Biochimica et Biophysica Acta, 1109 (1992) 161–171

BBAMEM 75719

Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug-resistant Chinese hamster ovary cells

Carl A. Doige and Frances J. Sharom

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry and Biochemistry, University of Guelph, Guelph (Canada)

(Received 10 April 1992)

Key words: P-glycoprotein; Glycoprotein; Colchicine transport; Vinblastine transport; Plasma membrane; Multidrug resistance; Chemosensitizer; (CHO cell)

Multidrug resistant (MDR) cells overexpress a 170–180 kDa membrane glycoprotein, the P-glycoprotein, which is believed to export drugs in an ATP-dependent manner. Plasma membrane vesicles from the MDR CH^RC5 cell line, but not the AuxB1 drug-sensitive parent, showed uptake of [^{3}H]colchicine and [^{3}H]vinblastine that was stimulated by the presence of ATP and an ATP-regenerating system. Steady-state uptake of drugs was achieved by 10 min and was stable for greater than 30 min. Non-hydrolysable ATP analogues were unable to support drug uptake, indicating that ATP hydrolysis is essential for transport. ATP-stimulated drug uptake appeared to result from drug transport into inside-out vesicles, since uptake was osmotically sensitive and could be prevented by detergent permeabilization. Steady-state uptake was half-maximal at 100 μM colchicine and 200 nM vinblastine and was inhibited by a 10–100-fold excess of MDR drugs and chemosensitizers, in the order vinblastine > verapamil > daunomycin > colchicine. In addition to being vanadate-sensitive, drug uptake was inhibited by 10–200 μM concentrations of several sulfhydryl-modifying reagents, suggesting that cysteine residues play an important role in drug transport. Vesicular colchicine was rapidly exchanged by an excess of unlabelled drug, demonstrating that drug association is the net result of opposing colchicine fluxes across the membrane.

Introduction

Pgp is a 1280 amino-acid glycoprotein overexpressed in the plasma membrane of multidrug resistant cells in vitro. Pgp is believed to cause multidrug resistance through its ability to actively export drugs from the cell, thus maintaining intracellular drug concentrations at subtoxic levels. Pgp can be labelled by photoaffinity analogues of vinblastine [1,2], verapamil [3] and colchicine [4] and by azido-ATP [5], and two domains of Pgp are highly homologous to the ATP-binding domains of bacterial transport systems [6,7], supporting the hypothesis that Pgp serves as an ATP-dependent drug-efflux pump.

It is important to understand how Pgp recognizes its structurally diverse substrates and how ATP hydrolysis supports their transport across the membrane. Many transport studies have been carried out on intact cells (for examples, see Refs. 8–12). While these investigations demonstrated differences in bulk drug levels between drug-sensitive and resistant cells, they could not readily be used to characterize the transport properties of Pgp. In intact cells, drug transport across the plasma membrane is complicated by the existence of several other interacting processes such as drug binding, sequestration and metabolism [13]. A more useful approach is to conduct drug association studies using plasma membrane vesicles isolated from MDR cells. Of the studies of this type that have been carried out to date, some have reported ATP-dependent drug transport [14–16], while others purport to have measured both ATP-dependent [17–19] and independent [20,21] drug binding.

One observation consistently found in most of the studies of membrane-drug association has been that, while other MDR-type drugs (e.g., vinblastine, vin-

Correspondence to: Dr. F.J. Sharom, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

Abbreviations: AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; pCMBS, *p*-chloromercuribenzenesulfonate; DMSO, dimethylsulfoxide; MDR, multidrug resistant; NEM, *N*-ethylmaleimide; Pgp, P-glycoprotein; PMSF, phenylmethylsulfonyl fluoride, Tris, tris(hydroxymethyl)aminomethane; WGA, wheat germ agglutinin.

cristine, daunomycin) or chemosensitizers (e.g., verapamil) compete strongly for the interaction of radiolabelled Vinca alkaloids with membrane vesicles, colchicine is a relatively poor competitor [14,18,20]. Colchicine has also been shown to be a poor inhibitor of photoaffinity labelling of Pgp by vinblastine and verapamil analogues [1,3]. Yet many MDR cell lines are highly resistant to colchicine, and a colchicine analogue has been shown to photoaffinity label Pgp [4].

In this study, we have examined the association of colchicine with plasma membrane vesicles from a MDR cell line selected for resistance to colchicine, the CH^RC5 Chinese hamster ovary cell line. In addition, we compared certain aspects of colchicine association with that seen for the Vinca alkaloid vinblastine. Our objectives were to characterize this drug association and determine how it is affected by other MDR-type drugs and chemosensitizers. We have been able to demonstrate osmotically-sensitive drug transport that requires ATP and a regenerating system for optimum activity. Results show that CH^RC5 plasma membrane vesicles are a very useful system for characterizing the drug transport properties of Pgp.

Materials and Methods

Materials. Colchicine, cytosine arabinoside, daunomycin, CHAPS, Triton X-100, pCMBS, NEM, mercuric chloride, verapamil hydrochloride, vinblastine sulfate, 5,5'-dithiobis(2-nitrobenzoic acid), acetylthiocholine chloride, acetylcholinesterase and ATP were purchased from Sigma (St. Louis, MO). Creatine kinase, creatine phosphate, ATPγS, AMP-PNP, dATP and GTP were obtained from Boehringer-Mannheim Canada (Dorval, Québec). Sodium metavanadate was obtained from Aldrich (Milwaukee, WI). Tissue culture supplies were purchased from Gibco Canada (Burlington, Ontario) and supplemented/defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT).

Cell culture and plasma membrane preparation. The drug-sensitive parent CHO cell line (AuxB1) and the MDR cell line selected for colchicine resistance (CH^RC5) have been described previously [22]. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in α-minimal essential medium supplemented with 10% heat-inactivated bovine calf serum, penicillin (1000 U/ml), streptomycin (1 mg/ml) and 2 mM L-glutamine.

The procedure for isolation of plasma membrane vesicles from AuxB1 and CH^RC5 has been described in detail elsewhere [23] and is an adaptation of a method by Lever [24]. Briefly, cells were harvested using trypsin-citrate-saline, washed with Dulbecco's phosphate-buffered saline and disrupted using a Yeda press in homogenization buffer consisting of 10 mM Tris-HCl, 0.25 M sucrose, pH 7.3 at 23°C, containing 0.2 mM $CaCl_2$, 0.02% NaN_3 and 1 mg/ml of each of aprotinin, PMSF, pepstatin A and leupeptin (all from Sigma). The homogenate was centrifuged for 10 min at $1000 \times g$ to remove nuclei and unlysed cells and the supernatant was layered onto a 35% (w/w) sucrose cushion and centrifuged for 1 h at $95000 \times g$. The interface layer was removed and washed twice with 10 mM Tris-HCl, 0.25 sucrose (pH 7.3) at 23°C and collected by centrifugation for 30 min at $100000 \times g$. The final pellet was resuspended in the same buffer using a 25 gauge needle and stored at −70°C for no more than 3 months before use. Plasma membrane vesicles were labelled with ^{125}I using Iodobeads (Pierce, Rockford, IL) and $Na^{125}I$ (ICN Biochemicals, St. Laurent, Québec), as described previously [25].

Colchicine and vinblastine transport into plasma membrane vesicles. Drug transport was measured by a modification of the method of Horio et al. [14]. All measurements were made at 23°C. An aliquot of membrane vesicles (20 μl, 30–50 μg protein) diluted in transport buffer (50 mM Tris-HCl, 0.25 M sucrose, 5 mM Mg^{2+}, pH 7.4) was combined with 30 μl of transport buffer containing ATP and a regenerating system (final concentrations were 1 mM ATP, 30 μg/ml creatine kinase and 3 mM creatine phosphate). Transport was initiated by the addition of either 0.2 μCi [^{3}H]colchicine (15.6–25.7 Ci/mmol, DuPont Canada, Mississauga, Ontario) or 0.025 μCi [^{3}H]vinblastine (17 Ci/mmol, Amersham Canada, Mississauga, Ontario) in 50 μl transport buffer. At various times, transport was terminated by filtration of the entire reaction mixture through a Whatman GF/F filter (soaked overnight in 10% bovine serum albumin at 37°C for vinblastine studies) on a Hoeffer filtration manifold, followed immediately by a wash with 5 ml of cold transport buffer. Filters were oven-dried and counted for ^3H by liquid scintillation counting using Ready-Safe (Beckman Canada, Mississauga, Ontario). All data were corrected for the amount of radioactivity bound to the filter in the absence of membrane vesicles, which was usually 5–10% of the total. Vesicle recovery on the filters was about 50%, as determined by carrying out the assay with ^{125}I-labelled membrane vesicles, followed by gamma counting of the dried filters. Hydrophobic drugs (vinblastine, verapamil, pCMBS and NEM) used in this study were prepared as stock solutions in DMSO and controls contained the appropriate DMSO concentration, which never exceeded 0.5% (w/v). This level of DMSO had no inhibitory effects on ATP-dependent colchicine transport. NEM and pCMBS were prepared fresh daily, while other drugs were stored as stock solutions at −20°C. For comparison, some experiments were performed without ATP, or with ATP, but without a regenerating system, as indicated in the figures or figure legends. All other experiments were performed

in the presence of both ATP and a regenerating system. Drug uptake was normalized to the protein content of the membrane vesicle preparation, as determined by a microwell adaptation of the Bradford assay [26] using bovine serum albumin (crystallized and lyophilized, Sigma) as a standard. Data are presented as the means of triplicate determinations ± S.E.

Determination of plasma membrane vesicle orientation. Plasma membrane vesicle orientation was assessed by measuring the latent acetylcholinesterase activity in a microwell adaptation of the method by Steck and Kant [27]. Briefly, plasma membrane vesicles (150–180 μg) were suspended in 200 μl of reaction buffer (10 mM Tris-HCl, 0.25 M sucrose, 0.5 mM acetylthiocholine chloride, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.02% azide, pH 7.6) and incubated for 30 min at 37°C. The reaction was terminated by the addition of 50 μl of 5% SDS and the absorbance of each well at 405 nm was measured using an ELISA plate reader. Acetylcholinesterase activity was determined by comparison to a series of acetylcholinesterase standards which were assayed simultaneously (1 U will hydrolyze 1 μmol of acetylcholine per min at pH 8.0 at 37°C). Total acetylcholinesterase activity was determined in the presence of 0.1% Triton X-100.

Enrichment of inside-out vesicles by WGA agglutination. All operations were performed at 4°C. An aliquot of CH^RC5 plasma membrane (4 mg/ml) was mixed with an equal volume of WGA (Boehringer-Mannheim, 1 mg/ml) in transport buffer and incubated overnight. Agglutinated vesicles were collected by centrifugation for 3 s at 14000 × *g*. The supernatant, enriched in inside-out vesicles, was collected and pelleted at 164000 × *g* for 15 min. The resulting pellet was then washed with deagglutination buffer (50 mM Tris-HCl, 0.27 M *N*-acetylglucosamine, 5 mM $MgCl_2$, 0.02% azide, pH 7.4), centrifuged at 164000 × *g* for 15 min and resuspended in transport buffer. The agglutinated vesicle pellet, enriched in right-side-out vesicles, was suspended in deagglutination buffer for at least 10 min, followed by centrifugation at 164000 × *g* for 15 min. The vesicles were washed once more with deagglutination buffer and then resuspended in transport buffer.

Calculation of transmembrane drug concentration gradient. Quasi-elastic light scattering measurements were carried out as described previously [25], to determine the size distribution of plasma membrane vesicles from CH^RC5 cells. Results (not shown) indicated that the vesicles were a relatively homogeneous population, with a mean diameter of 0.31 ± 0.03 μm (three different preparations). Assuming a membrane thickness of 10 nm, the internal volume of a vesicle of this size was estimated to be $1.28 \cdot 10^{-14}$ ml. This is in reasonable agreement with Mimms et al. [28], who estimated the intenal volume of a 0.23 μm diameter vesicle to be $0.5 \cdot 10^{-14}$ ml. The amount of drug taken up into plasma membrane vesicles at 100 μM colchicine and 200 nM vinblastine (drug concentrations producing half-maximal uptake) in the absence of ATP and a regenerating system, is 816 pmol/mg and 159 pmol/mg respectively (see Fig. 2A and B). Assuming a plasma membrane protein content of 50%, a membrane density of 1.16 mg/ml and accounting for vesicle recovery in the filtration assay, this corresponds to $1.25 \cdot 10^{-12}$ pmol colchicine/vesicle, giving an effective internal concentration of 195 μM colchicine, about twice the external drug concentration. This agrees well with the idea that the uptake of colchicine into membrane vesicles in the absence of energization represents equilibration across the membrane by passive diffusion. The small amount of additional colchicine taken up over that expected likely represents binding to the membrane surface, as well as partitioning into the membrane itself. Corresponding calculations for 200 nM vinblastine gave an effective internal concentration of 38 μM. This result is consistent with the high hydrophobicity of vinblastine, which would be expected to partition into the membrane. Assuming that only inside-out vesicles (29%, see Results) can take up colchicine actively, the effective intravesicular colchicine concentration at 100 μM drug in the presence of ATP and a regenerating system was calculated to be 3.65 mM. Similar calculations for vinblastine lead to the estimation of an effective intravesicular concentration of 376 μM at an external concentration of 200 nM.

Results

ATP stimulates drug uptake into membrane vesicles from drug-resistant cells

Uptake of [^{3}H]colchicine and [^{3}H]vinblastine into plasma membrane vesicles prepared from drug-sensitive AuxB1 or drug-resistant CH^RC5 was determined at various times, either in the absence of ATP, in the presence of ATP alone, or in the presence of ATP and a regenerating system (Fig. 1A and B). In the absence of ATP, drug uptake was similar in vesicles from both AuxB1 and CH^RC5. This uptake likely represents diffusion of drug into the membrane vesicles, as well as binding to both external and internal sites and partitioning into the membrane bilayer (see below for more discussion on this point). In the presence of 1 mM ATP, there was a small but transient increase in the uptake of [^{3}H]colchicine into CH^RC5 vesicles, which was not observed in AuxB1 vesicles (data not shown). The transient nature of the uptake into CH^RC5 vesicles may be related to the rapid depletion of ATP by the ATP-hydrolysing activity of Pgp and other membrane-associated ATPases. In support of this view, when a higher concentration of ATP (3 mM) was used, colchicine uptake was greater and more sustained,

reaching a maximum at 10 min. When both ATP and an ATP regenerating system were used, a significant increase in [^{3}H]colchicine uptake into CH^{R}C5 vesicles occurred, which was not observed in AuxB1 vesicles. This enhanced uptake achieved a maximum steady-state level at 5–10 min, after which it decreased slowly up to 45 min. Uptake of [^{3}H]vinblastine into CH^{R}C5 membrane vesicles followed a similar pattern, but ATP

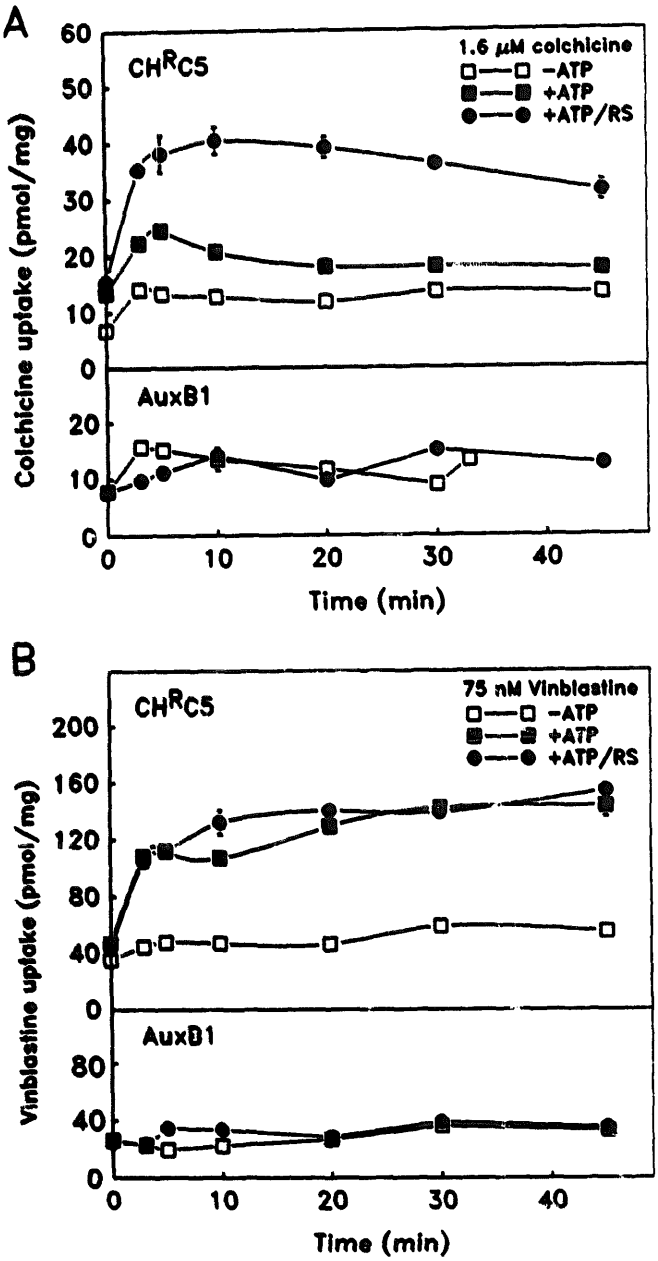

Fig. 1. Time course of (A) [^{3}H]colchicine uptake and (B) [^{3}H]vinblastine uptake into plasma membrane vesicles from drug-sensitive AuxB1 (lower panel) and drug-resistant CH^{R}C5 (upper panel). Vesicles were incubated for the indicated times in transport buffer alone (□), or buffer containing 1 mM ATP (■), or 1 mM ATP plus a regenerating system (RS,●) consisting of 30 μg/ml creatine kinase and 3 mM creatine phosphate. Concentrations of colchicine and vinblastine were 1.6 μM and 78 nM, respectively. Data points represent means ± S.E. (n = 3).

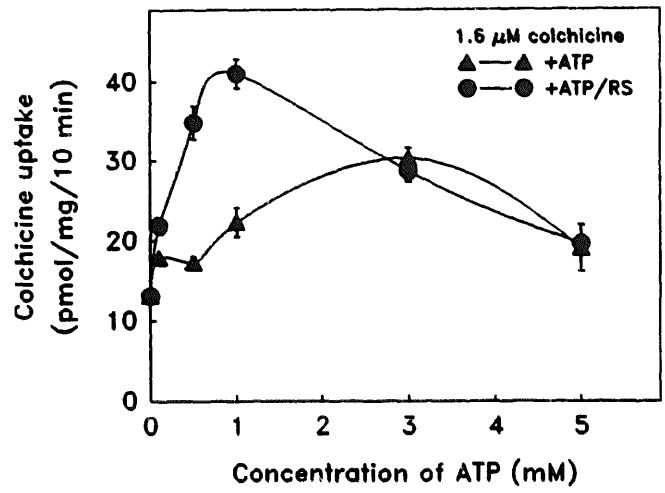

Fig. 2. Dependence of [^{3}H]colchicine uptake into CH^{R}C5 vesicles on ATP concentration. Membrane vesicles were incubated with various concentrations of ATP alone (▲), or ATP with a regenerating system (RS, ●). Steady-state colchicine uptake into the vesicles was determined at 10 min. Colchicine concentration was 1.6 μM. Data points represent means + S.E. (n = 3).

alone was sufficient to sustain a high level of uptake, which did not decline over a period of 45 min. At the drug concentrations used in Fig. 1, maximal colchicine uptake at 10 min for numerous experiments (n = 31) was 53.8 ± 3.6 pmol/mg (several different membrane preparations), while maximal vinblastine uptake was 227 ± 46 pmol/mg (n = 5, for two different preparations). On the basis of these results, unless otherwise indicated, further characterization of drug uptake was carried out using measurements made at 10 min.

To ensure that the optimum ATP concentration was used, we assessed the ATP dependence of [^{3}H]colchicine uptake into CH^{R}C5 vesicles at 10 min (Fig. 2). When ATP alone was used, maximum uptake occurred at 3 mM, however when ATP was used with a regenerating system, maximum uptake was considerably higher and occurred at 1 mM ATP. Colchicine uptake in the presence of 3 mM ATP alone and 3 mM ATP plus a regenerating system was identical, suggesting that at concentrations of ATP higher than 1 mM, either the transporter or the regenerating system is inhibited.

To test the nucleotide dependence and whether ATP hydrolysis was required for drug uptake, [^{3}H]colchicine uptake was measured in the presence of different nucleotides (Fig. 3). Two different nucleotide concentrations were used; 1 mM (corresponding to the maximum uptake for ATP plus a regenerating system) and 3 mM (corresponding to the maximum uptake for ATP alone). The enhanced [^{3}H]colchicine uptake appeared to require ATP hydrolysis, since non-hydrolysable analogues of ATP (ATPγS and AMP-PNP) were unable to support increased colchicine uptake. Enhanced drug uptake is nucleotide specific; dATP produced only a small increase in [^{3}H]colchicine uptake,

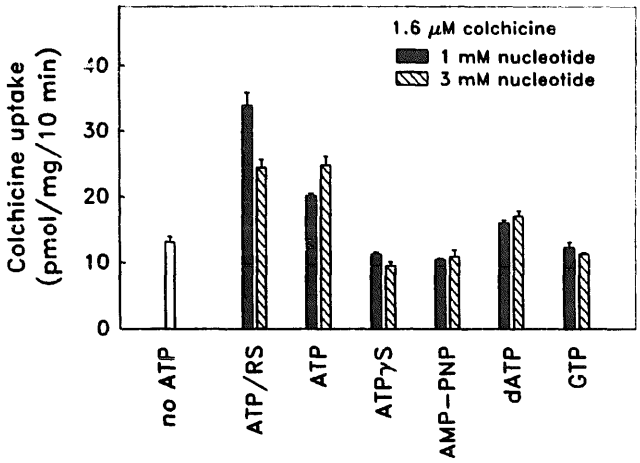

Fig. 3. Ability of various nucleotides to support [^{3}H]colchicine uptake into CH^{R}C5 plasma membrane vesicles. Drug uptake was measured after 10 min in the absence of ATP (open bar), or in the presence of 1 mM (solid bars) and 3 mM (hatched bars) concentrations of various nucleotides. ATP was tested both with and without a regenerating system (RS). Colchicine concentration was 1.6 μM. Data points represent means ± S.E. (n = 3).

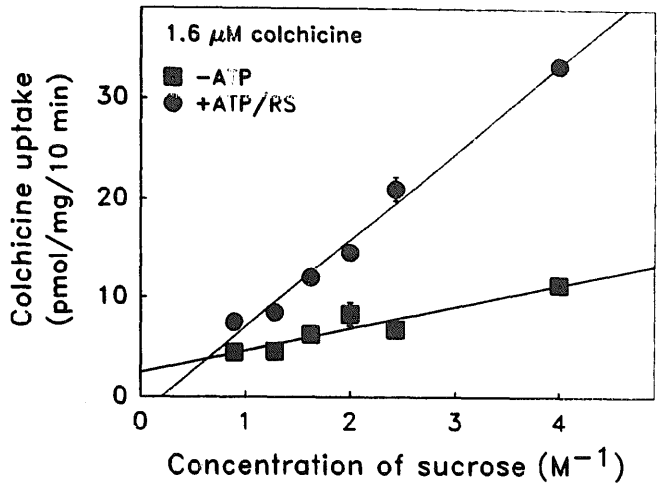

Fig. 4. Osmotic sensitivity of colchicine uptake. CH^{R}C5 membrane vesicles were preincubated for 10 min in transport buffer containing sucrose concentrations ranging from 0.25 M (isotonic) to 1.12 M. Steady-state [^{3}H]colchicine uptake was measured at 10 min in the absence (■) and presence (●) of 1 mM ATP and a regenerating system (RS). Colchicine concentration was 1.6 μM. Data points represent means ± S.E. (n = 3).

while uptake in the presence of GTP was not significantly different from the control with no ATP.

Enhanced uptake represents drug transport into membrane vesicles

An important criterion for distinguishing membrane transport from binding is the demonstration that steady-state uptake is proportional to the intravesicular space. This space can be changed by altering the sucrose concentration outside the vesicles; as the sucrose concentration increases, the vesicles will shrink progressively by osmosis, reducing the intravesicular space and thus reducing accumulation of the substrate. CH^{R}C5 membrane vesicles were preincubated for 10 min with sucrose concentrations ranging from 0.25 to 1.12 M. Steady-state ^{3}H-labelled drug uptake in the absence and presence of ATP and a regenerating system was then measured. As shown in Fig. 4, ATP-dependent [^{3}H]colchicine uptake was osmotically sensitive and, therefore, represents transport into the vesicle interior. Vinblastine uptake also showed osmotic sensitivity (not shown). A small fraction of the ATP-independent uptake appeared to be sensitive to the intravesicular space and therefore also represents accumulation inside the vesicles. This is expected for drug uptake resulting from simple diffusion.

A further indication of transport is the disruption of uptake by permeabilizing concentrations of detergent. When CH^{R}C5 vesicles were incubated with CHAPS, ATP-dependent uptake of both [^{3}H]colchicine and [^{3}H]vinblastine was almost eliminated (Table I). The concentrations of CHAPS used in these experiments were sufficient to produce membrane permeabilization [29], but did not affect the recovery of ^{125}I-labelled membrane vesicles in the filtration assay, indicating that decreased drug uptake was not due to vesicle solubilization.

Based on the predicted membrane topology of Pgp, our results are consistent with ATP-dependent colchicine transport into inside-out vesicles, a situation analogous to drug export from the cytosol. Disruption of CH^{R}C5 cells produces a mixture of right-side-out and inside-out membrane vesicles. We determined the orientation of these vesicles by measuring the activity of the ecto-enzyme acetylcholinesterase in the absence and presence of a permeabilizing amount of detergent. The difference in activity between these two treatments represents sealed inside-out vesicles. Acetylcholinesterase activity in the absence and presence of 0.1% (v/v) Triton X-100 was $(1.47 \pm 0.04) \cdot 10^{-4}$ and $(2.06 \pm 0.06) \cdot 10^{-4}$ U/mg/min (n = 6), respectively, indicating that around 29% of the vesicle population was inside-out.

TABLE I

Effect of the detergent CHAPS on drug uptake into CH^{R}C5 plasma membrane vesicles

Uptake of [^{3}H]colchicine and [^{3}H]vinblastine into CH^{R}C5 plasma membrane vesicles was measured after 10 min in 1.6 μM colchicine or 76 nM vinblastine, in the presence or absence of 1 mM ATP and a regenerating system. The ATP-dependent drug uptake was determined and expressed as the means ± S.E. (n = 3).

CHAPS concentration (% (w/v))	ATP-dependent colchicine uptake (pmol/mg per 10 min)	ATP-dependent vinblastine uptake (pmol/mg per 10 min)
0	36.1	106.1
0.04	13.1	70.9
0.1	1.1	18.3

We next attempted to separate inside-out from right-side-out vesicles by agglutinating right-side-out vesicles bearing exposed surface carbohydrate with the lectin WGA [30]. We initially tested several WGA concentrations and monitored the distribution of vesicles in the supernatant and agglutinated pellet by both protein assay and counting of ^{125}I-labelled vesicles. A plot of membrane protein remaining in the supernatant at increasing ratios of WGA:protein gave a biphasic curve, with a steep drop up to a WGA:membrane protein ratio of 0.25 (w/w), at which point around 75% of the vesicles were agglutinated, followed by a gradual decrease reaching 85% agglutination at a WGA:membrane protein ratio of 0.5. Since this second phase likely represents nonspecific agglutination, we used a WGA:membrane protein ratio of 0.25 in subsequent experiments. To determine whether inside-out vesicles were able to carry out ATP-dependent drug transport, we measured [^{3}H]colchicine uptake into the vesicle fractions separated by WGA agglutination (Table II). The non-agglutinated inside-out vesicle fraction displayed a clear enrichment in ATP-dependent drug uptake. The residual transport activity in the right-side-out agglutinated fraction likely indicates an incomplete separation of inside-out and right-side-out vesicles by this method. As a control, drug uptake into a sample containing the separated fractions remixed in their original proportions was measured. Kamimoto et al. also measured transport activity in antibody-separated inside-out vesicles and reported that inside-out vesicles demonstrated transport, but with 2-fold lower activity than the original vesicle mixture [15].

Drug uptake is specific and saturable

For active transport, it is important to demonstrate that the process is saturable at high substrate concentrations. Fig. 5A and B shows the dependence of transport on drug concentration. In the presence of ATP and a regenerating system, [^{3}H]colchicine uptake was saturable at colchicine concentrations of approximately 400–500 μM, with half-maximal uptake occurring at 100 μM (Fig. 6A). When an excess of unlabelled colchicine (10 mM) was included with ATP, [^{3}H]colchicine uptake was approx. 4–5-fold lower and was linear with colchicine concentration, suggesting a nonspecific uptake process. The difference between colchicine uptake in the presence and absence of excess colchicine represents specific ATP-dependent transport into membrane vesicles. Colchicine uptake in the absence of ATP was approximately linear with

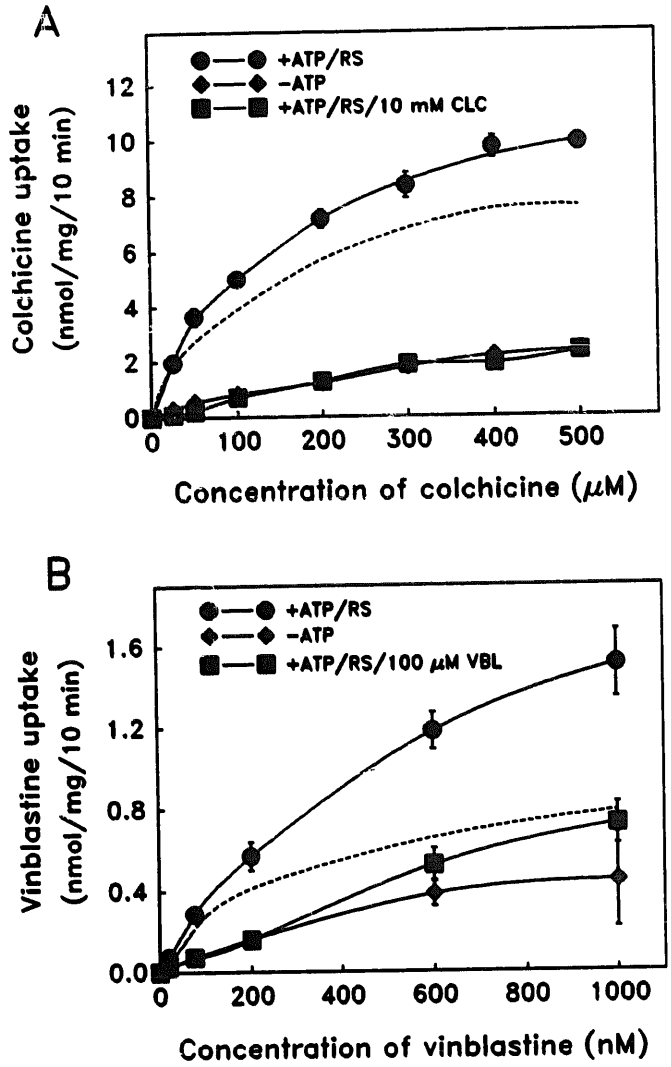

Fig. 5. Dependence of (A) colchicine and (B) vinblastine transport on drug concentration. [^{3}H]Colchicine and [^{3}H]vinblastine uptake into CH^{R}C5 membrane vesicles were measured at 10 min at various drug concentrations in the absence (◆) and presence (●, ■) of 1 mM ATP and a regenerating system (RS). (●) shows total drug uptake and (■) shows nonspecific uptake in the presence of 10 mM unlabelled colchicine (CLC) or 100 μM vinblastine (VBL). The dashed line indicates specific drug transport in the presence of 1 mM ATP and a regenerating system, calculated by subtraction of nonspecific from total drug uptake. Data points represent means ± S.E. (n = 3).

TABLE II

Drug uptake into CH^{R}C5 plasma membrane vesicles fractionated by WGA agglutination

CH^{R}C5 membrane vesicles were agglutinated using WGA and fractions enriched in inside-out and right-side-out vesicles were isolated as described in Materials and Methods. A mixed fraction was prepared by recombining the agglutinated and non-agglutinated fractions in proportions equal to the original membrane composition. Drug uptake was determined after 10 min in 1.6 μM colchicine in the presence or absence of 1 mM ATP and a regenerating system. Results are shown as the means ± S.E. (n = 3).

Treatment	ATP-dependent colchicine uptake (pmol/mg per 10 min)
Non-agglutinated	37.4 ± 2.4
Agglutinated	22.7 ± 2.1
Mix	27.0 ± 2.0

colchicine concentration and was at a level similar to that observed for nonspecific ATP-dependent colchicine uptake. A similar pattern was observed for uptake of [^{3}H]vinblastine, however nonspecific uptake in the presence of excess drug was higher, likely as a result of the more hydrophobic nature of the molecule. Vinblastine uptake approached saturation at 1000 nM, with half-maximal uptake at 200 nM. It should be noted that this data cannot be expressed in terms of Michaelis-Menten parameters, since steady-state uptake values are being measured, rather than initial rates of transport. Maximal steady-state levels of ATP-dependent colchicine uptake approached 7.5 nmol/mg, whereas for vinblastine, they only reached 0.75 nmol/mg. Thus, although the multidrug transporter appears to have a higher affinity for vinblastine relative to colchicine (as evidenced by the 500-fold lower drug concentration needed to achieve half-maximal uptake), it apparently has a much higher transport capacity for colchicine relative to vinblastine (indicated by the 10-fold higher steady-state uptake at saturation). Other researchers have reported maximal steady-state uptake values for vinblastine in the range 0.02–0.70 nmol/mg [14,16,18,31].

Steady-state uptake of drug likely represents a balance between active ATP-dependent pumping by Pgp and passive outward diffusion through the bilayer [31]. If this is the case, trapped intravesicular drug should be exchangeable with drug in the external milieu. To examine this possibility, membrane vesicles were incubated in a standard reaction mixture containing [^{3}H]colchicine in the absence or presence of ATP and a regenerating system. After 5 min, 10 mM unlabelled colchicine was added and the amount of labelled drug remaining in the vesicles was measured over time. Fig. 6 shows that accumulated [^{3}H]colchicine was rapidly displaced by the addition of excess unlabelled drug. These results show that, at steady-state, the intravesicular pool of [^{3}H]colchicine can be rapidly exchanged with (and diluted by) the large excess of unlabelled colchicine present on the vesicle exterior. [^{3}H]Colchicine accumulated in the absence of ATP was also displaced by an excess of unlabelled colchicine, implying that the drug might also interact specifically with the membrane vesicles in an ATP-independent fashion. Since the level of ATP-independent accumulation was similar in vesicles from both drug-sensitive and MDR cells (see Fig. 1), it is unlikely to arise from a specific interaction with Pgp.

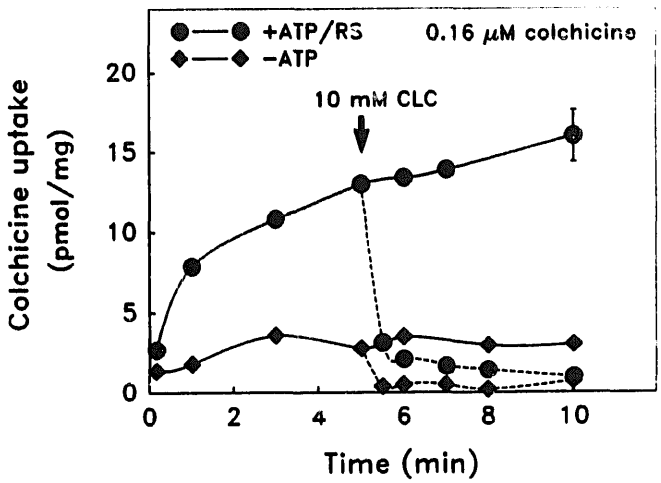

Fig. 6. Rapid exchange of accumulated [^{3}H]colchicine by the addition of unlabelled colchicine. CH^{R}C5 membrane vesicles were incubated in transport buffer containing 0.16 μM [^{3}H]colchicine in the absence (♦) or presence (●) of 1 mM ATP and a regenerating system (RS). Unlabelled colchicine (CLC, 10 mM) was added as indicated and the amount of labelled colchicine remaining in the vesicles was measured over time, as indicated by the dashed lines. Data points represent means ± S.E. (n = 3).

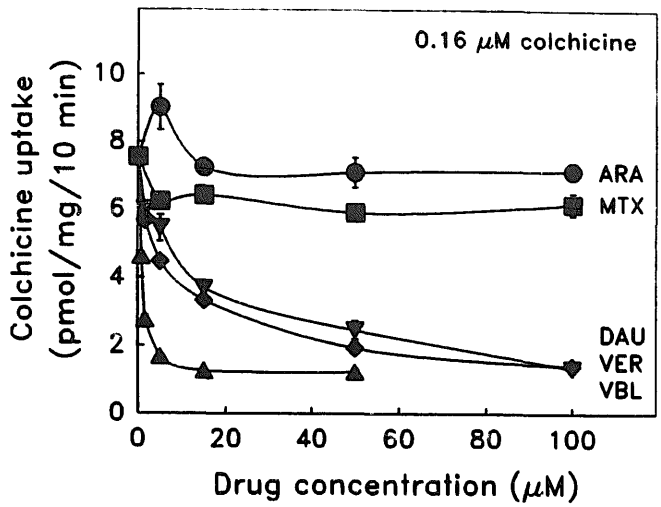

Fig. 7. Competition by cytotoxic drugs and chemosensitizers for ATP-dependent colchicine transport. [^{3}H]Colchicine uptake into CH^{R}C5 membrane vesicles was measured at 10 min in the presence of 1 mM ATP and a regenerating system, together with the indicated concentrations of cytosine arabinoside (ARA, ●), methotrexate (MTX, ■), daunomycin (DAU, ▼), verapamil (VER, ♦) and vinblastine (VBL, ▲). Colchicine concentration was 0.16 μM and uptake in the absence of ATP was 1.82 ± 0.08 pmol/mg per 10 min. Data points represent means ± S.E. (n = 3).

Drug uptake is inhibited by other MDR-type compounds and chemosensitizers

Experiments were performed to determine whether ATP-dependent drug uptake could be inhibited by other anticancer drugs and chemosensitizers. As shown in Fig. 7, other MDR spectrum drugs such as daunomycin and vinblastine and the chemosensitizer verapamil, inhibited ATP-dependent [^{3}H]colchicine uptake into membrane vesicles. The order of potency of inhibition was vinblastine > verapamil > daunomycin. Drugs to which the CH^{R}C5 cells are not cross-resistant, such as cytosine arabinoside and methotrexate, did not compete for [^{3}H]colchicine uptake, indicating that this effect is specific for drugs which interact with

Pgp. It is interesting to note that although colchicine has been reported to be a poor inhibitor of vinblastine uptake, our studies showed that the reverse was not true; vinblastine was an excellent inhibitor of colchicine uptake into CH^RC5 membrane vesicles. All drugs tested had IC_{50} values lower than that of colchicine (Table III).

Vanadate and sulfhydryl-modifying reagents inactivate ATP-dependent colchicine transport

Vanadate is known to be an inhibitor of E_1E_2-type ion-motive ATPases, such as the Na^+/K^+- and Ca^{2+}-ATPases. Low concentrations of vanadate also inhibited ATP-dependent [^{3}H]colchicine uptake (Table III), suggesting that Pgp may employ a transport mechanism involving a phosphorylated intermediate. Vanadate inhibition of colchicine uptake in the presence of ATP, but without a regenerating system, yielded similar results, confirming that the observed effect was due to inhibition of the transporter itself and not creatine kinase. It should be noted, however, that up to 1 mM ouabain, a specific inhibitor of the Na^+/K^+-ATPase, had no effect on ATP-dependent colchicine transport by Pgp (data not shown). The well-characterized mechanism of action of the E_1E_2 ion-motive ATPases may provide some insight into the functioning of Pgp.

Sulfhydryl groups on cysteine residues have been shown to be essential for ATP-dependent organic cation [32] and organic anion [33] transport in renal brush-border membranes and for glutathione-S-conjugate transport in membrane vesicles from human erythrocytes [34]. Since it seems possible that these transport systems might share functional similarities with Pgp, we tested the effect of sulfhydryl-modifying reagents on the active transport of colchicine into CH^RC5 membrane vesicles. As shown in Fig. 8, [^{3}H]colchicine transport was completely inhibited by all three agents tested, in the concentration range 10–200 μM, in the order of potency $HgCl_2$ > pCMBS > NEM. IC_{50} values for the inhibitors are shown in Table III. These experiments were conducted using ATP without a regenerating system, since sulfhydryl reagents inhibit creatine kinase [35] at concentrations similar to those used in this study.

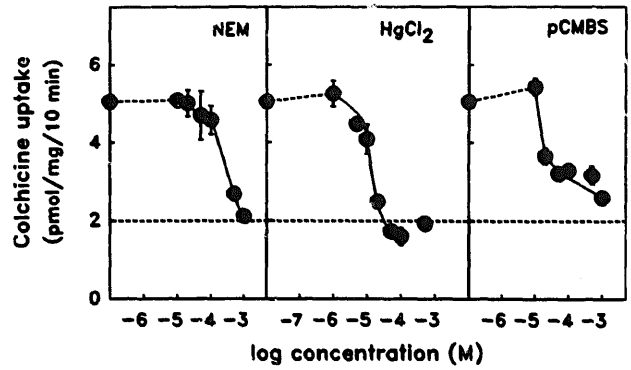

Fig. 8. Inhibition of ATP-dependent colchicine transport by sulfhydryl-modifying reagents. [^{3}H]Colchicine uptake into CH^RC5 membrane vesicles was measured at 10 min in the presence of 1 mM ATP and a regenerating system, together with the indicated concentrations of NEM, pCMBS or $HgCl_2$. Data points on the left-hand vertical axis represent uptake in the absence of inhibitors. Colchicine concentration was 0.16 μM and uptake in the absence of ATP was 1.98 ± 0.11 pmol/mg per 10 min, as indicated by the dashed line. Data points represent means ± S.E. (n = 3).

TABLE III

Inhibition of colchicine uptake

[^{3}H]Colchicine uptake into CH^RC5 plasma membrane vesicles was determined after 10 min in 1.6 μM colchicine, in the presence of 1 mM ATP and a regenerating system, together with various concentrations of drugs and inhibitors. IC_{50} values were determined by plotting % inhibition of colchicine uptake vs. inhibitor concentration and interpolating the concentration which produced 50% inhibition of the ATP-stimulated transport.

Agent	IC_{50} (μM)
Vinblastine	1.0
Verapamil	10
Daunomycin	14
Colchicine	195
Vanadate	30
$HgCl_2$	15
pCMBS	20
NEM	200

Discussion

Pgp is believed to confer multidrug resistance by maintaining intracellular drug concentrations at sublethal levels through active drug export. However, it is not understood how Pgp recognizes a multitude of substrates and how ATP energizes their export. Cornwell et al. [20] first showed that vinblastine binding to vesicles from human MDR KB carcinoma cells was higher than to vesicles from drug-sensitive cells. This association did not require exogenously added ATP, nor was it osmotically sensitive. More recently, Horio et al. [14] demonstrated osmotically-sensitive transport of vinblastine into KB plasma membrane vesicles that required ATP and a regenerating system. Naito et al. [18], on the other hand, reported ATP-dependent binding of vincristine to membrane vesicles from MDR K562 cells. Although studies by other researchers have all indicated the existence of a specific, saturable interaction of drugs with MDR membrane vesicles [15–17,19,21], there is general disagreement as to whether drug binding or transport is being measured and whether ATP, or both ATP and a regenerating system, is required. Unfortunately, these studies are difficult to compare to one another, since the experimental condi-

tions and the type of experiments performed were not standardized. Furthermore, although some researchers claim to have measured drug binding, they failed to test whether drug association was sensitive to osmotic strength or detergent permeabilization [18,21].

In this study, we have used a MDR CHO cell line, CH^RC5, that was selected for resistance to colchicine. SDS-PAGE and Western immunoblot analysis indicated high levels of Pgp (approx. 5–10% of the total membrane protein) in plasma membrane from these cells, whereas virtually no Pgp was found in the membranes of drug-sensitive AuxB1 cells [23]. ATP-dependent colchicine and vinblastine uptake was observed only in vesicles from CH^RC5 cells and not in vesicles from the drug-sensitive parent, suggesting that Pgp is directly involved. Furthermore, this enhanced drug uptake was osmotically sensitive and could be abolished by the presence of detergent, demonstrating that the uptake represents true active transport into membrane vesicles.

Transport was not supported by non-hydrolysable analogues of ATP (ATPγS and AMP-PNP), suggesting that ATP hydrolysis and not simply ATP binding, was required for colchicine transport. In addition, transport was saturable with increasing concentrations of drug, demonstrating the specificity of the process. Sustained colchicine accumulation required both ATP and a regenerating system, while vinblastine accumulation could be supported only by ATP. Further studies will be needed to understand the difference in ATP requirements for transport of the two drugs. Although the multidrug transporter in CH^RC5 membrane vesicles demonstrated a 10-fold higher capacity for transport of colchicine relative to vinblastine, its apparent affinity for vinblastine was 500-fold higher than for colchicine. Our results extend the work of Horio et al. [14,31], which demonstrated ATP-dependent, osmotically-sensitive vinblastine transport into plasma membrane vesicles from KB cells and confirm two other reports of ATP-energized drug transport into membrane vesicles containing Pgp [15,16].

Based on the predicted amino-acid sequence and hydropathy analysis, Pgp is believed to consist of a tandem duplication, with each half containing six transmembrane domains and a cytoplasmic domain with a putative ATP binding site [6,7]. Disruption of CH^RC5 cells produced a mixture of approx. 70% right-side-out and 30% inside-out membrane vesicles, as shown by measurements of the activity of the ectoenzyme acetylcholinesterase in the absence and presence of detergent. A membrane fraction enriched in inside-out vesicles by WGA agglutination showed higher levels of drug transport than the right-side-out fraction. Our measurements are thus consistent with ATP-dependent drug transport into inside-out vesicles, a situation analogous to drug export from the cytosol.

It is interesting to note that other research groups have reported measuring enhanced ATP-dependent Vinca alkaloid binding [18,19] to MDR membrane vesicles, using methodology similar to that used in this study to measure transport. The differences in results may be explained in a number of ways. For instance, the drug-uptake system may not have been sufficiently characterized in previous studies, so that drug transport was actually being measured instead of drug binding. Alternatively, whether transport or binding is measured may depend on the properties of the vesicles, which, in turn, may depend on the method of preparation.

In this report, we have shown that steady-state levels of accumulated [^{3}H]colchicine were rapidly exchanged with an excess of unlabelled drug added to the vesicle exterior. We interpret our data to mean that, at steady state, the level of accumulated drug is the net result of rapid and opposing fluxes across the membrane. ATP-energized drug transport into the vesicle is balanced against simple diffusion out of the vesicle, driven by a concentration gradient. When an excess of unlabelled drug was added, it competed with radiolabelled drug for transport into the vesicle and thus diluted the steady-state levels of accumulated [^{3}H]colchicine. This was perceived as a rapid loss of [^{3}H]colchicine from the vesicle as new steady-state levels were established. Similar experiments using inside-out vesicles from *Salmonella typhimurium* showed that steady-state levels of accumulated radiolabelled histidine could be rapidly exchanged via histidine permease by the external addition of excess unlabelled substrate [36].

Both colchicine and vinblastine are accumulated inside the membrane vesicles against a concentration gradient in the presence of ATP. Calculations indicate that at the concentration required for half-maximal uptake, the drug concentration associated with the vesicles in the presence of ATP and a regenerating system is 37-fold greater than that in the extravesicular medium for colchicine and 1900-fold greater for vinblastine (see Materials and Methods for details). All intravesicular drug concentrations are calculated based on the assumption that vesicle-associated drug is entirely intravesicular. Clearly this will not be true for hydrophobic drugs like vinblastine, where partitioning into the membrane will be substantial. This effect can be accounted for by comparing intravesicular drug concentrations in the absence and presence of ATP. Calculations on drug uptake in the absence of ATP are consistent with equilibration of drug across the membrane by simple diffusion in the case of colchicine (2-fold gradient) and additional partitioning in the case of vinblastine (19-fold gradient). Thus ATP and a regenerating system stimulated an 18-fold increase in the concentration gradient for colchicine and a 10-fold increase for vinblastine. Reconstituted histidine per-

mease vesicles concentrate histidine 100-fold [37] and glutathione-S-conjugates are accumulated against an 8-fold gradient in erythrocyte membrane vesicles [34], both ATP-dependent processes.

Colchicine has consistently been found to be a poor competitor for photoaffinity labelling of Pgp by azidopine [38] and photoactive analogues of vinblastine [1] and verapamil [3]. Similar observations have been made for Vinca alkaloid binding or transport in MDR membrane vesicles [14,18,20]. Since MDR cells show high levels of colchicine resistance, the existence of an independent binding site for colchicine has been postulated [3,14]. An alternative explanation is that MDR-type drugs possess significantly different affinities for Pgp [4,20]. Our results are also consistent with this idea and Fig. 5A and B shows that Pgp has a higher affinity for vinblastine than colchicine. In addition, vinblastine, verapamil and daunomycin readily competed for colchicine transport and required lower concentrations for half-maximal inhibition than colchicine itself. Thus, although the CH^RC5 line was selected for resistance to colchicine, the Pgp expressed appears to have greater affinity for other MDR drugs. Our results also showed that verapamil is a good competitor of colchicine transport, confirming the report of Horio et al., who demonstrated inhibition of vinblastine transport by chemosensitizers [31]. Further studies are being directed towards determining the initial rate kinetics of drug transport in CH^RC5 membrane vesicles, in an effort to characterize the nature of the competition observed between MDR-type drugs and chemosensitizers.

Our results indicate that Pgp sulfhydryls may be important in ATP-dependent drug transport. The three sulfhydryl-modifying agents used in this study differed in their ability to inactivate drug transport in the order $HgCl_2$ > pCMBS > NEM. Similar results were noted for the organic anion exchanger [33], where the higher reactivity of $HgCl_2$ was attributed to the enhanced capability of the small molecule to penetrate sensitive sites. pCMBS is relatively membrane impermeant [39], suggesting that it interacts with cysteine residues located on the cytoplasmic face of Pgp. There are three candidate cysteines found in the C-terminal cytoplasmic domain of Pgp (one within the Walker A motif), which are conserved in the cDNA of hamster *pgp1* and *pgp2* [40] and in the corresponding *mdr* cDNAs from human [6] and mouse [7]. Although NEM has been shown to inhibit the ATPase activity of purified Pgp [41 and C.A. Doige and F.J. Sharom, unpublished data], there have been no reports of inhibition of drug transport or binding by sulfhydryl-modifying reagents. Little is currently known concerning the interaction of ATP with the nucleotide binding domain, or the coupling of ATP hydrolysis to substrate translocation and further investigation in this area may provide some insight into the mechanism of drug transport.

Acknowledgements

The authors would like to thank Dr. Victor Ling, of the Ontario Cancer Institute, for providing the multidrug resistant cell lines used in this study and Dr. Ross Hallett, of the Department of Physics at the University of Guelph, for use of the quasielastic light scattering apparatus. This research was supported by a grant from the National Cancer Institute of Canada. C.A.D. was supported by a graduate student scholarship from the Natural Sciences and Engineering Research Council of Canada.

References

1 Cornwell, M.M., Safa, A.R., Felsted, R.L., Gottesman, M.M. and Pastan, I. (1986) Proc. Natl. Acad. Sci. USA 83, 3847–3850.
2 Safa, A.R., Glover, C.J., Meyers, M.B., Biedler, J.L. and Felsted, R.L. (1986) J. Biol. Chem. 261, 6137–6140.
3 Safa, A.R. (1988) Proc. Natl. Acad. Sci. USA 85, 7187–7191.
4 Safa, A.R., Mehta, N.D. and Agresti, M. (1989) Biochem. Biophys. Res. Commun. 162, 1402–1408.
5 Cornwell, M.M., Tsuruo, T., Gottesman, M.M. and Pastan, I. (1987) FASEB J. 1, 51–54.
6 Chen, C.-j., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M. and Roninson, I.B. (1986) Cell 47, 381–389.
7 Gros, P., Croop, J.M. and Housman, D.E. (1986) Cell 47, 371–380.
8 Skovsgaard, T. (1978) Cancer Res. 38, 1785–1791.
9 Inaba, M., Kobayashi, H., Sakurai, Y. and Johnson, R.K. (1979) Cancer Res. 39, 2200–2203.
10 Johnson, B.A., Cheang, M.S. and Goldenberg, G.J. (1986) Cancer Res. 46, 218–223.
11 Cano-Gauci, D.F. and Riordan, J.R. (1987) Biochem. Pharmacol. 36, 2115–2123.
12 Inaba, M., Watanabe, T. and Sugiyama, Y. (1987) Jpn. J. Cancer Res. 78, 397–404.
13 Riordan, J.R. and Ling, V. (1985) Pharmacol. Therap. 28, 51–75.
14 Horio, M., Gottesman, M.M. and Pastan, I. (1988) Proc. Natl. Acad. Sci. USA 85, 3580–3584.
15 Kamimoto, Y., Gatmaitan, Z., Hsu, J. and Arias, I.M. (1989) J. Biol. Chem. 264, 11693–11698.
16 Tamai, I. and Safa, A.R. (1990) J. Biol. Chem. 265, 16509–16513.
17 Tamai, I. and Safa, A.R. (1991) J. Biol. Chem. 266, 16796–16800.
18 Naito, M., Hamada, H. and Tsuruo, T. (1988) J. Biol. Chem. 263, 11887–11891.
19 Sehested, M., Bindslev, N., Demant, E.J.F., Skovsgaard, T. and Jensen, P.B. (1989) Biochem. Pharmacol. 38, 3017–3027.
20 Cornwell, M.M., Gottesman, M.M. and Pastan, I. (1986) J. Biol. Chem. 261, 7921–7928.
21 Yang, C.-p.H., Cohen, D., Greenberger, L.M., Hsu, S.I.-H. and Horwitz, S.B. (1990) J. Biol. Chem. 265, 10282–10288.
22 Kartner, N., Everdnen-Porelle, D., Bradley, G. and Ling, V. (1985) Nature 316, 820–823.
23 Doige, C.A. and Sharom, F.J. (1991) Protein Express. Purif. 2, 256–265.
24 Lever, J. (1987) Methods Enzymol. 146, 376–383.
25 Chicken, C.A. and Sharom, F.J. (1983) Biochim. Biophys. Acta 729, 200–208.
26 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
27 Steck, T.L. and Kant J.A. (1974) Methods Enzymol. 31, 172–180.
28 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, G. and Reynolds, J.A. (1981) Biochemistry 20, 833–840.
29 Hjelmeland, L.M. (1990) Methods Enzymol. 182, 253–264.
30 Charuk, J.H.M, Howlett, S. and Michalak, M. (1989) Biochem. J. 264, 885–892.

31 Horio, M., Lovelace, E., Pastan, I. and Gottesman, M.M. (1991) Biochim. Biophys. Acta 1061, 106–110.
32 Sokol, P.P., Holohan, P.D. and Ross, C.R. (1986) J. Biol. Chem. 261, 3282–3287.
33 Sokol, P.P., Holohan, P.D. and Ross, C.R. (1986) Biochim. Biophys. Acta 862, 335–342.
34 LaBelle, E.F., Singh, V., Srivastava, S.K. and Awasthi, Y.C. (1986) Biochem. J. 238, 443–449.
35 Ennor, A.H. and Rosenberg, H. (1954) Biochem. J. 57, 203–212.
36 Prossnitz, E., Gee, A. and Ames, G.F.-L. (1989) J. Biol. Chem. 264, 5006–5014.
37 Bishop, L., Agbayani, R., Ambudkar, S.V., Maloney, P.C. and Ames, G.F.-L. (1989) Proc. Natl. Acad. Sci. USA 86, 6953–6957.
38 Safa, A.R., Glover, C.J., Sewell, J.L., Meyers, M.B., Biedler, J.L. and Felsted, R.L. (1987) J. Biol. Chem. 262, 7884–7888.
39 Rothstein, A. (1970) Curr. Top. Membr. Transp. 1, 135–176.
40 Endicott, J.A., Juranka, P.F., Sarangi, F., Gerlach, J.H., Deuchars, K.L. and Ling, V. (1987) Mol. Cell. Biol. 7, 4075–4081.
41 Hamada, H. and Tsuruo, T. (1988) Cancer Res. 48, 4926–4932.