Linear and cyclic peptides as substrates and modulators of P-glycoprotein: peptide binding and effects on drug transport and accumulation

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One cause of multidrug resistance (MDR) in human cancers is the overexpression of the P-glycoprotein multidrug transporter, a member of the ABC superfamily of membrane proteins. Natural products and chemotherapeutic drugs are pumped out of the cell by P-glycoprotein in an ATP-dependent fashion. There is growing evidence that many hydrophobic peptides are also P-glycoprotein substrates. With the use of a fluorescence-quenching assay, we have shown that some linear and cyclic hydrophobic peptides interact with P-glycoprotein, whereas others do not. The measured values of the quenching constant, K_q , for interaction of peptides with P-glycoprotein ranged from 200 nM for cyclosporine A to 138 μ M for the tripeptide *N*-acetyl-leucyl-leucyl-norleucinal. Peptides that interacted with P-glycoprotein in the fluorescence assay also blocked colchicine transport into plasma membrane vesicles from MDR cells. The values of D_m , the

peptide concentration causing 50% inhibition of drug uptake, were highly correlated with the values of K_q , over three orders of magnitude. The P-glycoprotein ATPase stimulation/inhibition profile of the peptides was not helpful in making a quantitative assessment of the ability of a peptide to interact with P-glycoprotein or to block drug transport. Some hydrophobic peptides were able to restore accumulation in MDR cells of the chemotherapeutic drug daunorubicin and the fluorescent dye rhodamine 123 to the levels observed in the drug-sensitive parent. Peptides that interacted with P-glycoprotein also displayed a relatively low overall toxicity to intact MDR cells, and inhibited drug transport at concentrations below the toxic range. Hydrophobic peptides should be given serious consideration for development as clinical chemosensitizing agents.

INTRODUCTION

The resistance of human tumours to multiple chemotherapeutic drugs (multidrug resistance, MDR) is a major reason for the failure of cancer therapy. Overexpression of P-glycoprotein (Pgp) is a primary cause of multidrug resistance in many human cancers. Pgp is a member of the ABC (ATP-binding cassette) superfamily of membrane transporters [1]. The structure of Pgp, as inferred from sequence and hydropathy plot analysis, consists of two homologous halves, each comprising six membrane-spanning segments and a nucleotide-binding domain (NBD). The structure and function of the Pgp multidrug transporter have been the subject of intensive investigation over the past decade (reviewed in [2–5]). Reconstitution studies have shown that Pgp acts as an active transporter, driven by the hydrolysis of ATP [6,7]. The transporter is unusual in that it displays a high level of constitutive ATPase activity, which can be further modulated by drugs [8–10]. The K_m for ATP hydrolysis [8–10] and the K_d for ATP binding [11] are both in the range 0.4–0.8 mM, which is atypically high compared with other ATP-dependent membrane transporters. The mechanism by which Pgp pumps its substrates across the plasma membrane is currently unknown, although evidence is accumulating that it might act as a ‘flippase’, translocating drug molecules from the inner to the outer leaflet within the membrane (reviewed in [5]).

Substantial evidence now exists that Pgp interacts with compounds other than natural product drugs. MDR cells are cross-

resistant to several hydrophobic peptides and ionophores [12–14]. Some bioactive peptides, including ionophores, protease inhibitors, chemoattractants and immunosuppressants, stimulate Pgp ATPase activity and inhibit drug transport in both intact cells and membrane vesicle systems [15,16]. Prenylated cysteine methyl esters also enhance Pgp ATPase activity [17], indicating that they are likely to be Pgp substrates. The ionophores valinomycin and gramicidin D [18], and the yeast α -factor mating peptide [19] also seem to be transported by Pgp. Studies in our laboratory have recently shown that a simple linear tripeptide, NAc-LLY-amide (in which, as below, single-letter amino acid codes are used) is transported by Pgp in an active, ATP-dependent manner in plasma membrane vesicles from MDR cells and reconstituted Pgp proteoliposomes [20], which suggests that all of the Pgp-interacting peptides identified so far might be transport substrates.

Compounds known as chemosensitizers, MDR modulators or reversers are able to inhibit the drug-pumping action of Pgp and restore drug sensitivity in MDR cells. Many clinical trials have been performed, or are continuing, that combine chemosensitizing agents with chemotherapeutic drugs [21,22]. Some cyclic peptides have already been identified as chemosensitizing agents for MDR, including valinomycin [23], and cyclosporine A and related compounds such as SDZ PSC 833 [24]; the latter are currently being used clinically.

Mapping and site-directed mutagenesis studies point to the transmembrane segments, especially TM5, TM6, TM11 and

Abbreviations used: ALLM, *N*-acetyl-leucyl-leucyl-methioninal; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; DTE, dithioerythritol; MDR, multidrug resistant/resistance; α -MEM, α -minimal essential medium; MIANS, 2-(4'-maleimidyl)anilino)naphthalene-6-sulphonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBD, nucleotide-binding domain; norL, norleucine; Pgp, P-glycoprotein; t-boc, t-butyloxycarbonyl.

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TM12, as the location within Pgp where drugs bind [25–27]. This is in agreement with evidence that Pgp takes its substrates directly from the lipid bilayer rather than from the aqueous compartment [28,29]. Both the N-terminal and C-terminal halves of Pgp seem to contribute to formation of the drug-binding site(s) [30]. The ability of Pgp to interact with so many structurally diverse compounds has generated much controversy about the number and nature of the substrate-binding sites. It has been reported that multiple dihydropyridine molecules are able to bind to Pgp simultaneously [31]. Recent studies in our laboratory also indicated that a hydrophobic tripeptide and colchicine can bind to Pgp concurrently [20]. It seems possible that all Pgp substrates (drugs, chemosensitizers and peptides) interact with different overlapping regions of a flexible binding site that is large enough to accommodate more than one compound.

In the present study we examined the interaction of hydrophobic peptides and ionophores with Pgp, using a variety of experimental systems. The structures of these compounds range from simple linear tripeptides to more complex cyclic structures; they include both natural products of microbial and fungal origin, and synthetic peptides. Interaction of the peptides with highly purified Pgp has been characterized by using a fluorescence-quenching technique. We show that the value of the quenching constant, K_q , for a particular peptide is an excellent predictor of its ability to inhibit Pgp-mediated drug transport in a plasma membrane vesicle system. Finally we demonstrate that some linear and cyclic peptides are able to restore the accumulation of a chemotherapeutic drug and a fluorescent dye to normal levels in intact MDR cells. Chemosensitization by peptides took place within a concentration range over which they were relatively non-toxic to intact cells.

MATERIALS AND METHODS

Materials

Asolectin was supplied by Fluka (Ronkonkoma, NY, U.S.A.), and 2-(4'-maleimidylanilino)naphthalene-6-sulphonic acid (MIANS) was purchased from Molecular Probes (Eugene, OR, U.S.A.). CHAPS, disodium ATP, dithioerythritol (DTE), colchicine, rhodamine 123, *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), *N*-acetyl-leucyl-leucyl-methioninal (ALLM), leupeptin, *N*-t-boc-deacetyl-leupeptin (in which t-boc stands for t-butyloxycarbonyl), *N*-acetyl-phenylalanyl-norleucyl-arginyl-phenylalanyl-amide (NAc-FnorLRF-amide), pepsinostreptin, chymostatin, pepstatin A, valinomycin, beauvericin, melittin, gramicidin S, alamethicin, nigericin, monensin and vinblastine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Acetyl-LLY-amide was synthesized by Queen's University Core Facility for Protein/DNA Chemistry (Kingston, ON, Canada). The cyclic peptide cyclosporine A was provided by Pfizer Central Research (Groton, CT, U.S.A.).

MDR cell lines and plasma membrane preparation

The drug-sensitive parent cell line AuxB1, and the MDR Chinese hamster ovary cell lines CH^RB30 [32] and CH^RC5 [33] were grown as described previously [13,34]. Cells were cultured at 37 °C in a humidified air/CO₂ (19:1) atmosphere in α -minimal essential medium (α -MEM) supplemented with 10 % (v/v) heat-inactivated iron-supplemented/defined bovine calf serum (Hyclone Laboratories, Logan, UT, U.S.A.), penicillin (1000 i.u./ml), streptomycin (1 mg/ml) and L-glutamine (2 mM). CH^RB30 cells were grown in the presence of 30 μ g/ml colchicine. The isolation of plasma membrane vesicles from CH^RC5 and

CH^RB30 cells has been described [35]. Membrane vesicles were stored at –70 °C for up to 3 months before use.

Purification of Pgp

A modification of the method previously described for CH^RC5 [8] was used to purify Pgp from CH^RB30 plasma membrane [11]. After the plasma membrane had first been extracted with CHAPS, the S₁ pellet was solubilized in 8 mM CHAPS buffer at a final protein concentration of 1 mg/ml. After incubation and centrifugation a soluble CH^RB30 S₂ supernatant was obtained that was highly enriched in Pgp. A single chromatographic run through a column of either lentil lectin–Sephacrose or concanavalin A–Sephacrose (Pharmacia, Dorval, QC, Canada) was used to remove contaminating glycoproteins from the S₂ fraction. The final Pgp preparation (90–95 % pure Pgp at a concentration of 0.1–0.2 mg/ml in 2 mM CHAPS buffer [2 mM CHAPS/50 mM Tris/HCl/0.15 M NaCl/5 mM MgCl₂/0.02 % NaN₃ (pH 7.5)]) was kept on ice and used within 24 h. Protein in plasma membrane was quantified by the method of Bradford [36]; the method of Peterson [37] was employed for purified Pgp, with BSA (crystallized and freeze-dried; Sigma) as a standard.

Labelling of Pgp with MIANS, and fluorescence measurements

Purified Pgp was fully modified with MIANS and isolated by gel-filtration chromatography as described previously [11]. The soluble thiol-containing molecule DTE was also coupled to MIANS to act as a control for the effect of peptides on the fluor in aqueous solution. Fluorescence-quenching studies with various peptides and ionophores were performed on solutions of 50 μ g/ml Pgp in 2 mM CHAPS buffer at 22 °C, in the presence of 0.5 mg/ml asolectin (soybean phospholipids), which was added as 100 nm extruded unilamellar vesicles [11]. The working solutions of peptides and ionophores were prepared in DMSO. Quenching experiments were performed by successively adding 5 μ l aliquots of peptide/ionophore solution to 500 μ l of MIANS-labelled Pgp in a 0.5 cm quartz cuvette. After each addition the steady-state fluorescence was measured at 420 nm (excitation at 322 nm). Corrections were made to the measured fluorescence intensities for dilution, scattering and the inner filter effect [38]. Control titrations were performed with DMSO alone and showed no effects on MIANS-Pgp fluorescence. To check that peptides did not affect the fluorescence of the MIANS probe alone, MIANS-DTE in solution was also titrated against selected compounds.

The experimental data were computer-fitted to the following equation:

$$100\Delta F/F_0 = \{(100\Delta F_{\max}/F_0) \times [S]\} / (K_q + [S])$$

where $100\Delta F/F_0$ is the percentage quenching (percentage change in fluorescence relative to the initial value), after the addition of peptide at a concentration $[S]$, and K_q is the quenching constant (equivalent to the apparent dissociation constant for binding, K_d). SIGMAPLOT for Windows (SPSS, Chicago, IL, U.S.A.) was used to fit the data and extract values of K_q and $100\Delta F_{\max}/F_0$ by non-linear regression with the Marquardt–Levenberg algorithm.

Photoaffinity labelling

Photoaffinity labelling of purified native Pgp and MIANS-labelled Pgp with [³H]azidopine was performed with a modification of the method used earlier for plasma membrane vesicles [16,34]. A series of samples of purified Pgp or MIANS-Pgp (2.5,

5.0, 7.5 and 10 μg in 2 mM CHAPS buffer) were incubated with 200 nM (0.3 μCi) [^3H]azidopine (47 Ci/mmol; Amersham, Oakville, ON, Canada) at 25 °C for 1 h in the dark, followed by irradiation with UV for 30 min. Photolabelled samples were then separated by SDS/PAGE on 10% (w/v) polyacrylamide gels and either subjected to autoradiography or stained with Coomassie Blue to reveal the protein loading. To assess the inhibition of Pgp azidopine photoaffinity labelling by MDR substrates after reaction with MIANS, photolabelling of 2 μg of purified native Pgp and MIANS-Pgp was performed in the presence of increasing concentrations of vinblastine.

Inhibition of drug transport in plasma membrane vesicles

Steady-state uptake of [^3H]colchicine into CH^RB30 plasma membrane vesicles was determined as described previously [16,20]. In brief, membrane vesicles (30 μg of protein) were mixed in a 100 μl final volume of transport buffer with 1 μM [^3H]colchicine, 5 mM Mg^{2+} and 1 mM ATP, together with an ATP-regenerating system (creatine phosphate/creatine kinase). Peptides and ionophores were added as stock solutions in DMSO and controls contained the appropriate DMSO concentration. After 30 min at 23 °C, vesicles were harvested by rapid filtration with Whatman GF/F filters, and immediately washed with 5 ml of ice-cold buffer. Filters were dried and radioactivity was quantified by liquid-scintillation counting. Colchicine binding to filters, and non-specific uptake into vesicles, were determined in the absence of membrane vesicles and in the absence of ATP and a regenerating system respectively. The transport inhibition data were analysed with the median effect equation [39], as previously reported [16,40].

Measurement of Pgp ATPase activity

Pgp ATPase activity (Mg^{2+} -dependent) was determined as described previously [8,41] by measuring the release of P_i from ATP in the presence of 1 mM ATP and 5 mM Mg^{2+} . Membrane vesicles were preincubated with peptides for 5 min before the addition of ATP. Peptides were added as stock solutions in DMSO; controls contained the appropriate DMSO concentration, which did not exceed 0.2% (v/v). This level of DMSO had no effect on Pgp ATPase activity.

Daunorubicin uptake in intact MDR cells

The MDR cell line CH^RC5 or the parent drug-sensitive cell line AuxB1 was plated at 10^5 cells per well in 24-well plates and cultured for 3 days as described previously [34]. Wells were rinsed twice with 1 ml of serum free α -MEM before the start of the assay. Peptides were added to wells as serial dilutions in a 150 μl volume of α -MEM, followed by a 20 min incubation at 37 °C. A 150 μl aliquot of 0.25 μM [^3H]daunorubicin (1.9 Ci/mmol; Dupont-NEN, Boston, MA, U.S.A.), at a concentration of 0.1 $\mu\text{Ci}/\text{ml}$, was then added to each well, and the plate was incubated for a further 30 min at 37 °C. 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% SDS in 20 mM Tris/HCl, pH 7.7, and the radioactivity in the cell lysates was determined by scintillation counting. The protein content of each well was measured with the Pierce bicinchoninic acid assay system (Pierce, Rockford, IL, U.S.A.).

Rhodamine 123 uptake in intact MDR cells

CH^RC5 or AuxB1 cells were plated at 2×10^4 cells per well in 96-well plates and cultured for 2 days. Wells were rinsed twice with

200 μl of serum-free α -MEM, and peptides (100 μl per well) were added in α -MEM buffer at the required concentration. Plates were incubated at 37 °C for 20 min before the addition of 25 μM rhodamine 123 (100 μl per well) in α -MEM buffer. After incubation of the plates for a further 30 min at 37 °C, dye uptake was terminated by rinsing the plate three times with ice-cold PBS (200 μl per well). Cells were lysed with 20 mM Tris/HCl, pH 7.7, containing 0.2% SDS, and total cellular rhodamine 123 accumulation was measured with a fluorescence plate reader (excitation at 485 nm; emission at 530 nm).

Assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for peptide cytotoxicity

The effect of various peptides on the proliferation of MDR CH^RC5 cells was assayed by a modification of the dye-reduction assay with MTT, as described previously [34,42]. Cells were incubated with the peptide for 72 h before the addition of MTT. All compounds were dissolved in DMSO and dispersed in culture medium containing 10% (v/v) bovine calf serum to reduce non-specific interactions with plastic surfaces. Relative cell growth was normalized by comparison with absorbance in the absence of peptide; the results were plotted as relative cell growth (percentage of control) against the logarithm of peptide concentration. IC_{50} values were determined by interpolation from cytotoxicity plots.

RESULTS

Binding of peptides to purified Pgp

In the present study we explored the interaction with Pgp of a panel of linear and cyclic hydrophobic bioactive peptides, peptide-like compounds and ionophores of diverse structure (Table 1). Many of the compounds were originally of microbial or fungal origin, some were synthetic analogues of a natural product, and some were entirely synthetic. Although many of the peptides were expected to be uncharged at physiological pH, several contained positively charged residues, and two were negatively charged (see Table 1). Interaction of the peptides with highly purified Pgp was determined with a fluorescence-quenching assay developed recently in our laboratory [11]. Pgp was covalently modified at two conserved cysteine residues (Cys-428 and Cys-1071), one within each Walker A motif of the NBD, with the fluorescence probe MIANS. Fluorescence resonance energy transfer experiments previously showed that the labelled Cys residues are in close proximity to the site where ATP binds within the NBD [43].

Photoaffinity labelling of Pgp with the drug substrate azidopine has often been used as a measure of drug binding to the protein. A direct comparison of purified native Pgp and MIANS-Pgp showed that the ability of the protein to be photolabelled by [^3H]azidopine was fully retained after covalent modification with MIANS (results not shown), indicating that azidopine binding was not impaired. To further assess whether MIANS affects the ability of Pgp to bind substrates, azidopine photolabelling of unmodified Pgp and MIANS-Pgp was performed in the presence of increasing concentrations of the competing transport substrate vinblastine. As shown in Figure 1, labelling of unmodified Pgp by azidopine was progressively inhibited at increasing concentrations of vinblastine, with a vinblastine concentration of 1–2 μM decreasing photolabelling by 50% as assessed by densitometric quantification of the labelled Pgp bands. Inhibition of azidopine photolabelling of MIANS-Pgp by vinblastine showed a very similar pattern, with an IC_{50} of 0.5–1 μM . Taken together, the results of these photolabelling experiments indicate that

Table 1 Properties of peptides and ionophores used in this study

Abbreviations: Sta, statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid]; Cpd, capreomycinide {[*S*,*S*]- α -(2-imino-6-hydroxy-4-pyrimidinyl)-Gly}; Xaa = Leu, Val or Ile; Hylva, α -hydroxyisovalerate; Lac, lactate.

Compound	Type	Original source	Charge	Comments
ALLN (NAC-LLNorL-al)	Linear	Microbial	0	Calpain inhibitor I
ALLM (NAC-LLM-al)	Linear	Microbial	0	Calpain inhibitor II
Leupeptin (NAC-LLR-al)	Linear	Microbial	+	Protease inhibitor
Pepsinostreptin (isobutyl-Val-Val-Sta-Ala-Sta)	Linear	Microbial	0	Pepsin inhibitor
Chymostatin [<i>N</i> -(α -carbonyl-Cpd-Xaa-Phe-al)-Phe]	Linear	Microbial	+	Enzyme inhibitor
Pepstatin A (isovaleryl-Val-Val-Sta-Ala-Sta)	Linear	Microbial	0	Acid protease inhibitor
NAC-LLY-amide	Linear	Synthetic	0	Increases drug transport via Pgp
<i>N</i> -t-boc-deacetyl-leupeptin	Linear	Semi-synthetic	+	Modified analogue of leupeptin
NAC-FnorLRF-amide	Linear	Synthetic	+	Molluscan cardioexcitatory neuropeptide analogue
Alamethicin	Linear	Fungal	0	Membrane-active antibiotic
Melittin	Linear	Bee venom	+	Membrane-active toxin
Nigericin	Linear	Microbial	−	K ⁺ /H ⁺ -selective ionophore
Monensin	Linear	Microbial	−	Na ⁺ -selective ionophore
Valinomycin [cyclo-(L-Val-D-Hylva-D-Val-L-Lac) ₃]	Cyclic	Microbial	0	K ⁺ -selective ionophore
Beauvericin [cyclo-(D- α -Hylva-L-NMePhe) ₃]	Cyclic	Fungal	0	Toxic to insects and crustaceans
Cyclosporine A	Cyclic	Fungal	0	Immunosuppressant
Gramicidin S	Cyclic	Microbial	+	Membrane-active antibiotic

Figure 1 Inhibition by vinblastine of azidopine photoaffinity labelling of native Pgp and MIANS-Pgp

Photoaffinity labelling of purified native Pgp or MIANS-Pgp (2 μ g in 2 mM CHAPS buffer) was performed with [³H]azidopine (250 μ Ci; 200 nM) at 25 °C for 1 h in the presence of increasing concentrations of vinblastine. After irradiation with UV for 30 min, photolabelled samples were separated by SDS/PAGE [10% (w/v) gel] and subjected to autoradiography. The only visible band was Pgp, with a molecular mass of 170–180 kDa. The vinblastine concentration (in μ M) is indicated at the top.

labelling of Pgp with MIANS does not interfere with its interaction with either azidopine or vinblastine, suggesting that the substrate-binding properties of the protein remain intact.

Previous work in our laboratory showed that the interaction of drugs and chemosensitizers (including daunorubicin, vinblastine and verapamil) with MIANS-Pgp resulted in saturable quenching of the probe's fluorescence, which was interpreted as arising from a conformational change in the NBD induced by substrate binding [11]. The apparent affinity of interaction of each substrate with the transporter can be quantitatively estimated from the concentration dependence of the quenching. The addition of many peptides to MIANS-labelled Pgp also led to concentration-dependent, saturable quenching of the MIANS fluorescence (Figure 2). The fluorescence-quenching data for this group of peptides fitted well to an equation describing the interaction with a single type of binding site (see Figure 2, and the Materials and methods section), which was used to determine the value of the quenching constant for each species, K_q (Table 2). K_q can be considered an apparent K_d for binding. The cyclic

peptides beauvericin, cyclosporine A (Figure 2A) and valinomycin (Figure 2B) interacted with Pgp with very high affinity, with K_q values in the sub-micromolar range. Simple tripeptides and tetrapeptides with blocked termini, such as *N*-t-boc-deacetyl-leupeptin (Figure 2B), leupeptin (Figure 2D) and NAC-FnorLRF-amide (Figure 2C), had affinities in the low μ M range. The K_q values for the interaction with Pgp of the natural product peptide-like compounds chymostatin (Figure 2C), pepsinostreptin (Figure 2D) and leupeptin (Figure 2D) were in the range 33–78 μ M. The K_q values for other peptides tested ranged from 28 μ M for NAC-LLY-amide to 138 μ M for ALLN (Table 2). These results indicate that these 12 peptides interact with Pgp with affinities that vary over an approx. 700-fold range. The maximal fluorescence quenching as saturation was approached was between 7% and 15% (Table 2). To exclude possible direct effects of the peptides and ionophores on probe fluorescence, selected peptides of different structural classes (valinomycin, pepstatin A, and *N*-t-boc-deacetyl-leupeptin) were titrated with MIANS-DTE in aqueous solution over the same concentration range used in Figure 2. None of the peptides showed significant effects on MIANS-DTE fluorescence relative to a DMSO control (results not shown), indicating that the peptides do not interact with and quench the MIANS probe in solution, only within the context of the NBD of Pgp.

Several other hydrophobic peptides and ionophores did not display saturable quenching of MIANS-Pgp fluorescence, including the linear peptides alamethicin and melittin, the linear ionophores nigericin and monensin, and the cyclic ionophore gramicidin S (Figures 2B and 2C). For these species, MIANS-Pgp fluorescence either remained essentially unchanged or increased slightly, giving a negative value for $100\Delta F/F_0$. MDR cells are not cross-resistant to these compounds [13,16], which suggests that they are not Pgp substrates, and the results obtained here confirm that they do not interact specifically with Pgp.

Inhibition of drug transport in plasma membrane vesicles by peptides

Plasma membrane vesicles from MDR cells transport drugs in a saturable, ATP-dependent manner, to generate a drug concentration gradient. Simple membrane systems such as these

Figure 2 Interaction of peptides and ionophores with purified MANS-labelled Pgp as assessed by fluorescence quenching

Increasing concentrations of peptides were added to 50 $\mu\text{g}/\text{ml}$ MANS-labelled Pgp (in 2 mM CHAPS buffer) at 22 $^{\circ}\text{C}$ in the presence of 0.5 mg/ml asolectin, and the fluorescence emission at 420 nm was recorded. Symbols: (A) beavericin (●), cyclosporine A (○); (B) *N*-t-boc-deacetyl-leupeptin (■), valinomycin (□), monensin (◇), alamethicin (●); (C) NAc-FnorLRF-amide (◆), chymostatin (◇), nigericin (▲), melittin (△), gramicidin S (●); (D) pepsinostreptin (▼), leupeptin (▽). The percentage quenching of fluorescence, $100\Delta F/F_0$, was calculated relative to the fluorescence of MANS-labelled Pgp in the absence of peptides, at increasing peptide concentrations. For all peptides except monensin, alamethicin, nigericin, melittin and gramicidin S, the continuous line represents the best computer-generated fit of the data points (shown by the symbols) to an equation describing the interaction of peptide with a single type of binding site (see the Materials and methods section). Results are means $\pm$ ranges for duplicate determinations.

have proved to be very useful tools in assessing the ability of various compounds to block drug transport by Pgp *in vitro*. Equilibrium uptake of [^3H]colchicine into CH $^{\text{R}}$ B30 plasma membrane vesicles was determined in the presence of increasing concentrations of various peptides (Figure 3). Beavericin abolished drug transport completely at 5 μM (Figure 3A), whereas a 5–10-fold higher concentration of NAc-FnorLRF-amide (Figure 3A) or *N*-t-boc-deacetyl-leupeptin (Figure 3B) was required for complete inhibition. Chymostatin and pepsinostreptin blocked colchicine transport in the concentration range 150–250 μM (Figure 3B). Transport inhibition data for the various peptides fitted well to the median effect equation [39], to give linear plots of $\log(f_u/f_u)$ against $\log D$ (Figure 4), where f_u is the fractional inhibition of colchicine uptake at a peptide concentration D , and f_u is the fraction of drug uptake that is uninhibited ($f_u = 1 - f_u$) [16,40]. The value of the median effect dose, D_m , i.e. the peptide concentration causing 50% inhibition of colchicine transport, was determined from each plot, together

with the slope, m , which is a parameter describing the sigmoidal nature of the median effect plot (Table 3).

The D_m values can be used to rank the various peptides in terms of their efficacy in blocking drug transport via Pgp. They varied from less than 1 μM , for the cyclic peptides beavericin and cyclosporine A, to 130 μM for ALLN and ALLM (Table 3). Comparison of the D_m values for the various peptides with their K_q values for interaction with Pgp shows a close correspondence between the two. Linear regression analysis of a plot of $\log D_m$ against $\log K_q$ gave a straight line for which $r = 0.933$ (Figure 5). Thus the ability of a peptide to inhibit drug transport via Pgp is highly correlated with its apparent binding affinity for the purified protein, over about three orders of magnitude. These results indicate that measurement of the K_q for interaction of a particular peptide with purified Pgp is an excellent predictor of the ability of that peptide to compete with drug transport. In general, the peptide concentration required to cause 50% inhibition of drug transport *in vitro* was always approx. 3–4-fold the K_q value (see

Table 2 Parameters for interaction of peptides with MIANS-labelled Pgp

Saturable fluorescence quenching data, of the type shown in Figure 2, were computer-fitted to an equation describing an interaction with a single type of binding site by using non-linear regression (see the Materials and methods section). Values of K_d and $100\Delta F_{\max}/F_0$ shown are means $\pm$ S.E.M. ($n = 2$ or 3) extracted by computer fitting from at least two separate independent experiments with different batches of MIANS-labelled Pgp. The dashes indicate that no parameters could be measured, because these compounds do not bind to P-glycoprotein.

Peptide	K_d (μ M)	$100\Delta F_{\max}/F_0$
ALLN	138 ± 20	7.0 ± 0.4
ALLM	83.1 ± 15.2	7.3 ± 0.4
Leupeptin	77.6 ± 12.6	12.8 ± 0.8
Pepsinostreptin	62.8 ± 5.3	11.9 ± 0.4
Pepstatin A	35.8 ± 2.8	10.3 ± 0.3
Chymostatin	32.7 ± 5.5	10.7 ± 0.8
NAc-LLY-amide	28.4 ± 4.4	10.0 ± 0.3
<i>N</i> -t-boc-deacetyl-leupeptin	11.3 ± 1.0	12.7 ± 0.4
NAc-FnolRf-amide	10.3 ± 0.8	12.4 ± 0.3
Valinomycin	0.78 ± 0.08	15.0 ± 0.3
Beauvericin	0.36 ± 0.03	8.00 ± 0.2
Cyclosporine A	0.20 ± 0.01	15.4 ± 0.2
Alamethicin	—	—
Melittin	—	—
Nigericin	—	—
Monensin	—	—
Gramicidin S	—	—

Tables 2 and 3). This difference might reflect the presence of higher levels of lipid in the plasma membrane vesicles than in the purified Pgp fluorescence-quenching assay system. Partitioning of a hydrophobic peptide from a fixed volume of aqueous phase into a larger volume of membrane lipid would be expected to decrease the effective concentration of peptide in the lipid phase. For example, consider a hydrophobic peptide with a lipid/water partition coefficient of 50, in a system in which the aqueous volume is 100-fold the volume of the lipid phase. If the volume of the lipid phase is increased 4-fold relative to the aqueous phase, calculations show that the concentration of the peptide in the lipid phase is halved. This would result in a decrease in

Figure 4 Median effect analysis of CH^B30 plasma membrane vesicle transport inhibition data

Median effect plots of $\log(f_a/f_u)$ against $\log D$ (results presented in Figure 3) are shown for beauvericin (●), NAc-FnolRf-amide (◆), *N*-t-boc-deacetyl-leupeptin (■), chymostatin (▲) and pepsinostreptin (▼). Each data set was fitted to a straight line by linear regression analysis.

peptide concentration being experienced by Pgp, which has been proposed to extract its substrates from the lipid phase in accordance with the ‘vacuum cleaner’ model [44].

The m values for the various peptides were generally close to 1, although three compounds (ALLN, valinomycin and cyclosporine A) displayed values of m close to 3 (Table 3). The m value is an indicator of the sigmoidal nature of the drug transport inhibition plot, but its significance in terms of the molecular nature of the peptide–Pgp interaction remains unclear.

Peptide effects on Pgp ATPase activity

The ATPase activity of Pgp is known to be modulated, often in a bimodal fashion, by various substrates and chemosensitizers. The effects of selected peptides on Pgp ATPase activity were examined in native plasma membrane vesicles from MDR

Figure 3 Inhibition of colchicine transport in native CH^B30 plasma membrane vesicles by peptides

The equilibrium uptake of 1μ M [³H]colchicine into CH^B30 plasma membrane vesicles was measured in the presence of 1 mM ATP and a regenerating system, together with increasing concentrations of beauvericin (●), NAc-FnolRf-amide (◆), *N*-t-boc-deacetyl-leupeptin (■), chymostatin (▲) or pepsinostreptin (▼). Results are presented as percentages of control ATP-dependent [³H]colchicine uptake relative to plasma membrane vesicles in the absence of peptides (means $\pm$ S.E.M., $n = 3$).

Table 3 Parameters for inhibition by peptides of Pgp-mediated colchicine transport

D_m and m were determined from the median effect plots shown in Figure 4, as described previously [16,40]. Results for the peptide NAc-LLY-amide could not be determined because it stimulates, rather than inhibits, drug transport [20].

Peptide	D_m (μ M)	m
ALLN*	130	3.0
ALLM*	130	1.3
Pepsinostreptin	81	1.2
Chymostatin	47	0.86
Leupeptin*	45	1.1
Pepstatin A*	10	1.0
NAc-LLY-amide	—	—
<i>N</i> -t-boc-deacetyl-leupeptin	6.0	1.3
NAc-FnolLRF-amide	5.7	1.4
Valinomycin*	4.0	2.9
Beauvericin	1.0	0.94
Cyclosporine A*	0.7	3.0

* Median effect parameters for these peptides were reported previously [16].

Figure 5 Correlation of D_m with K_q for peptides interacting with Pgp

The results were fitted to a straight line by linear regression ($r = 0.933$). Note that the peptide NAc-LLY-amide is not included because it stimulates drug transport [20].

CH^RC5 cells (Figure 6). The cyclic compound beauvericin produced a large stimulation of ATPase activity up to 5 μ M but inhibited activity at higher concentrations. NAc-FnolLRF-amide also stimulated activity at relatively low concentrations, but to a smaller extent. The other peptides tested caused only modest stimulation of ATPase activity, with no inhibition at higher concentrations. In general, the ability of a particular peptide to either stimulate or inhibit Pgp ATPase activity was not correlated in an obvious way with either K_q or D_m . Pgp ATPase stimulation/inhibition profiles are therefore not particularly helpful in making a quantitative assessment of the ability of a peptide to interact with Pgp or to inhibit drug transport.

Restoration of daunorubicin and rhodamine 123 accumulation by peptides

Uptake of the chemotherapeutic drug daunorubicin by drug-resistant and drug-sensitive Chinese hamster ovary cells reached a maximum after a 30 min incubation at 37 °C in the presence of

Figure 6 Stimulation of the ATPase activity of native membrane-bound Pgp by selected peptides

CH^RB30 plasma membrane vesicles (1.5–2.0 μ g of protein) were assayed for Mg²⁺-dependent ATPase activity in the presence of increasing concentrations of beauvericin (●), NAc-FnolLRF-amide (◆), *N*-t-boc-deacetyl-leupeptin (■), chymostatin (▲) or pepsinostreptin (▼). Results are presented as percentages of control Mg²⁺-ATPase activity measured in the absence of peptides (mean $\pm$ S.E.M., $n = 3$).

radiolabelled drug. Intact cells of the MDR line CH^RC5 took up approx. one-sixth of the [³H]daunorubicin that the drug-sensitive parent cells AuxB1 did (Figure 7). Some peptides were able to restore cellular accumulation of daunorubicin in CH^RC5 to normal levels. When CH^RC5 cells were treated with the cyclic peptide beauvericin (Figure 7A) or the linear peptide pepstatin A (Figure 7B), daunorubicin uptake returned to levels seen for the drug-sensitive cells. Chemosensitization of the cells by peptides was concentration-dependent. Drug uptake approached that observed for drug-sensitive cells at a beauvericin concentration of approx. 4 μ M, whereas a pepstatin A concentration of approx. 800 μ M was required to achieve the same effect. The concentrations causing 50 % restoration of maximal daunorubicin uptake were 3.5 and 450 μ M for beauvericin and pepstatin A respectively, which is consistent with the 100-fold difference in their K_q values for binding to Pgp (Table 2). For comparison, the concentration of cyclosporine A required for 50 % restoration of maximal daunorubicin uptake was 4 μ M (results not shown).

Similar results were obtained for the uptake of the fluorescent dye rhodamine 123, which is a Pgp substrate. Beauvericin (Figure 7C) and pepstatin A (Figure 7D) were able to restore rhodamine 123 uptake in CH^RC5 cells to the levels associated with drug-sensitive cells, in a concentration-dependent fashion. An approx. 10-fold difference in efficacy was evident when comparing beauvericin to pepstatin A. Cyclosporine A also restored levels of rhodamine 123 in MDR cells; the concentration required for dye uptake to reach 50 % of the levels observed in AuxB1 cells was 5 μ M (results not shown).

The cytotoxicity of the various peptides alone to CH^RC5 cells was determined with an MTT assay system. As a group, the linear peptides were relatively non-toxic, even at very high concentrations, whereas the three cyclic peptides (valinomycin, beauvericin and cyclosporine A) displayed substantially higher toxicity (Table 4). However, all the peptides were generally effective at blocking Pgp-mediated drug transport in membrane vesicles *in vitro* in the concentration range in which they were non-toxic (see Tables 3 and 4).

Figure 7 Restoration of uptake of $[^3\text{H}]$ daunorubicin and rhodamine 123 into intact MDR cells by peptides

CHRC5 cells were preincubated for 20 min at 37 °C with various concentrations of beauvericin (A, C) or pepstatin A (B, D), followed by the addition of 0.25 μM $[^3\text{H}]$ daunorubicin or 25 μM rhodamine 123. After 30 min at 37 °C, uptake was terminated by rapidly rinsing each well of the plate with ice-cold PBS. Cellular uptake of daunorubicin or rhodamine 123 in the presence of peptide was determined as described in the Materials and methods section. Uptake of $[^3\text{H}]$ daunorubicin and rhodamine 123 in the drug-sensitive parent cell line AuxB1 was determined under the same conditions (broken lines). Results are presented as means $\pm$ S.E.M. ($n = 3$).

Table 4 Cytotoxicity of peptides

The cytotoxicity of selected peptides to CHRC5 cells was determined with the MTT assay, as outlined in the Materials and methods section. IC_{50} was defined as the peptide concentration resulting in 50% inhibition of cellular viability.

Peptide	IC_{50} (μM)
ALLN	400
ALLM	> 200
Pepsinostreptin	> 1000
Chymostatin	> 850
Leupeptin	500
Pepstatin A	750
NAC-LLY-amide	> 1000
N-t-boc-deacetyl-leupeptin	> 1000
NAC-FnorLRF-amide	> 1000
Valinomycin	30
Cyclosporine A	25
Beauvericin	7.5

DISCUSSION

Previous studies in our laboratory [13,16] and others [12,14,15,18,19] have suggested that several hydrophobic peptides and peptide-like compounds, both linear and cyclic, interact with Pgp. In addition we have recently demonstrated that a simple synthetic tripeptide with modified N- and C-termini, NAc-LLY-amide, is transported by Pgp in proteoliposomes and plasma membrane vesicles [20]. However, the inference on whether a particular peptide specifically interacts with Pgp has previously been made indirectly, from results such as cross-resistance to the peptide [12,13] or the ability of the peptide to block azidopine photoaffinity labelling of Pgp [14] or to inhibit drug transport in MDR cells [15] and membrane vesicle systems [16]. MIANS-labelled Pgp retains its ability to be photoaffinity-labelled by azidopine, and the similar profiles obtained for competition of photolabelling by vinblastine indicate that the drug-binding properties of the protein are unimpaired after covalent modification. This feature allowed the development of a fluorescence-

quenching technique that can demonstrate the interaction of Pgp with putative substrates in a highly purified system *in vitro*. Results of the present study indicated that many peptides interact with Pgp, and allowed their binding affinity to be quantified. The measured K_q values (which can be considered equivalent to K_d values) for peptides range from a very high affinity for cyclic peptide-like compounds (beauvericin, valinomycin and cyclosporine A) to a relatively low affinity for simple linear tripeptides such as ALLN and ALLM. The 12 peptides used in this study that interacted with Pgp displayed a 700-fold range of affinities. Clearly, Pgp is able to interact with a wide variety of peptides and peptide-like structures with hydrophobic residues as their main constituents. A positively charged nitrogen atom has been proposed to be important for the ability of a compound to interact with Pgp [45]. However, this structural feature is absent from most of the peptides in the panel, and is clearly not essential for high-affinity binding to Pgp. Several membrane-active peptides and ionophores are not Pgp substrates and do not interact with Pgp in the fluorescence-quenching assay; some of these carry a net positive charge (melittin and gramicidin S).

All of the peptides that showed saturable quenching of MIANS-Pgp also inhibited the uptake of [3 H]colchicine into plasma membrane vesicles from MDR cells. The median effect analysis was applied to the transport inhibition curves to generate a value for D_m , the concentration at which the peptide inhibited drug transport by 50 %. The ranking of the peptides in order of D_m (Table 3) was almost identical with the ranking in order of their binding affinity, K_q (Table 2); the values of D_m and K_q were highly correlated (Figure 5). Measurement of the K_q for a particular peptide is therefore an excellent predictor of its ability to inhibit the drug transport function of Pgp *in vitro*. The ability of a compound to stimulate Pgp ATPase activity has been suggested as a screen for the identification of Pgp substrates and chemosensitizers. However, the profile of stimulation or inhibition of Pgp ATPase (Figure 6) could not be related in a useful way to either K_q or D_m . There was a weak correlation between D_m or K_q and the peptide concentration causing half-maximal stimulation of activity. However, no satisfactory method for the quantitative analysis of ATPase profiles of the varied types shown in Figure 6 has yet been developed. Given the good correlation between K_q and D_m , we suggest that the determination of K_q alone is an excellent initial rapid screen for the identification of high-potency peptide chemosensitizers for the reversal of drug resistance.

As expected, cyclosporine A, a known powerful chemosensitizer of Pgp-mediated MDR, was able to restore uptake by CH^RC5 cells of [3 H]daunorubicin and rhodamine 123 to levels similar to those observed in drug-sensitive cells. We have also demonstrated the ability of a cyclic peptide (beauvericin) and a linear peptide (pepstatin A) to restore uptake of the chemotherapeutic drug daunorubicin in intact CH^RC5 MDR cells. These peptides were able to increase total cellular levels of the drug to values equal to or exceeding those seen for the parent cell line AuxB1, at concentrations that were related in a predictable way to their K_q and D_m values. The cyclic peptide beauvericin was similar in efficacy to cyclosporine A, which is in current use clinically as a Pgp-chemosensitizing agent for MDR cancers.

None of the other peptides in the panel was able to restore accumulation of [3 H]daunorubicin or rhodamine 123 in intact MDR cells, within the concentration ranges accessible on the basis of their solubility. However, all of the peptides were able to inhibit drug transport by Pgp in a simple membrane vesicle system *in vitro*. It seems that additional factors, not applicable to

the system *in vitro*, come into play when blocking Pgp function in intact cells. One such factor is that of accessibility of the putative peptide chemosensitizer to the relevant binding site within the Pgp molecule. Many attempts have been made to determine the nature and number of the binding sites for drug substrates and chemosensitizers, but no consensus has been reached and many of the results in the literature are contradictory. It is possible that a single, large, flexible binding site exists that is large enough to accommodate many different drugs and chemosensitizers within overlapping regions. It has recently been suggested that the substrate-binding site of Pgp is located close to the cytoplasmic face of the molecule and is accessible from the inner face of the plasma membrane [46]. If this is indeed so, the ability of peptides to traverse (i.e. flip-flop across) the plasma membrane when added to the extracellular face of an intact cell is crucial in their ability to reach this site. This factor is not applicable to the systems *in vitro* used in this study; both faces of the Pgp molecule are accessible in detergent solution, and only the Pgp molecules with their NBD accessible at the outer surface of the vesicle (i.e. Pgp located in inside-out membrane vesicles) are able to transport drug into the vesicle lumen. It therefore seems likely that those peptides that are unable to reverse daunorubicin and rhodamine 123 accumulation in intact cells, but are able to inhibit drug transport in inside-out membrane vesicles, have the property of crossing slowly to the inside face of the plasma membrane in whole cells. In contrast, beauvericin, cyclosporine A and pepstatin A are probably able to cross rapidly to the cytoplasmic membrane face.

This proposal is consistent with the hypothesis of Eytan et al. [47], who presented evidence linking the flip-flop rate of a particular compound across lipid bilayers with its ability to act as a chemosensitizer. It is assumed that both drug 'substrates' and chemosensitizers are handled the same way by Pgp, i.e. they are transported, with ATP hydrolysis. Substrates were found to flip-flop across lipid bilayers slowly, on a timescale of minutes to hours, whereas chemosensitizers equilibrated across lipid bilayers rapidly, in seconds [47]. After Pgp has 'flipped' a drug molecule to the outer leaflet, it can 'flop' back into the inner leaflet before interacting with the transporter again (i.e. some futile cycling would take place). The slow flip-flop rate of a typical drug substrate would allow Pgp to keep pace and build up a drug concentration gradient; the cell would consequently display MDR. For chemosensitizers with a rapid flip-flop-rate, the rate of re-equilibration with the inner leaflet is so high that Pgp cannot establish a concentration gradient (futile cycling would occur), and the cell would consequently not display resistance to this or any other compound (discussed in [5]).

These considerations suggest that there are two criteria necessary for a particular peptide to be an effective Pgp chemosensitizer: high-affinity binding to Pgp, and a high rate of transmembrane equilibration or flip-flop. Peptides with a high membrane flip-flop rate would potentially be able to act as chemosensitizers, in accordance with their affinity of binding to Pgp. Peptides with high binding affinity but a low flip-flop rate would inhibit drug transport effectively in a vesicle system, but would be unable to block Pgp action in intact cells. Little is known about the relationship between the ability of a peptide to cross a lipid bilayer or membrane, and its chemical structure. Because linear peptides generally seem to be of very low toxicity, and cyclic peptides are effective at blocking drug transport in a relatively non-toxic concentration range, peptides should be given serious consideration for development as clinical chemosensitizing agents. Systematic investigation is needed in this area, leading to the development of peptides with both high Pgp-binding affinity and high flip-flop rates.

This work was supported by a grant to F.J.S. from the Cancer Research Society (Canada) and the National Cancer Institute of Canada (with funds from the Canadian Cancer Society).

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