 μL/well of ice-cold PBS. Cells were lysed with 20 mM Tris–HCl, pH 7.7, containing 0.2% SDS, and total rhodamine 123 accumulation was measured using a fluorescence plate reader (λ_{ex} = 485 nm, λ_{em} = 530 nm).

Fluorescent Dye Transport

Calcein fluorescence was measured [26, 29, 41] by incubating 2–5 $\times 10^5$ cells/mL in HPMI medium, containing 0.25 μM calcein AM (Molecular Probes), at 37° with gentle stirring in a Hitachi F-4000 fluorescence spectrophotometer (λ_{ex} and λ_{em} for calcein were 493 and 515 nm, respectively, with a band width of 5 nm). NIH 3T3 cells were trypsinized before the measurements and kept in suspension by gentle stirring. Due to MDR1-dependent calcein AM extrusion, free calcein accumulation was markedly slower in MDR1-expressing cells than in the control MDR1-negative cells, and inhibitors increased the rate of calcein accumulation to a large extent, up to the level seen in the control cells. In each experiment, two different concentrations of a modulator agent were applied, and then a maximum inhibition of calcein AM extrusion was achieved by the addition of 50 μM verapamil. Percent inhibition of MDR1 by the different modulator concentrations was calculated relative to the maximum inhibition value. In the MDR1-negative cells, neither verapamil nor the reversin concentrations employed had a significant effect on the rate of calcein accumulation (for details, see Ref. 29).

Tumor Cell Lines and Cytotoxicity

The toxicity of the reversins alone to CH^RC5 cells was determined using a MTT assay system [24, 35]. Cytotoxicity assays in tumor cells were carried out in 72-hr cultures in 96-well plates in the appropriate culture media [41, 49]. In the NCI tests for MDR1 antagonists, six cell lines from the anticancer drug screening panel (HCT-15, UO 31, CAKI-1, ACHN, OVCAR-5, and H-23), which represent a variety of levels of MDR1 expression and function, were utilized. None of these cell lines was artificially selected for drug resistance. The *mdr1* expression of each of the cell lines was measured by quantitative PCR using previously reported primers [50]. Reverse transcription and amplification of 125 ng of RNA were used to obtain an initial estimate of *mdr1* expression. Precise quantitation then was determined using serially diluted samples. Next, all levels of *mdr1* detected were expressed relative to a reference standard cell line, the colon carcinoma line SW620 (not from the NCI panel), which was included and amplified in every experiment, and was assigned an arbitrary value of 10. β_2-Microglobulin measurement by quantitative PCR also was included as an internal standard.

Rhodamine 123 efflux was measured [26] by allowing logarithmically growing cells to accumulate rhodamine 123 (0.5 μg/mL) for 30 min in the presence or absence of cyclosporin A (3 μg/mL). Efflux was then initiated by sedimentation and resuspension of the cells in rhodamine-free medium, with or without cyclosporin A, and was measured over 90 min. Both accumulation and efflux phases were measured by flow cytometric analysis of the rhodamine 123 green fluorescence. The rhodamine efflux value was taken to be the difference in channel number in the presence or absence of cyclosporin A [26].

In each set of experiments, the modulatory effects of reversins were compared with the effectiveness of two known modulating agents, verapamil and cyclosporin A, in increasing the toxicity of four different cytotoxic agents, 5-fluorouracil (serving as negative control for MDR1 modulation), vincristine, taxol, and DINIB as MDR1-interacting drugs. For these studies, cells were grown in RPMI with 5% FBS, plated in 96-well tissue culture plates, and incubated for 24 hr at 37°. MDR1 modulators were added at four or five different concentrations, the highest concentrations representing approximately the IC_{30} values. Four

hours later, six dilutions of the cytotoxic drugs were added, and then the cells were incubated for a further 48 hr. After this time, the cells were fixed and stained with sulforhodamine-B; absorbances representing the amount of cellular protein were recorded, and concentration–responses calculated [31]. To assess the effects of modulating agents, the Relative Curve Shift Factor (RCSF) values were calculated according to the following equation:

$$\mathrm{RCSF} = 100 \times \frac{((GD_1 - GA_1) + (GD_2 - GA_2) + \ldots + (GD_n - GA_n))}{\text{number of drug doses} \times 200}$$

where GD_n = percent growth of cells at cytotoxic drug concentration n in the control response, and GA_n = percent growth of cells at cytotoxic drug concentration n in the drug/modulator test response. A positive RCSF value indicates a shift towards increased toxicity in the case of a given drug, induced by the modulator agent. The sums of the RCSF values obtained for the five MDR1-positive cell lines for the different modulator agents also are presented.

RESULTS

Basic Data for Reversins

Reversins are di- or tripeptide derivatives consisting of L-amino acids containing protecting groups used in classical and solid-phase peptide chemistry. These hydrophobic side chains with their bulky aromatic or alkyl groups greatly enhance their interaction with the MDR1 transporter.* The chemical structures, and schematic views of possible three-dimensional structures, of R121 and R205 are shown in Fig. 1. This projection suggests that the atomic groups are not constrained. In a hydrophobic environment, intramolecular groups, e.g. aromatic rings, OtBu groups, or aliphatic side chains, may become closer because of their lipophilic character.

Binding of Reversins to Purified Pgp

Highly purified Pgp was modified covalently at two conserved cysteine residues (Cys 428 and Cys 1071), one within each Walker A motif of the nucleotide binding domains, using the fluorescence probe MIANS. Binding of reversins to labeled Pgp led to concentration-dependent quenching of the MIANS fluorescence (Fig. 2, A and B), as described previously for MDR drugs, chemosensitizers, and peptides [27, 43, 51]. Fitting of the fluorescence quenching titration data to an equation describing interaction with a single type of binding site (see Materials and Methods) led to estimation of the K_d values for the two reversin peptides. Both peptides bound to Pgp with very high affinity: 77 nM for R121 and 154 nM for R205 (Table 1). In both cases, the maximal fluorescence quenching as saturation was approached fell in the 7–8% range. These data indicate that the two reversin peptides bind directly to Pgp with very high affinity.

Inhibition of Azidopine Photoaffinity Labeling of Pgp by Reversins

Inhibition of photoaffinity labeling of Pgp by the drug substrate azidopine often has been used as an indicator that a compound competes for a common binding site on the multidrug transporter. Both R121 and R205 inhibited azidopine photolabeling of Pgp in CH^RC5 plasma membrane (Fig. 3), with IC_{50} values of 8 and 12 μM, respectively, compared with an IC_{50} of 50 μM for the chemosensitizer verapamil (Table 1). R121 and R205 inhibited azidopine photolabeling at concentrations considerably higher than those at which they were observed to bind to Pgp, stimulate ATPase activity, and block drug transport (see below).

Effect of Reversins on ATPase Activity of Pgp

Many Pgp substrates and chemosensitizers have been observed to modulate the ATPase activity of Pgp. When we examined the activation of MDR1 ATPase in isolated insect cell membranes expressing the MDR1 Pgp, we found that R121 and R205, similar to verapamil, strongly stimulated the ATPase (Fig. 4A). However, reversins acted at significantly lower concentrations than verapamil; the half-maximal activating concentration (K_a) of verapamil was around 2–3 μM, while the K_a values for R121 and R205 were about 80 and 40 nM, respectively. We also observed a strong inhibition of the Pgp ATPase activity at higher concentrations (above 1 μM) for R205, but not R121 (Fig. 4A). This is a clear-cut difference between the two reversins in terms of their interactions with Pgp that may be reflected in significant differences in their modulatory effects on drug transport.

The effects of reversins also were examined in native plasma membrane vesicles from CH^RB30 cells (which express hamster MDR1) (Fig. 4B) and proteoliposomes of 1:1 (w/w) egg PC:DPPE containing reconstituted Pgp purified from CHO cells (Fig. 4C). In both cases, a bimodal effect was observed, with stimulation of ATPase activity at lower reversin concentrations, which reached a maximum of 175–200% for plasma membrane and 150–175% for proteoliposomes. The concentration that led to maximal ATPase stimulation was slightly lower for R205 relative to R121 in both model systems, as was the concentration required for 50% maximal ATPase stimulation (Table 1). Higher reversin concentrations led to inhibition of Pgp ATPase, with background levels of activity approached at about 100 μM for both peptides in CH^RB30 membrane vesicles (Fig. 4B). In the proteoliposome system, R121 gave rise to less inhibition at high concentrations than it did in MDR cell plasma membranes (Fig. 4C), with the profile more closely resembling that for human MDR1 in Sf9 cell membranes.

* Seprodi J, Mezo I, Vadasz Z, Sarkadi B and Teplan I, Peptide derivatives against multidrug resistance. *24th European Peptide Symposium*, Abstr. P234, 1998.

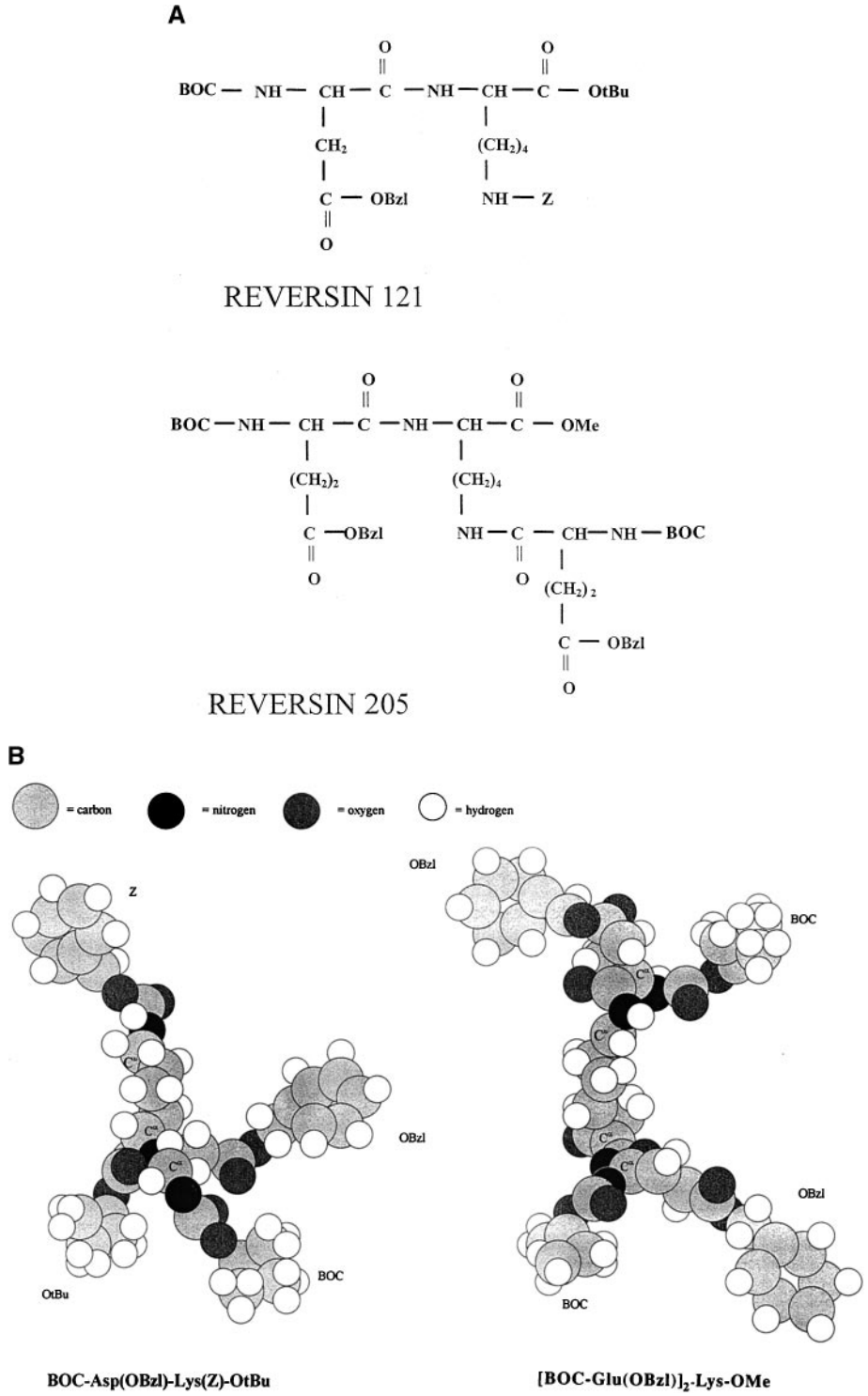

FIG. 1. Chemical structure (top panel) and schematic view (bottom panel) of one of the possible conformations of R121 and R205. The perspective projection of the structures of the reversins is based on the Stuart-Briegleb atomic model.

Inhibition of Colchicine Transport in Vesicle Systems by Reversins

MDR cell plasma membrane vesicles, and proteoliposomes containing reconstituted purified Pgp, have proven to be very useful tools in assessing the ability of various compounds to block drug transport by Pgp. Equilibrium uptake of [^{3}H]colchicine into CH^{R}B30 plasma membrane vesicles (Fig. 5A) and proteoliposomes containing purified CH^{R}B30 Pgp (Fig. 5B) was determined in the presence of increasing concentrations of the two reversins. In both systems, R121 and R205 showed very similar behavior; colchicine transport was 70–90% inhibited at 1 μM, and complete inhibition was observed at 5 μM. The ability of the simple protease inhibitor peptide calpeptin (Z-leucyl-

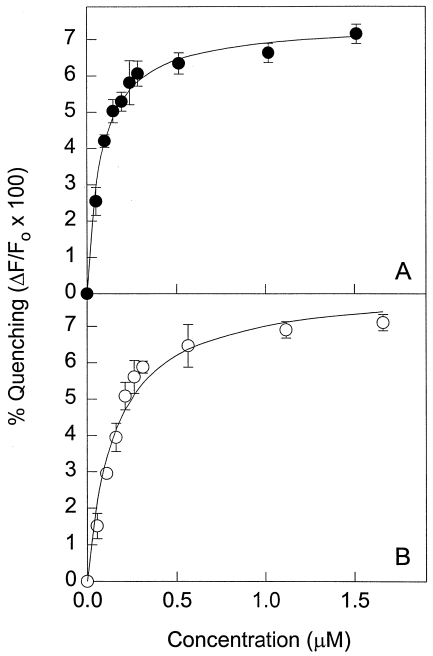

FIG. 2. Binding of reversins to purified MIANS-labeled Pgp as assessed by fluorescence quenching. Increasing concentrations of R121 (A) or R205 (B) were added to 50 μg/mL of MIANS-labeled Pgp (in 2 mM CHAPS buffer) at 22° in the presence of 0.5 mg/mL of asolectin, and the fluorescence emission at 420 nm was recorded. The percent quenching of fluorescence ($\Delta F/F_o \times 100$) was calculated at increasing peptide concentrations, relative to fluorescence of MIANS-labeled Pgp in the absence of reversins. The continuous line represents the best computer-generated fit of the data points (shown by the symbols) to an equation describing interaction of peptide with a single type of binding site (see Materials and Methods). Data are presented as means ± range for duplicate determinations.

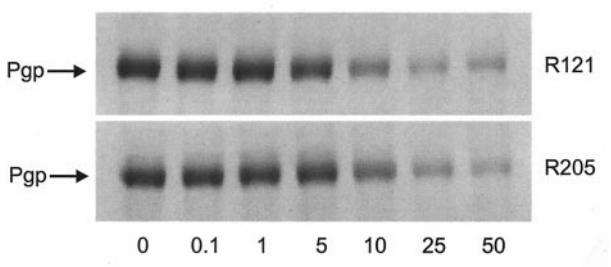

FIG. 3. Inhibition of azidopine photoaffinity labeling of native membrane-bound Pgp by reversins. Pgp in CH^{R}C5 plasma membrane vesicles was photolabeled with 200 nM [^{3}H]azidopine in the presence of increasing concentrations of R121 or R205, followed by SDS–PAGE/autoradiography. The only visible band was Pgp, with a molecular mass of 170–180 kDa (arrow). Concentrations of the reversins in micromolar are indicated for each lane along the bottom panel.

norleucinal) to inhibit colchicine uptake is shown for comparison (Fig. 5, A and B); much higher concentrations of calpeptin were needed to block drug transport.

We previously showed that median effect analysis can be used to evaluate Pgp transport inhibition data of this type [5, 25, 27, 48]. The median effect equation [47] describes the relationship between any concentration of a compound, D, and its effect on the system being studied, f_a. In this case, the inhibition of ATP-dependent [^{3}H]colchicine uptake into proteoliposomes containing purified Pgp was measured at various concentrations of the test peptides. Figure 6 shows median effect plots of log (f_a/f_u) versus log D for the proteoliposome transport data (presented in Fig. 5B) for the two reversins and calpeptin. In each case, the data fitted well to a straight line, and could be used to obtain the values of D_m (the peptide concentration causing 50% inhibition of drug transport; Table 1). The slope of the median effect plot gives the parameter m, which indicates the sigmoidicity of the dose–effect curve. The median effect plots for the reversins were closely parallel, and the value of m in each case was close to 1 (Table 1).

Restoration of Daunorubicin and Rhodamine 123 Uptake in Intact MDR Cells by Reversins

The uptake of the chemotherapeutic drug daunorubicin by drug-resistant and drug-sensitive CHO cells reached a maximum after a 30-min incubation at 37° in the presence of radiolabeled drug. Intact cells of the MDR1-expressing line CH^{R}C5 took up approximately 6-fold less [^{3}H]daunorubicin than the drug-sensitive parent line AuxB1. When CH^{R}C5 cells were treated with the two reversins, dauno-

TABLE 1. Parameters for interaction of reversins 121, 205, and verapamil with Pgp

Parameter	System	R121	R205	Verapamil
K_d (50% maximal fluorescence quenching)	Purified Pgp	0.077	0.154	2.4
IC_{50} (50% of inhibition of photolabeling)	CH^{R}C5 plasma membrane	8.0	12.0	50
K_a (50% maximal stimulation of ATPase activity)	Sf9 insect cell membranes	0.080	0.040	2.5
K_a	CH^{R}B30 plasma membrane	0.30	0.22	10.0
D_m (50% injhibition of colchicine uptake)	CH^{R}B30 plasma membrane	0.56	0.44	2.9
D_m	Pgp proteoliposomes	0.24	0.32	3.2
m	Pgp proteoliposomes	1.2	1.1	0.51

Parameters derived from the data presented in Figs. 2–6 are summarized. K_a, IC_{50}, K_a, and D_m values are given in μM. The slope of the median effect plot, m, is dimensionless. The experimental system used for determination of the various parameters is indicated for each.

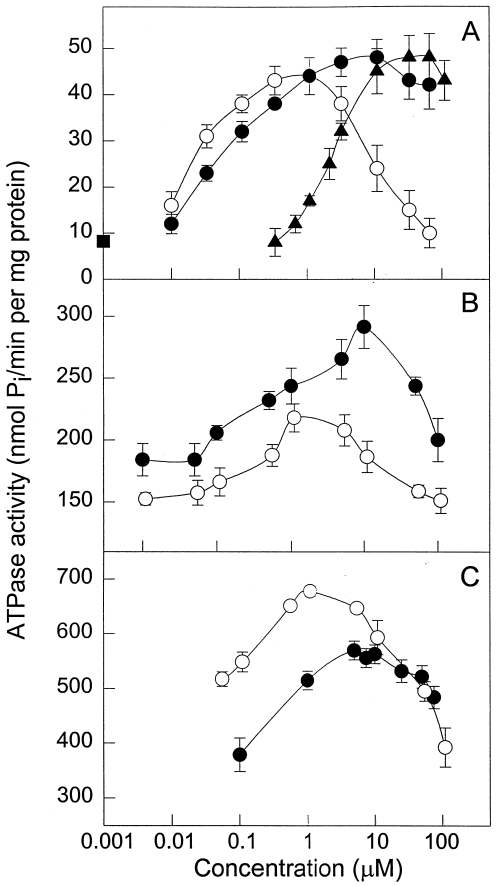

FIG. 4. Modulation of Pgp ATPase activity by reversins. (A) Vanadate-sensitive MDR1 ATPase activity in isolated membranes of Sf9 insect cells. The ATPase activity was determined for untreated membranes (■), and in the presence of verapamil (▲), R121 (●), and R205 (○). The differences between the ATPase activities measured in the absence and presence of 100 μM vanadate are plotted. Isolated membranes of uninfected or β-galactosidase infected Sf9 cells had no drug or reversin-stimulated ATPase activity. (B) ATPase activity of Pgp in CHO cell plasma membrane. CH^RB30 plasma membrane vesicles (1.5 to 2.0 μg protein) were assayed for Mg^{2+}-dependent ATPase activity in the presence of increasing concentrations of R121 (●) or R205 (○). (C) ATPase activity of purified reconstituted Pgp. Proteoliposomes of 1:1 (w/w) egg PC: DPPE containing purified Pgp (55 μg/mL of protein) were assayed for Mg^{2+}-ATPase activity in the presence of increasing concentrations of R121 (●) or R205 (○). Data points represent means ± SEM for triplicate determinations in representative experiments.

rubicin uptake was restored to levels seen for the drug-sensitive cells in a concentration-dependent manner (Fig. 7, A and B). R121 was substantially more effective than R205 at restoring drug uptake; drug accumulation approached that seen for drug-sensitive cells at a R121 concentration of ~ 5 μM, whereas a concentration of ~ 50 μM R205 was required to achieve the same effect. The concentrations producing 50% restoration of daunorubicin uptake were 2 and 11 μM for R121 and R205, respectively (Table 2).

In the next series of experiments, the uptake of another Pgp substrate, rhodamine 123, was assessed in CH^RC5 and

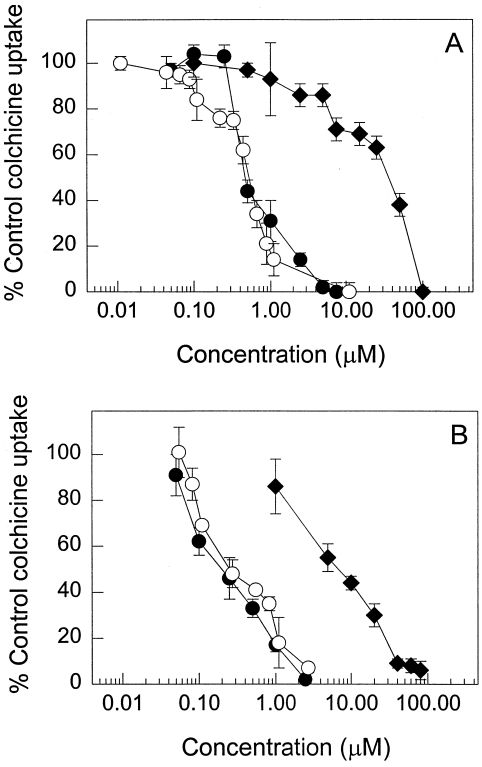

FIG. 5. Inhibition of colchicine transport by reversins. (A) Equilibrium uptake of 1 μM [^{3}H]colchicine into CH^RB30 plasma membrane vesicles was measured in the presence of 1 mM ATP with a regenerating system, and increasing concentrations of R121 (●), R205 (○), or calpeptin (♦). (B) Equilibrium uptake of 1 μM [^{3}H]colchicine into proteoliposomes containing purified reconstituted Pgp was measured in the presence of 1 mM ATP with a regenerating system, and increasing concentrations of R121 (●), R205 (○), or calpeptin (♦). Data are presented as percent of control ATP-dependent [^{3}H]colchicine uptake relative to membrane vesicles or proteoliposomes in the absence of peptides (means ± SEM, N = 3). One hundred percent values were 26.1 pmol/mg in (A) and 12.6 pmol/mg in (B).

AuxB1 cells using a fluorescence-based assay. In this assay similar results were observed: both R121 (Fig. 7C) and R205 (Fig. 7D) increased cellular uptake of the fluorescent

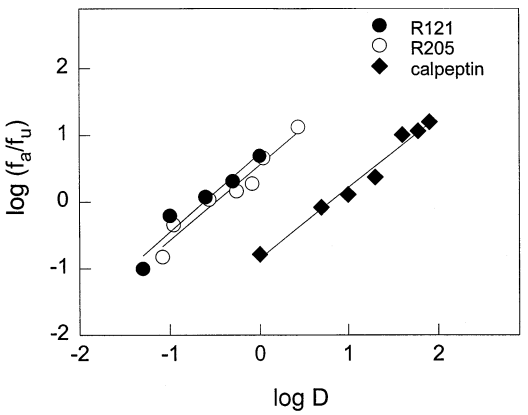

FIG. 6. Median effect analysis of proteoliposome transport inhibition data presented in Fig. 5. Median effect plots of log (f_a/f_u) versus log D are shown for R121, R205, and calpeptin. Each data set was fitted to a straight line by linear regression analysis.

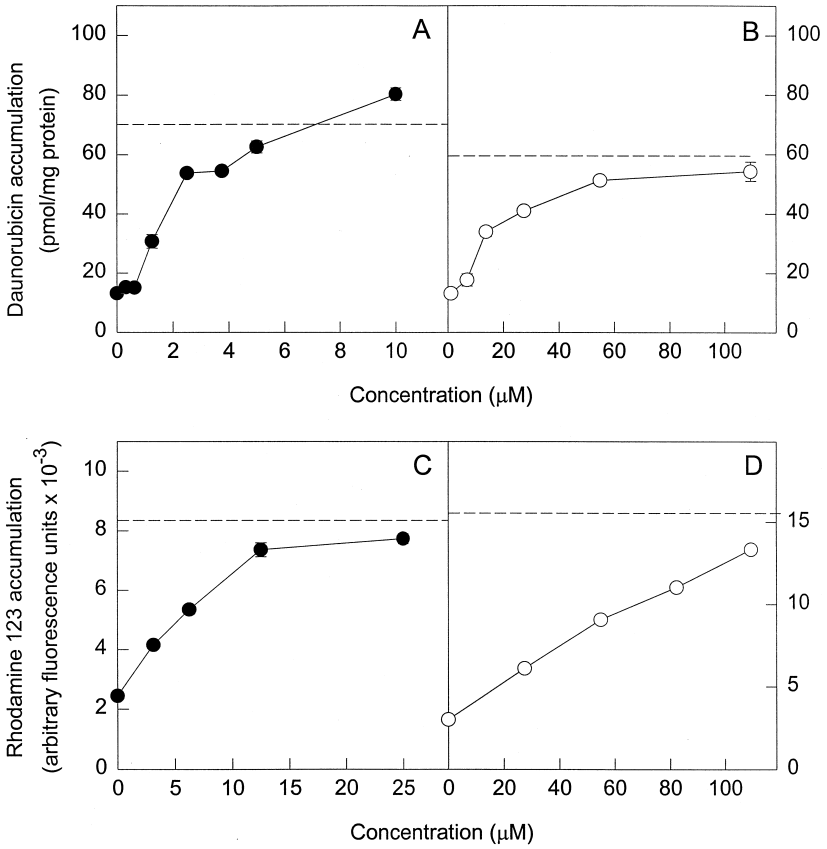

FIG. 7. Restoration of uptake of daunorubicin (A, B) and rhodamine 123 (C, D) in MDR1-expressing cells by reversins. CH^RC5 cells cultured in a 24-well plate were preincubated with various concentrations of R121 (●) or R205 (○), followed by the addition of [^{3}H]daunorubicin or rhodamine 123. Uptake of the compounds into intact cells was determined as described in Materials and Methods. Data are presented as means ± SEM (N = 3). Drug accumulation in the parent cell line AuxB1 was determined under the same conditions for each experiment, and is shown by the dashed lines (A, 70.2 ± 9.3; B, 59.5 ± 4.3; C, 8.34 ± 0.44 × 10^{-3}; D, 15.5 ± 1.0 × 10^{-3}; N = 3).

dye to levels approaching that of the drug-sensitive cell line in a concentration-dependent fashion. R121 again appeared to be more effective than R205 in reversing the dye accumulation defect in MDR cells (Table 2).

R121 had no effect on CH^RC5 cell viability at concentrations up to ~ 18 μM, at which it restored > 90% of the daunorubicin accumulation. R121 had an LC_{50} value of 33 μM, over 16-fold higher than the concentration required to restore drug accumulation to half of the value observed for drug-sensitive cells. Cell viability was unaffected by R205 at

TABLE 2. Parameters for reversal of drug accumulation and drug resistance in MDRI- expresisng cell lines by reversins 121 and 205

Cell line	Parameter	R121	R205
CH^RC5	Concentration restoring 50% uptake of [s3RH]daunorubicin	3.0	11
CH^RC5	Concentration restoring 50% uptake of rhodamine 123	2.5	40
NIH 3T3/MDR1	Concentration inhibiting calcein AM extrusion by 50%	0.4	0.3
KBV1	Chemosensitization index* at 2.5 μM	286	67
	Chemosensitization index at 5 μM	2000	333
HCT-15	Concentration producing		
	RCSF = 15 for vincristine	1.4	3.0
	RCSF = 15 for taxol	2.0	3.0
	RCSF = 15 for DINIB	1.7	3.0
Panel of four	Concentration producing		
	RCSF = 20 for vincristine	0.8	4.4
	RCSF = 10 for taxol	1.0	3.4
	RCSF = 50 for VINIB	1.7	4.2

Parameters given in Figs. 7–11 are summarized; all concentrations are given in micromolar. The cell line used for determination of the various parameters is indicated for each.
*Ratio of (IC_{50} without reversin)/(IC_{50} with reversin) [24, 35].

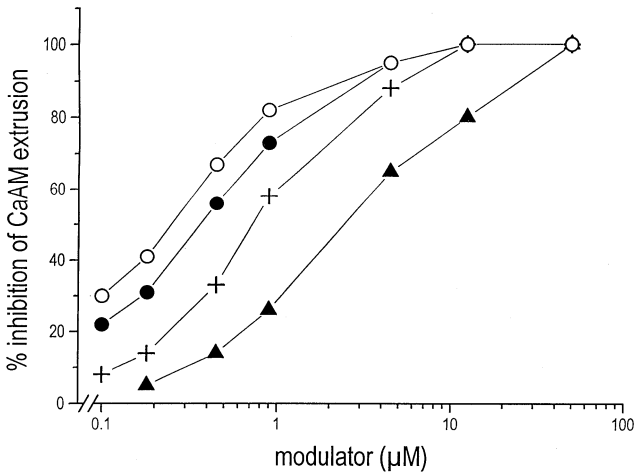

FIG. 8. Inhibition of calcein AM extrusion by reversins in NIH 3T3/MDR1 fibroblasts expressing the human MDR1 protein. Free calcein accumulation was measured as described in Materials and Methods. One hundred percent inhibition of calcein AM extrusion was achieved in each experiment by the addition of 50 μM verapamil. Data are shown as mean values obtained in 3 independent experiments. Modulators used were verapamil (▲), cyclosporin A (+), R121 (●), and R205 (○).

concentrations as high as 44 μM, at which daunorubicin accumulation was restored by > 50%, and the LC_{50} for cytotoxicity was 110 μM. The two reversin peptides are, therefore, relatively nontoxic in the concentration range within which they reverse drug resistance in CH^RC5 cells.

Inhibition of MDR1-Dependent Fluorescent Dye Extrusion by Reversins in Intact Cells

In mammalian cells expressing the MDR1 Pgp, the multidrug transporter actively extrudes the hydrophobic AM derivatives of certain fluorescent dyes, e.g. calcein. The accumulation of fluorescent free calcein under these conditions is modeling the uptake of cytotoxic drugs into the tumor cells, and the prevention of fluorescent dye accumulation directly reflects the function of the MDR1 protein [29, 30, 41]. Drug substrates and modulators of MDR1-dependent multidrug resistance, e.g. vinblastine, verapamil, or cyclosporin A, all were found to inhibit this MDR1-catalyzed dye extrusion in a concentration-dependent manner [30, 41].

As shown in Fig. 8, in mouse NIH 3T3 fibroblasts transfected with human *MDR1* cDNA and expressing human MDR1 protein, R121 and R205 showed effectiveness similar to that of verapamil for the inhibition of calcein AM extrusion, but acted at significantly lower concentrations. Verapamil inhibited dye extrusion with a K_i of about 3 μM; the K_i value for R121 was 0.4 μM, and for R205 this value was 0.3 μM (Table 2). For comparison, cyclosporin A, one of the better reversing agents currently known, had a K_i value of 0.8 to 1 μM in this assay system. In their effective concentration range, none of the above modulator agents had any effect on calcein accumulation in control MDR1-negative cells. We have repeated these

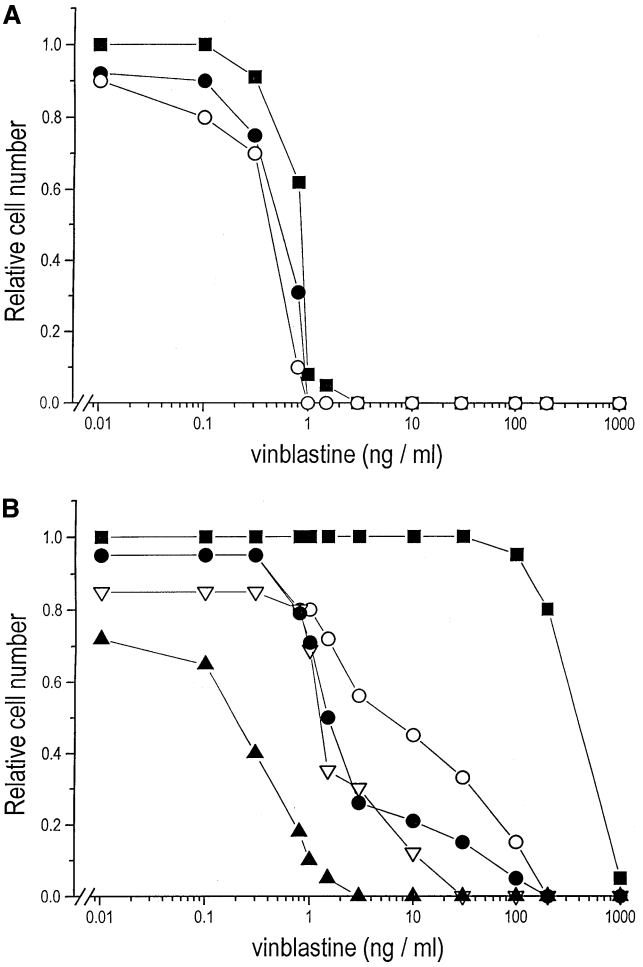

FIG. 9. Effects of R121 and R205 on the vinblastine sensitivity of drug-sensitive KB3 (A) and multidrug-resistant KBV1 (B) human tumor cells. Vinblastine cytotoxicity was measured as described in Materials and Methods; relative cell numbers after 72 hr are expressed as a function of vinblastine concentration in the medium. The data represent mean values obtained in 3 parallel experiments. (A) control (■); 5 μM R121 (●); and 5 μM R205 (○). (B) control (■); 2.5 μM R121 (●); 5 μM R121 (▲); 2.5 μM R205 (○); and 5 μM R205 (▽).

experiments using several different MDR1-positive human cell lines (K562 MDR1 leukemia cells, KBV1 epidermoid carcinoma cells, and MDR1-positive clinical samples of leukemic patients), and the results were essentially similar in all these cell types (data not shown). These experiments strongly suggest that R121 and R205 interacted with the MDR1 protein with high affinity and prevented MDR1-dependent dye extrusion in intact cells at submicromolar to micromolar concentrations.

Sensitization of Multidrug Resistance by Reversins in Cultured Tumor Cells

The *in vitro* multidrug resistance modulating action of reversins was examined by following the cytotoxic action of various drugs in MDR1-expressing cells. Figure 9 presents such an experiment using the drug-sensitive KB3 human

TABLE 3. Characterization of the cell lines used for modulator studies in the NCI test for MDR1 expression and function

Cell line	MDR1 expression*	Rhodamine efflux†
HCT-15	457	414
UO 31	749	244
CAKI-1	177	171
ACHN	31	120
OVCAR-5	0.1	13
H-23	0.1	−2

*Measured by quantitative PCR and expressed in arbitrary units (see Materials and Methods and [50]).

†Measured by rhodamine 123 efflux and expressed in artitrary units (see Materials and Methods and [26]).

tumor cell line and its multidrug-resistant, MDR1-expressing subline KBV1. As shown in Fig. 9A, at a concentration of 5 μM, neither R121 nor R205 had any measurable effect on the normal growth rate of KB3 cells or their sensitivity to vinblastine. In contrast, in the KBV1 cells (Fig. 9B) both R121 and R205 markedly increased the vinblastine sensitivity of the tumor cells, even at a concentration of 2.5 μM, and this effect was even more pronounced at a 5-μM modulator concentration (Table 2). We have repeated these studies with doxorubicin, etoposide, and vincristine as cytotoxic drugs, and the results were similar (data not shown). It is worth mentioning that whereas R205 had no effect on the growth of KBV1 cells in the absence of vinblastine, 5 μM R121 measurably (by about 25%) inhibited the growth of the MDR1-expressing cells in the absence of the cytotoxic agent.

These cytotoxicity experiments were extended and reinforced by detailed studies carried out using the National Cancer Institute drug resistance panel. We have examined the effects of R121 (NSC No. 667658) and R205 (NSC No. 667659) in six different cell lines, in combination with three different cytotoxic agents (vincristine, taxol, and DINIB). The cells were not artificially selected for drug resistance, and they expressed variable amounts of the MDR1 protein. The relative amounts of MDR1 expression and rhodamine efflux for the panel of cell lines are shown in Table 3. As discussed in detail [31, 32], the particular type of cumulative assay developed at the NCI has been specifically designed to avoid possible misleading effects of different amounts of Pgp, differences in the plasma membrane, and/or intracellular differences in the various cell lines. Thus, a significant modulatory effect by a given compound, when observed by this method of evaluation, can be considered a most promising indicator for the initiation of *in vivo* studies for MDR-reversing effects. We have compared the effectiveness of the reversins with that of verapamil and cyclosporin A in each case. Summaries of the NCI results obtained with the reversins are shown in Figs. 10 and 11 and Table 2.

HCT-15 cells express a large amount of functional MDR1 protein (see Table 3) and previously have been used successfully to test the drug resistance modulating effect of

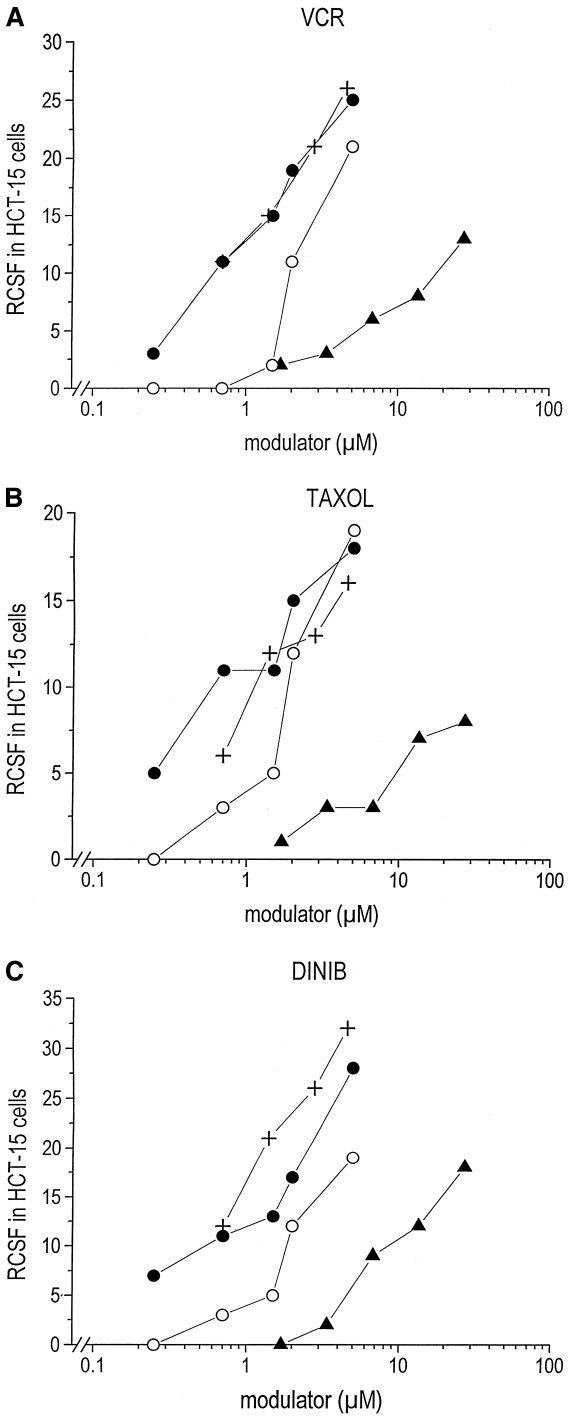

FIG. 10. Effects of verapamil, cyclosporin A, R121, and R205 as drug-resistance modulator agents on the growth of HCT-15 tumor cells (highly MDR1-positive; see Table 3) in the presence of various cytotoxic agents. The effects of modulators are represented as RCSF (see Materials and Methods; a positive change in the RCSF indicates an increased cytotoxic effect of the drug applied). The data show a representative experiment (repeated several times) with quadruplicate growth control and background wells, and duplicate drug test wells. (A) Modulation of the cytotoxic effect of vincristine (VCR). (B) Modulation of the cytotoxic effect of taxol. (C) Modulation of the cytotoxic effect of DINIB. Key: verapamil (▲); cyclosporin A (+); R121 (●); and R205 (○).

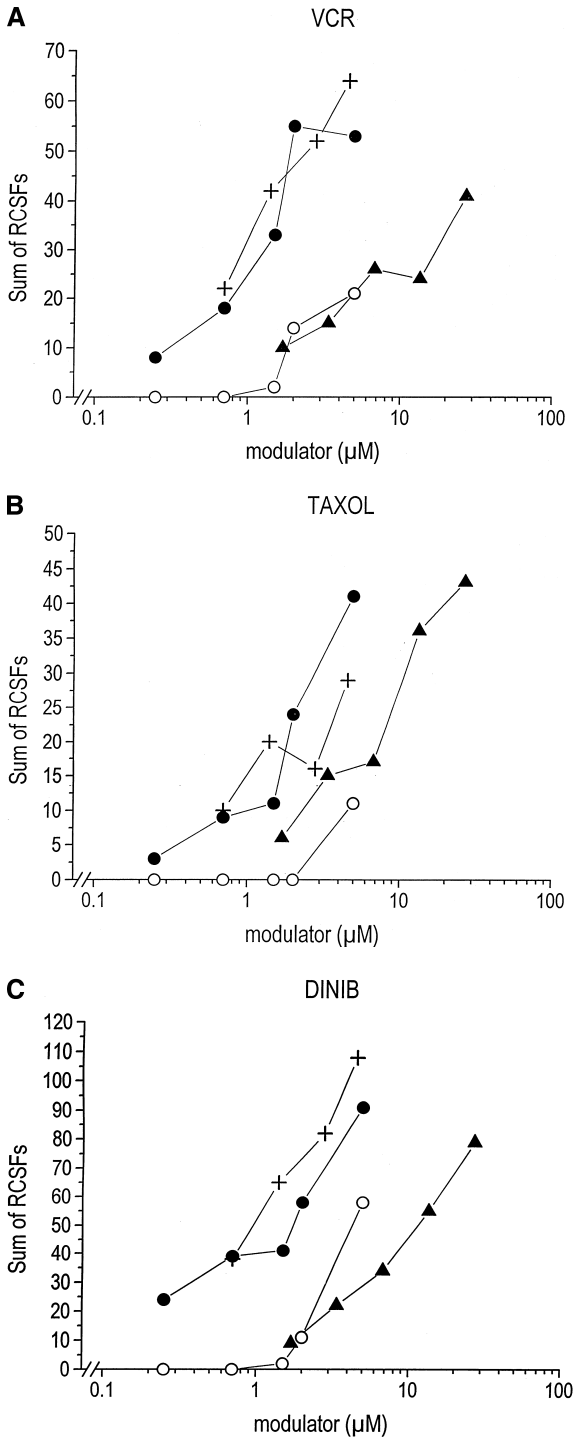

FIG. 11. Effects of verapamil, cyclosporin A, R121, and R205 as drug-resistance modulatory agents on the growth of different MDR1-positive tumor cells in the presence of the cytotoxic agents vincristine, taxol, and DINIB. The data show a representative experiment (repeated several times) with quadruplicate growth control and background wells, and duplicate drug test wells. Effects of modulators on the cytotoxicity of (A) vincristine (VCR), (B), taxol, and (C) DINIB, represented as the sum of RCSF in four different MDR1-positive cell lines (see Table 3). A positive change in the RCSF indicates an increased cytotoxic effect of the drug in the given cell type. Key: verapamil (▲); cyclosporin A (+); R121 (●); and R205 (○).

various agents [32, 52]. As shown in Fig. 10, the values for the RCSF, indicating drug sensitivity evoked by a modulator agent, were increased significantly by R121 and R205 with all three different cytotoxic drugs examined (vincristine, Fig. 10A; taxol, Fig. 10B; DINIB, Fig. 10C; Table 2). Verapamil was much less effective, whereas cyclosporin A was in most cases similarly effective to R121, which produced a large increase in RCSF in the 1–5 μM range. R205 usually was found to be less effective in these test systems than R121, although in the case of taxol, R205 had a modulating effect almost similar to those of cyclosporin A or R121.

Figure 11 provides a summary of the RCSF obtained in the four MDR1-positive cell lines studied (HCT-15, UO 31, CAKI-1, and ACHN; see Table 3), in which the expression levels of MDR1 varied greatly. Again, the same cytotoxic agents were tested as above (vincristine, Fig. 11A; taxol, Fig. 11B; DINIB, Fig. 11C), and in all cases verapamil and cyclosporin A served as known resistance modulating agents. As shown in Fig. 11, R121 was found to be a modulating agent with effectiveness similar to that of cyclosporin A with all the drugs tested, and the effective concentration range for R121 was between 0.5 and 5 μM (Table 2). R205 was found to be less effective in most cell types, although in the case of vincristine and DINIB it was still more effective than verapamil. Neither R121 nor R205 had a measurable effect on drug resistance in the essentially MDR1-negative cell line (H-23) examined in this panel. In addition, the two peptides had absolutely no modulating influence on the effects of the non-MDR spectrum agent 5-fluorouracil in either control or Pgp-expressing cell lines.

These data clearly demonstrate that the reversins (R121 and R205) were found to be effective modulating agents in a large variety of drug-resistant tumor cells. R121 had a drug resistance-reversing effect comparable to that seen by cyclosporin A, that is, at considerably lower concentrations than verapamil, whereas R205 in these studies was less effective, and had an effect comparable to that seen for verapamil.

DISCUSSION

In a preliminary screen, we tested a large number of peptide derivatives using the MDR1 ATPase assay, and found that the inclusion of hydrophobic side chains and an increased molecular size significantly increased their interaction with the multidrug transporter (Sarkadi B, unpublished data). From about 500 similar peptide derivatives examined, we selected two lead compounds, R121 and R205, for further investigation. The reversins are a family of novel di- and tripeptides with additional N- and C-terminal modifications designed to increase their affinity for the Pgp multidrug transporter. R121 is a simple dipeptide containing standard protecting groups used in classical peptide chemistry, and R205 is a similarly modified branched-chain tripeptide. Both compounds have relatively simple structures, which allows their synthesis on a large scale, using

classical peptide synthetic methods, at relatively low cost. R121 and R205 have somewhat different features with respect to their Pgp ATPase inhibition profiles (described above).

Both of these peptides proved to be effective chemosensitizing agents in MDR tumor cells *in vitro*, and also in transplantable tumor tests with rodents (see below), so it was of great interest to characterize their interactions with Pgp at the molecular level. In recent years, defined model systems have been developed that make it possible to examine the binding of chemosensitizers to Pgp, and their effects on both ATPase activity and drug transport. The various parameters describing the interaction of the reversins with Pgp in these systems are summarized in Table 1.

A novel fluorescence quenching technique developed in our laboratory was used to determine the K_d for binding of the reversins to purified Pgp. Binding affinity was very high, with K_d values of 77 and 154 nM for R121 and R205, respectively. These K_d values are substantially lower than those measured using the same technique for cyclosporin A (200 nM) [27], a chemosensitizer that has been used extensively in clinical trials in cancer patients. Very high-affinity binding to Pgp is clearly an important criterion for potential chemosensitizers, which is fulfilled in the case of the two reversins. The fluorescence quenching method has the potential to be a very useful tool in the identification of novel high-affinity chemosensitizers (for more details, see Ref. 51). The maximal fluorescence quenching observed for the two reversins was 7–8% of the initial fluorescence, which is at the low end of the range observed for over 60 Pgp substrates and chemosensitizers ([27, 51]; Liu R and Sharom FJ, unpublished data). It has been our general observation that both linear and cyclic peptides give maximal fluorescence quenching of less than 10% on binding to Pgp [27].

Pgp chemosensitizers and drug substrates have been observed to modulate the constitutive ATPase activity of Pgp. In general, chemosensitizers (e.g. verapamil) produce the largest stimulation of ATPase activity, whereas some chemotherapeutic drugs (e.g. vinblastine, daunorubicin) have little stimulatory effect, and may be inhibitory [2, 4, 6–8]. Many chemosensitizers give bimodal stimulation profiles. Stimulation occurs at low concentrations to reach a maximal level of ATPase activity, and inhibition takes place as the chemosensitizer concentration is increased [2, 4, 6–8]. It has been suggested that overlapping stimulatory (catalytic) and inhibitory sites exist within Pgp for substrates [53], and that the observed bimodal behavior is caused by interaction of a particular compound with the inhibitory site following occupancy of the stimulatory site. We have reported that many hydrophobic peptides identified as Pgp substrates, including simple tripeptides, produce only stimulation of ATPase activity, with no inhibition taking place at higher concentrations [5, 25, 27]. Using the two-site hypothesis, simple tripeptides would bind to the stimulatory site, but not the inhibitory site. The reversin peptides, especially R205, showed a bimodal ATPase activity profile in CHO cell membranes and reconstituted proteoliposomes, more closely resembling that of chemosensitizers than tripeptides. These results indicate that modification of the peptides with bulky hydrophobic functional groups may change the mode of interaction of the peptides with the transporter, so that they can occupy both the inhibitory and stimulatory sites. It is interesting that although the reversin ATPase stimulation profiles of human MDR1 in insect cell membranes resemble those of CHO cell membranes, the K_a values were markedly different (Table 1). Also, R121 gave rise to little inhibition of MDR1 ATPase activity at high concentrations, compared to hamster Pgp in native or reconstituted membrane systems. This may reflect differences in the interaction of the peptides with the human MDR1 protein relative to the hamster homolog, or it may result from the different lipid environment of the host membranes. Urbatsch and Senior [54] have already demonstrated that the profile of ATPase stimulation of purified Pgp by different drugs is affected greatly by the lipid mixture into which it is reconstituted.

The ability of the reversins to block drug transport was examined in plasma membrane vesicles and reconstituted proteoliposomes containing purified Pgp. In both native membrane vesicles and a simple lipid bilayer system, the reversins proved to be highly effective in blocking transport of [^{3}H]colchicine. The peptides appear to interact with Pgp to block drug transport in a very similar fashion in both native plasma membrane vesicles and reconstituted proteoliposomes, underscoring the fact that drug transport is due entirely to Pgp in these systems. Previous work in our laboratory has shown that transport inhibition data for many Pgp substrates (drugs, chemosensitizers, and peptides) can be analyzed using the median effect equation [5, 25, 27, 48]. The colchicine transport inhibition data for the reversins also fitted well to the median effect equation, allowing the extraction of D_m and m values. This analysis resulted in D_m values (50% inhibitory concentrations) in the range of 0.24 to 0.56 μM, which is much lower than D_m measured for the simpler modified dipeptide calpeptin. The additional structural modifications carried by the reversin peptides, therefore, appear to increase the strength of their interaction with Pgp. The D_m values for the reversins compare very favorably with the inhibitory concentration of 0.7 μM measured in the same system for cyclosporin A [25]. Values of m, which indicate the sigmoidicity of the interaction, were close to 1 for both reversins. Many of the linear and cyclic peptides previously examined also gave m values close to 1, although for cyclosporin A and *N*-acetyl-leucyl-leucyl-norleucinal, values close to 3 were obtained [25, 27]. The significance of the value of m is not currently clear, although it likely reflects the nature of the interaction between Pgp and the peptide at the molecular level.

We previously observed that cyclic peptides (e.g. cyclosporin A) were able to block azidopine photoaffinity labeling of Pgp in plasma membrane vesicles, whereas several linear peptides (e.g. *N*-acetyl-leucyl-leucyl-norleucinal, leupeptin) were unable to do so [5, 25]. This suggested the

existence of a separate binding site on Pgp for linear peptides which, unlike that for cyclic peptides, does not overlap or interact with the azidopine binding site. Both R121 and R205 inhibited azidopine photolabeling of Pgp. Therefore, modification of simple linear peptides by addition of aromatic rings, as in the case of the reversins, appears to modify their properties such that they no longer behave like simple linear peptides. However, inhibition of photolabeling by R121 and R205 took place at concentrations considerably higher than those at which they bind to Pgp, stimulate ATPase activity, and block drug transport in simple *in vitro* systems (Table 1). This difference in effective concentration range suggests that the site at which the reversins bind may be linked allosterically to the azidopine site, rather than overlapping with it directly.

The key mechanism of the MDR1 protein in causing multidrug resistance is the prevention of the uptake of cytotoxic agents into tumor cells. Therefore, the ability of the reversins to restore the uptake of chemotherapeutic drugs in intact cells was investigated in several transport-related assay systems. When examining [^{3}H]daunorubicin and rhodamine 123 uptake in MDR CHO cells, we found that R121 and R205, at concentrations of 5–50 μM, were able to increase total cellular levels of radiolabeled daunorubicin and the fluorescent dye rhodamine 123 to values equal to, or exceeding, those seen for the parent drug-sensitive cell line AuxB1.

MDR1-positive cells efficiently extrude the hydrophobic calcein AM molecule, and the presence of the multidrug transporter strongly reduces fluorescent calcein accumulation [28, 29]. When the effects of R121 and R205 were examined in this highly sensitive calcein accumulation assay, in mouse cells transfected with the human *MDR1* cDNA, or in drug-selected MDR1-positive human tumor cells, similar findings were obtained. The reversins in concentrations between 0.2 and 5 μM restored calcein accumulation to the level observed in the MDR1-negative parent cells. These experiments, reinforced by the data obtained in isolated membrane and reconstituted Pgp preparations, clearly show that the reversins directly and effectively inhibit the drug extrusion mechanism catalyzed by the multidrug transporter protein. Since both R121 and R205 activate Pgp ATPase and inhibit the binding and transport of other substrates, drug transport inhibition most probably is based on the interaction of reversins with the binding and/or transport mechanism of Pgp.

The most relevant *in vitro* experiments for assessment of the drug resistance-modulating actions of potential reversing agents are cytotoxicity assays in MDR1-expressing cells. The experiments presented here, using a variety of MDR tumor cell lines, clearly indicate that MDR1-substrate drugs become effectively cytotoxic in these cells in the presence of 0.5 to 10 μM concentrations of the reversins. The best characterized test system in this regard is provided by drug-selected or MDR1-transfected cell lines, in which the expression level of MDR1 protein is high, and thus any drug resistance modulating action is easy to follow and quantitate. As presented in Fig. 9, R121 and R205 were found to be highly effective modulators in such an assay. Moreover, in the KBV1 cells, which show extremely high drug resistance and MDR1 expression, a 5 μM concentration of R121 was found to be cytotoxic even in the absence of drugs. This phenomenon may be due to the potent activation of Pgp ATPase by R121, as found in direct ATPase assays, and the consequent depletion of ATP in tumor cells expressing high levels of the MDR1 protein.

To assess the potential clinical relevance of the modulation of drug resistance by R121 and R205, we have examined several drugs involved in the multidrug resistance phenomenon, and a number of non-drug-selected tumor cell types, expressing variable amounts of the MDR1 protein. Since the level of MDR1 in several clinical samples of MDR tumor cells was found to be relatively low [21, 22, 55], this wide range of MDR1 expression is a prerequisite to assess the pharmacological relevance of a modulator compound. Our experiments indicate that the reversins, especially R121, were effective drug resistance-modulating agents in all these *in vitro* panels. Together with the lack of effect of R121 and R205 in cell growth or cell activation assays, these data strongly suggest that the reversins have potential as multidrug resistance modulating agents.

R121 and R205 behaved very similarly in blocking drug transport in the cell-free *in vitro* systems, but in most experiments with intact cells R121 was substantially more effective than R205. This was the case when measuring daunorubicin or rhodamine 123 uptake in MDR1-expressing CHO cells (Fig. 7), or when studying direct drug resistance reversing effect in tumor cell lines (Figs. 9–11). In contrast, in the calcein accumulation assay in intact cells (Fig. 8), there was little difference between the effectiveness of R121 and R205. These results indicate that additional factors can come into play when chemosensitizing agents are used with intact cells under various conditions. One possibility is that vesicles or cells studied in suspension are affected differently by reversins than the cells studied in microplates, due to the different binding features of the hydrophobic reversin compounds to cells, proteins, and/or plastic materials. To examine the possible modulation of the reversin effects by their binding to serum proteins, in the cytotoxicity assays we varied the amount of FBS between 5 and 20% in the incubation media. However, this had no measurable effect on the modulation of drug sensitivity by reversins (data not shown). Another possibility is the different accessibility of the drug-binding sites of MDR1 in different preparations. In particular, it has been suggested that Pgp acts as a "flippase" for drugs and chemosensitizers, moving them from the cytoplasmic leaflet to the extracellular leaflet of the plasma membrane [56, 57]. If this is the case, then the ability of the peptides to traverse the plasma membrane would affect their efficacy at blocking Pgp function in intact cells. On this basis, one might suspect that R205 has a relatively lower ability than R121 to cross the plasma membrane to gain access to the binding site on Pgp. Such considerations would not be a factor

when carrying out experiments *in vitro* using either plasma membrane, which consists of a mixture of inside-out and right-side-out vesicles [46], or proteoliposomes, where Pgp is reconstituted symmetrically [2]. In these sealed vesicle systems, only the "inward-facing" Pgp, where the nucleotide-binding domains have access to ATP in the medium, is active in ATPase and drug transport assays [4]. The relative ability of the reversins to traverse the membrane would not play a role in these systems, since the binding sites would be quite easily accessible.

In summary, we found that in the MDR1-expressing intact cells, R121 and R205 had effects similar to those of known drug resistance reversing agents, e.g. verapamil or cyclosporin A, and in several systems acted at significantly lower concentrations. Moreover, reversins had little effect on non-MDR1 expressing tumor or normal cells. Preliminary *in vivo* experiments (Sarkadi B, unpublished data) indicate that the parenteral administration of reversins in mice and rats had no toxic effects even at relatively high doses (up to 100 mg/kg), whereas they significantly increased the effectiveness of cytotoxic agents in MDR tumor cell killing. Since the reversins have very low *in vitro* and *in vivo* toxicity, these results are encouraging for their possible *in vivo* efficacy in reversing multidrug resistance [58]. Further exploration of the relationship between the structure of the reversins and their ability to interact with Pgp may lead to the design of hydrophobic peptides in this class with even higher affinity for Pgp binding, and corresponding efficacy in reversing MDR *in vivo*, which would be promising candidates for clinical application in the treatment of tumors.

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