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The influence of P-glycoprotein on morphine transport in Caco-2 cells. Comparison with paclitaxel

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Abstract

In vitro monolayer studies using Caco-2 cells were employed here to explore P-glycoprotein mediated transport of morphine. Bi-directional transport studies of 10–75 μM morphine showed efflux to be twofold higher than influx (4×10^{-6} compared to 2×10^{-6} cm/s) and cellular accumulation in the efflux direction was eightfold higher. The cyclosporin analogue (PSC-833) equilibrated morphine transport in both directions. Depletion of intracellular glutathione had a greater effect on increasing cellular morphine accumulation than P-glycoprotein inhibitors, suggesting a role for glutathione in morphine transport. P-glycoprotein had a substantially greater effect on paclitaxel accumulation, efflux and bi-directional transport than for morphine. Paclitaxel transport was below detection ($< 0.1 \times 10^{-6}$ cm/s) in the influx direction, yet efflux was very high (18.4×10^{-6} cm/s) and P-glycoprotein inhibition increased accumulation >100-fold. These results reinforce the substantial role P-glycoprotein has in paclitaxel transport. Conversely, P-glycoprotein regulated morphine transport is weak. Nevertheless, morphine transport rates could be doubled when administered with P-glycoprotein substrates. Therefore, increased analgesia through P-glycoprotein inhibition should be possible.

Keywords: Active efflux; P-glycoprotein; MRP (multidrug resistance-associated protein); Glutathione; Taxol; PSC-833; Verapamil; MDR1

1. Introduction

The efflux proteins P-glycoprotein and multidrug resistance-associated protein (MRP1) are thought to be involved in chemotherapy resistance for many haematological cancers. They may also increase rejection episodes in transplantation patients. This is due to either a reduction of the entry rate into target cells and/or removal from the cytoplasm of a wide range of anticancer and immunosuppressant drugs known to be substrates for these plasma membrane transporters. Although these proteins are known to become upregulated in the tissues being targeted (Hu et al., 1999; Larsen et al., 2000), many organs contain barrier systems that have high endogenous expression levels of these proteins (Van de Vrie et al., 1998). In most organs, P-glycoprotein is expressed on endothelial or epithelial cells surrounding blood channels (Decleves et al., 2000; Pardridge et al., 1997). One of these is the blood–brain barrier (BBB) (Jetté et al., 1993; Schinkel, 1999). High levels of activity from P-glycoprotein at the BBB is a real threat to many pharmaceutical challenges to disease management. For example, human immunodeficiency virus (HIV) protease inhibitors blocked from the brain by P-glycoprotein mediated efflux (Lee et al., 1998) is an area receiving much attention.

P-glycoprotein and, to a lesser extent MRP1, have been shown to inhibit cellular uptake of hundreds of drugs. However, understanding the structural substrate requirements of P-glycoprotein and MRP1 in the protein active sites are yet to be resolved. There are no consistent structural similarities between substrates or inhibitors in order to develop theoretical models of likely substrates and their affinities for these proteins. Recent attempts to address this issue have proposed at least one significant model based on the distances between electron donor groups (Seelig, 1998) but there are still many substrates that fall outside these broad categories. Only direct measurements using P-glycoprotein or transcellular transport can currently determine the extent that drugs are substrates for these proteins.

These efflux pumps are thought to have evolved to protect sensitive areas of the body from xenotoxins present

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in our diets. Naturally derived drugs make up a high proportion of those effluxed by P-glycoprotein. These include cyclosporin A, paclitaxel, vinblastine and doxorubicin among others. Morphine is also a naturally occurring drug recently suspected of being another substrate for P-glycoprotein. Although not conclusive for active efflux involvement, variations in basal levels of P-glycoprotein activity and inter-patient variability of either absorption or efficacy are common for P-glycoprotein substrates. It is widely known that morphine efficacy displays vast inter-patient variability (Glare and Walsh, 1991), further supporting the notion of this drug as a P-glycoprotein substrate.

It has been known for some time that the rate of morphine transport into the brain appears lower than its physical properties would suggest. During postnatal development, morphine transport through the BBB decreases while di-acetyl morphine (heroin) transport does not (Loh et al., 1971). Addition of acetyl groups increases the log *P* of morphine from 0.89 to 1.58 (Avdeef et al., 1996) allowing BBB penetration up to 10-fold greater than morphine alone (Smith, 1993), but morphine should theoretically have a greater rate of brain uptake than has been shown experimentally (Xie et al., 1999).

The aim of this study was to evaluate the extent that morphine is transported by P-glycoprotein using a Caco-2 cell line, which is known for its naturally high levels of P-glycoprotein activity and its relatively tight junctions when grown in monolayers. Comparisons were made with paclitaxel, a cytotoxic compound known for its high P-glycoprotein specificity. This paper confirms the ability of morphine to be effluxed from a P-glycoprotein expressing cell line, while emphasizing the Caco-2 monolayer model as an ideal platform for sustained efflux analysis.

2. Materials and methods

2.1. Materials

Morphine was supplied by Fauldings (Melbourne, Australia). Cyclosporin A and its non-immunosuppressive analogue (PSC-833) were kindly donated by Novartis Pharmaceuticals (Basel, Switzerland). Paclitaxel, indomethacin and verapamil hydrochloride were purchased from ICN Biomedicals (Seven Hills, NSW, Australia). Probenecid, mammalian protease inhibitor cocktail and 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) liquid substrate were supplied by Sigma (Castle Hill, NSW, Australia). All other materials were of analytical grade.

Phosphate buffered saline (PBS), Hanks buffered salt solution (HBSS), non-essential amino acids (NEAA) and high glucose Dulbecco's Modified Eagle Medium (DMEM) were from Gibco BRL (Melbourne, Australia). Penicillin G and Streptomycin were from Trace Biosciences (Castle Hill, NSW, Australia), while the foetal calf serum was obtained from the Australian Commonwealth Serum Laboratories (Parkville, Vic, Australia).

2.2. Cell transport studies

Caco-2 cells were seeded onto Falcon polyethylene teraphthalate 0.9 cm^2 filter inserts in 12 well plates at 65,000 cells/cm^2. Cells were grown in 'growth medium' (high glucose DMEM with 25 mM HEPES, 2 mM glutamine, 1 mM NEAA, 100 U/ml penicillin–streptomycin and 10% foetal calf serum set to pH 7.4.

Monolayers were incubated for 21–25 days to allow full maturation of the cells, including P-glycoprotein expression and increased trans-epithelial electrical resistance formation (Bailey et al., 1996; Hosoya et al., 1996). The trans-epithelial electrical resistance was measured both before and immediately after the study using an EVOM meter and the ENDOHM 12 chamber (World Precision Instruments, Sarasota, FL, USA) with readings between 400 and 700 Ω cm^2 for all cells in this study.

Filter inserts were transferred to fresh 12 well plates for the studies. The studies were conducted using 'assay medium' consisting of HBSS supplemented with both glucose (Ajax chemicals, NSW, Australia) and HEPES (Gibco BRL) to give final concentrations of 25 and 10 mM, respectively. The pH was adjusted to 7.4 with NaOH.

Cells were incubated in prewarmed HBSS ± an efflux inhibitor for 30 min and then rinsed in the same medium. Trans-epithelial electrical resistance was measured and HBSS ± inhibitors were placed in the receiver chambers. Morphine at various concentrations between 5 and 150 μM was added to the donor chamber of each well. The apical and basolateral chambers received 0.5 and 1.4 ml of medium, respectively. Sample was removed from the receiver chamber at various times over a 3-h period. Constant volumes were maintained by adding prewarmed medium to the receiver chambers in order to maintain an equilibrium pressure differential between the volumes in the donor and receiver chambers. Transport studies in the apical to basolateral and basolateral to apical directions were simultaneously conducted with paclitaxel using concentrations between 10 and 50 μM.

In studies where morphine or paclitaxel transport were performed in conjunction with inhibition of P-glycoprotein or MRP, cells were preincubated in HBSS containing the inhibitors for 30 min before initiation of the study. The inhibitors included 4 μM PSC-833, 100 μM verapamil, 75 μM indomethacin and 500 μM probenecid. To determine the role of glutathione in morphine transport, which would indirectly implicate MRP, glutathione (1 mM) and DL-buthionine sulfoxamine (40 μM) were included with the DMEM growth medium for overnight monolayer incubation prior to the morphine studies. During the transport study, these modifying agents were also present in the donor chamber with morphine or paclitaxel and in the receiver chamber at the same concentrations as stated above.

2.3. Cell accumulation

Caco-2 cells were grown in 24 well culture plates using growth medium at an initial seeding density of 5000 cells/cm^2. The cells were used 21–25 days after seeding. Morphine was added at either 5, 10 or 20 μM in HBSS for various times up to 120 min. Paclitaxel accumulation was also measured, using either 10 or 50 μM concentrations. Cells were washed 3× in ice cold PBS, transferred to microcentrifuge tubes and disrupted in a sonicating water bath for 5 min before storing at −80 °C in H_2O. P-glycoprotein and MRP inhibition studies were also conducted using the same concentrations of modulators used in the transport studies.

2.4. Cell efflux

In parallel studies with cell accumulation, identical 24 well plates also seeded at 5000 cells/cm^2, were grown for 21–25 days. Initially, cellular accumulation of either morphine or paclitaxel was allowed to progress for 120 min. Caco-2 monolayers were washed 4× in ice cold PBS before incubating with 800 μl prewarmed HBSS containing either P-glycoprotein or MRP inhibitors. Aliquots were removed to glass high pressure liquid chromatography (HPLC) vials at various times up to 40 min after addition of the prewarmed HBSS. Medium was not replaced to allow drug concentrations to build in the remaining volume as the amount effluxed was close to detection limits in some instances.

Protein concentrations for all aspects of this study were determined using a micro-Lowry method on a Perkin Elmer EZ210 spectrophotometer.

2.5. Drug analysis

2.5.1. Morphine determinations

Samples collected in HBSS from efflux and transport studies were immediately collected into 2.0 ml HPLC vials with 350 μl low volume inserts. No extraction phases were required. The mobile phase consisted of 50 mM KH_2PO_4 (pH 7.0):acetonitrile (55:45 v/v). The HPLC system consisted of a Waters 501 isocratic pump running at 1.2 ml/min and a Perkin Elmer Series 200 autosampler injecting 50 μl of sample through an Econosphere RP-C_{18} column (5 μm pores, 25 cm × 4.6 mm I.D.) with mated guard column (Alltech, NSW, Australia). The effluent was detected fluorescently on an Agilent 1100 fluorescent detector. The excitation and emission wavelengths were 215 and 320 nm, respectively. A Hewlett Packard 3496A integrator provided peak area results, with a typical retention time of 6.4 min.

Morphine determinations from cell lysates were also performed without the need for complex extraction protocols. A 5-min sonication of cell monolayers in microcentrifuge tubes was adequate for liberation of morphine from the cells. Pelleting of cellular debris for 3 min at 2000 × *g* resulted in a visually clear solution that had clear chromatograms at the time morphine eluted. The detection limit for natural fluorescence detection of morphine was 30 nM using 50 μl injections.

Paclitaxel was determined through modifications of a recent protocol (Theodoris et al., 1998). As with morphine, no extraction phase was required for HPLC analysis. The mobile phase consisted of acetonitrile:water (60:40), run through a 25 cm × 4.6 mm ID, 5 μm pore RP-C_{18} Alltech Econosphere column at 1.0 ml/min using the same hardware as described above. The effluent was detected under UV light at 230 nm using a Waters 486 UV detector. Typical retention times were around 6 min. The limit of detection was 60 nM, when injecting 100 μl into the column.

2.6. Data analysis

Drug transport through cell monolayers was calculated both as a simple amount passing the monolayer per minute, which would vary depending on the initial concentration used, and as an apparent permeability co-efficient as calculated previously (Crowe and Lemaire, 1998).

The efflux of drugs after an accumulation phase for cells grown on the base of wells followed first-order kinetics. Maximal efflux was determined through the *Y* intercept on a linear double reciprocal plot of amount effluxed over time. Time to the half maximal efflux was determined by calculating the *X* intercept.

Results expressed in this study are presented as the mean ± S.E.M, standardised on individual well protein concentrations. Significant differences between values were examined using Student's two-tailed unpaired *t*-test. Results were considered significant if $P < 0.05$.

3. Results

3.1. Accumulation

Accumulation of morphine into Caco-2 cells was conducted with monolayers of these cells grown on the base of 24 well plates (growth area 2 cm^2). Within 45 min of incubation with morphine, Caco-2 cells had reached maximal accumulation levels for all seeding concentrations of morphine (Fig. 1). The time taken to reach equilibrium with the surrounding medium was relatively uniform for 5–20 μM concentrations of morphine. Higher (75–150 μM) concentrations reached equilibrium 15–20 min quicker (data not shown). Even at the lower 5–20 μM concentrations there were still significant differences in the way maximal accumulation was achieved. This suggested factors aside from pure diffusion were playing a role in the uptake of morphine. For example, cells incubated with 20 μM morphine accumulated around 1300 pmol/mg protein with a relatively uniform increase in the cellular morphine con-

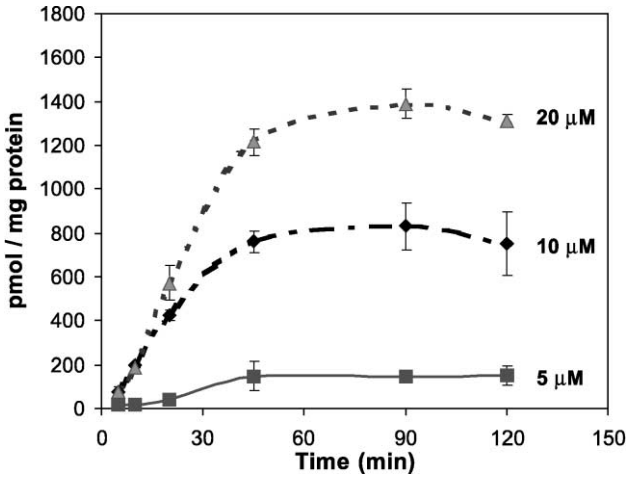

Fig. 1. Morphine accumulation in Caco-2 cell monolayers during 120 min incubation at either 5 μM (solid line), 10 μM (dashed line) or 20 μM (dotted line) concentrations. Results are the mean of triplicate samples ± S.E.M.

centration during the first 45 min. At 20 min over 50% of the equilibrium concentration has been taken up be the cells. However, at 5 μM, the equilibrium concentration was 150 pmol/mg protein, with only 25% of the final equilibrium levels present at 20 min. This is evident from 5 μM data in Fig. 1 where a long morphine accumulation lag was apparent. On a diffusion basis, it would be expected that incubation with 10 μM morphine would give half the amount of 20 μM morphine. Accumulation of around 700 pmol/mg was obtained with 10 μM morphine, very close to the theoretical diffusion value. However, 5 μM morphine should have given 300–350 pmol/mg, not the 150 pmol/mg determined experimentally. Coupled with the 5 μM accumulation lag phase, both parameters suggested a mechanism inhibiting uptake of physiologically relevant concentrations of morphine.

3.2. Modified accumulation

The next stage of this study involved modifying efflux activity of Caco-2 cells in order to determine whether increases in accumulation could be attained after this activity was increased or decreased. Addition of the various efflux protein modifiers 20 h prior to examining morphine uptake did not affect the structure of the monolayers. Cell protein concentrations were also consistent across all groups regardless of inhibitory compounds added, demonstrating that cell attachment and integrity remained intact throughout the washing steps.

Increases in cell accumulation were detected when overnight incubation with DL-buthionine sulfoxamine had depleted intracellular glutathione suggesting that glutathione may be involved in the efflux mechanism of morphine (Fig. 2). However, increasing glutathione levels did not result in a reduction of morphine in the cells compared to control cell cultures.

Incubation with 4 μM PSC-833 and 100 μM verapamil only had a small effect on increasing intracellular Caco-2 morphine levels using 5 μM morphine. Inhibitors were present during the 15–30 min accumulation section of this study as well as in the previous 20 h. Hence, both long-term and short-term effects of inhibitors on efflux proteins should have combined to exhibit an increase in morphine levels associated with the cells if efflux proteins were involved in expelling morphine from either the cytoplasm or inner cell membrane. Material adsorbing to the cells could increase the perceived accumulation results in our analysis of control cells and would not be differentiated from material entering the cytoplasm under current assay conditions. Hence, it was not possible to conclude that traditional P-glycoprotein inhibitors were not effective. However, it could be seen that depleting glutathione levels within the cells had a marked effect on morphine accumulation.

To further examine the role of P-glycoprotein mediated efflux at limiting morphine accumulation in Caco-2 cells, 4 μM PSC-833 was incubated with morphine for various times up to 120 min. There was no significant increase in the rate or final amount of morphine accumulated into these cells when used at 20 μM (Fig. 3A). However, it was clear that PSC-833 allowed significantly more paclitaxel to accumulate into Caco-2 cells (Fig. 3B).

As P-glycoprotein mediated transport of paclitaxel was very effective in this study, a variety of both P-glycoprotein and MRP inhibitors were incubated with paclitaxel for 2 h to examine differences between known P-glycoprotein modifying agents. Fig. 4 shows the accumulation of paclitaxel after co-incubation with these inhibitors. It is clear that known P-glycoprotein inhibitors have had a dramatic effect

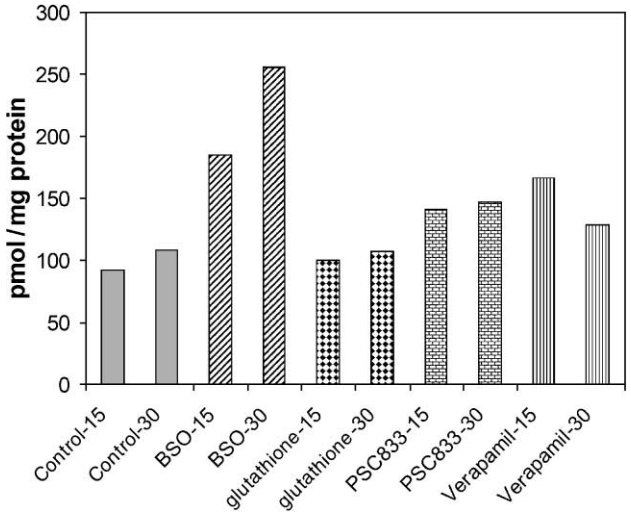

Fig. 2. Accumulation of morphine in Caco-2 cell monolayers grown on the base of 24 well plates after an overnight incubation with various P-glycoprotein or MRP inhibitors followed by 5 μM morphine for either 15 or 30 min. Inhibitors remained present in the medium during the accumulation phase of the study. Data represent the average of two measurements.

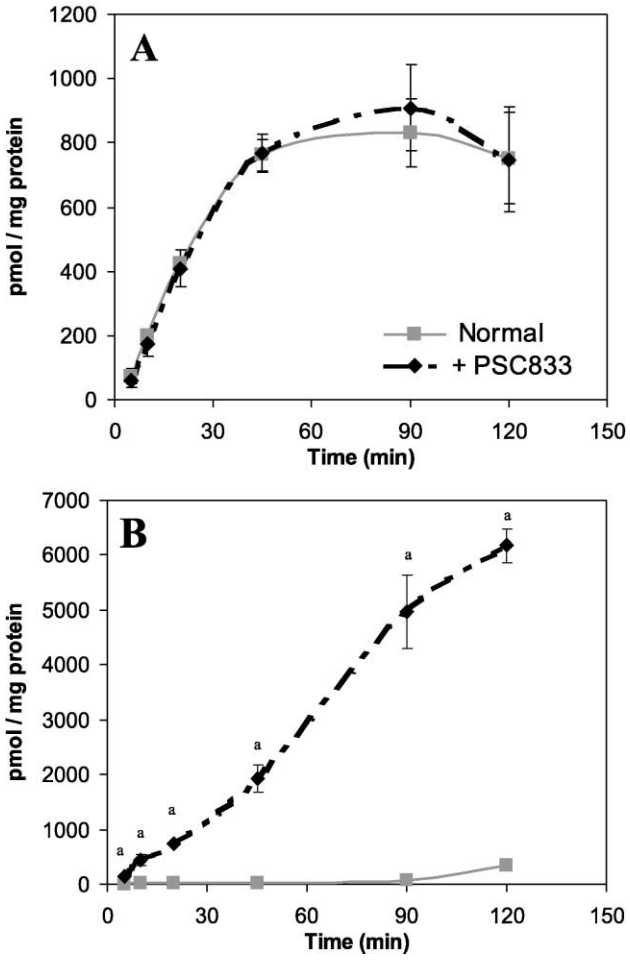

Fig. 3. Accumulation of drugs in Caco-2 cells over 120 min, with (dashed line) and without (full line) co-incubation with 4 μM PSC-833. (A) Use of 10 μM morphine in the medium for up to 120 min. (B) Use of 20 μM paclitaxel in the medium for up to 120 min. Each data point is the mean of triplicate samples ± S.E.M. [a]$P < 0.005$.

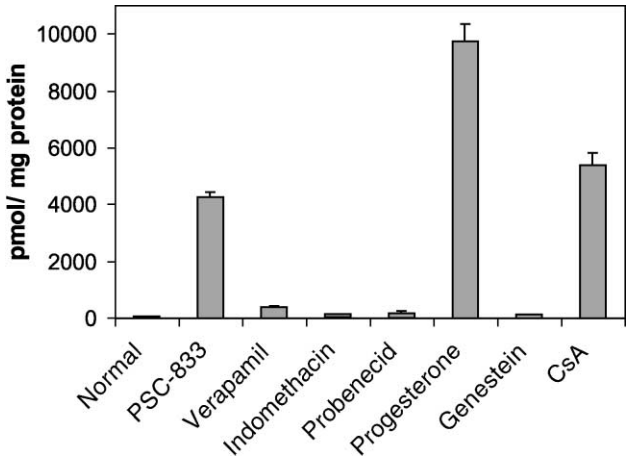

Fig. 4. Accumulation of 50 μM paclitaxel in Caco-2 cell monolayers grown on the base of 24 well plates. Paclitaxel was co-administered with P-glycoprotein and MRP inhibitors for 120 min. Results are the mean of triplicate samples ± S.E.M.

upon the cellular concentration of this drug. Control cells only accumulated 43 pmol/mg in the 2 h, while cells incubated with PSC-833, progesterone, and cyclosporin A all accumulated over 100 times the control amount. Surprisingly, 100 μM verapamil only increased cellular levels to 400 pmol/mg, so was 10-fold less effective than the other P-glycoprotein inhibitors.

The use of 10–20 μM morphine may have been too high for results of efflux modification to become apparent. Future work would concentrate on rates of accumulation in the first 5–10 min and the use of lower concentrations using [^{3}H]morphine.

3.3. Bi-directional transport

Transport of morphine through Caco-2 monolayers occurred in both apical to basolateral and basolateral to apical directions; however, basolateral to apical transport was of a greater rate than transport in the other direction (Fig. 5). Transport in the apical to basolateral direction is analogous to transport through the gut lumen to the blood supply. However, if the presence and activity of P-glycoprotein in Caco-2 monolayers are taken to represent a broader perspective of P-glycoprotein activity in the body then apical to basolateral transport can be equivalent to blood to brain transport though the BBB. Our results show two times the rate of transport in the basolateral to apical (brain to blood or blood to lumen) direction (Table 1), indicating the presence of an efflux pump to remove this compound from within cell membranes. Trans-epithelial electrical resistance values were consistently above 500 Ω cm^2 suggesting intercellular tight junctions were

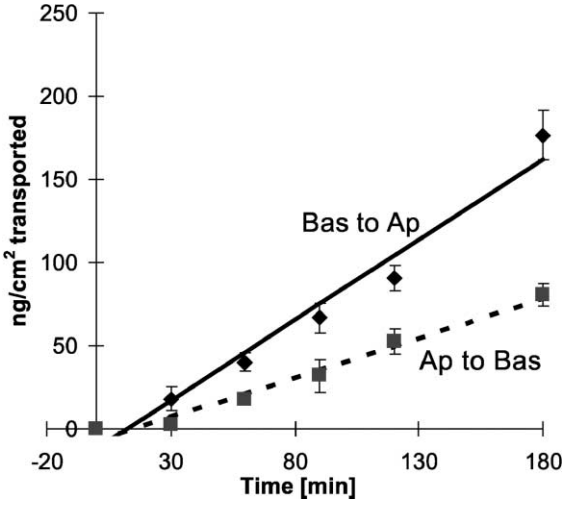

Fig. 5. Transport of 10 μM morphine through 3-week-old Caco-2 cells grown on filter inserts. Transport is expressed as ng/cm^2. Basolateral to apical transport is illustrated with diamonds (solid line), while apical to basolateral transport is shown with squares (dashed line). Results are the mean of six samples ± S.E.M.

Table 1
Transport rates of 10, 75 and 150 μM morphine through Caco-2 cell monolayers in both apical to basolateral and basolateral to apical directions from cells grown on filter inserts suspended in culture

Concentration (μM) ± modifier	Ap → Bas cm/s ($\times 10^{-6}$)	Bas → Ap cm/s ($\times 10^{-6}$)	Net Ap → Bas cm/s ($\times 10^{-6}$)
10	1.99 ± 0.17	4.06 ± 0.24	− 2.07[b]
10 + PSC 833[e]	1.89 ± 0.06	2.57 ± 0.05[d]	− 0.68[b]
10 + Cyclosporin A[e]	2.08 ± 0.06	2.59 ± 0.07[d]	− 0.51[a]
10 + Probenecid[e]	2.07 ± 0.10	4.05 ± 0.13	− 1.98[b]
10 + Glutathione[e]	1.26 ± 0.15[c]	3.28 ± 0.04[d]	− 2.02[b]
10 + BSO[e]	1.78 ± 0.07	3.80 ± 0.10	− 2.02[b]
75	1.5 ± 0.2	3.1 ± 0.3	− 1.6[b]
150	3.7 ± 0.1	4.8 ± 0.1	− 1.1[b]
150 + PSC	3.8 ± 0.2	3.6 ± 0.1	+ 0.2
Paclitaxel (μM) ± modifier			
10	0.0 ± 0.0	18.4 ± 0.2	− 18.4[b]
10 + PSC[e]	9.7 ± 0.8[d]	12.0 ± 0.2[d]	− 2.3[a]
150	3.7 ± 0.1[d]	4.8 ± 0.1[d]	− 1.1[b]

Results are presented as a clearance rate in cm/s. Transport was conducted during a 120-min period. Transport of 10 μM paclitaxel is shown as a positive control with PSC-833 used to inhibit P-glycoprotein activity. Significant differences between transport directions ([a,b]) and modifier addition in a given direction ([c,d]) are shown.

[a,c] $P < 0.05$.

[b,d] $P < 0.005$.

[e] PSC-833 used at 4 μM, cyclosporin A used at 10 μM, probenecid used at 500 μM, glutathione used at 1 mM and buthionine sulfoxamine (BSO) was used at 40 μM in all studies.

functional and thus paracellular transport would have been very low.

The use of a compound's clearance rate through the cell monolayers provides a concentration independent flux measurement (Crowe and Lemaire, 1998). The clearance rate for morphine in the basolateral to apical direction was greater than 4×10^{-6} cm/s, while apical to basolateral flux was around 2×10^{-6} cm/s (Table 1). As an amount, these clearance rates equated to 475 pg/cm^2/min in the apical to basolateral direction and 964 pg/cm^2/min in the basolateral to apical direction when using 10 μM morphine. These results are greater than those published previously and suggest that the effect of efflux proteins on reducing morphine uptake into organs such as the brain are more significant than previously realised.

Transport results from the more lipophilic paclitaxel showed almost no paclitaxel moving through the cells in the apical to basolateral direction, yet in the reverse direction, paclitaxel transport was very high (>18×10^{-6} cm/s) (Fig. 6, Table 1). Addition of PSC-833 significantly lowered basolateral to apical transport and increased apical to basolateral transport in a fashion indicative of P-glycoprotein activity suppression (Fig. 6).

To confirm that P-glycoprotein was involved in morphine efflux, 10 μM cyclosporin A and 4 μM PSC-833 were added to both sides of the transport system in different studies. MRP involvement was examined using 500 μM probenecid, 1 mM glutathione and 40 μM DL-buthionine sulfoxamine. Only the P-glycoprotein inhibitors reduced basolateral to apical transport, reducing rates from 4×10^{-6} to around 2.5×10^{-6} cm/s (Table 1) which equated to a reduction from 964 to 620 pg/cm^2/min using 10 μM morphine.

Usually, P-glycoprotein substrates are only expelled up to a threshold concentration before the binding sites become saturated, or the re-entry rate of a given drug equals or surpasses the ability of P-glycoprotein to remove it from the cell. Morphine transport conducted at 75 and 150 μM showed that a 150-μM concentration of morphine was required before significant reductions in the net basolateral to apical transport were noted (Table 1). Even at 150 μM, the ratio of efflux to influx was 1.3:1 compared to 2:1 for all lower concentrations, suggesting equilibrium had still not been reached. Co-incubation of 4 μM PSC-833 with 150 μM morphine was required before complete equilibration in both apical to basolateral and basolateral to apical directions was noted (Table 1).

Flux in the apical to basolateral direction remained quite consistent regardless of the transport inhibitor added. Thus, only basolateral to apical transport showed significant changes when agents such as PSC 833 and cyclosporin A were used, which was slightly different to the pattern shown by PSC-833 on paclitaxel transport. Once equilibrium was reached, it was estimated that morphine would have a human permeability of around 30%, having removed the influence of P-glycoprotein.

3.3.1. Drug association with cells after transport

An interesting observation from the transport studies came from drug accumulation within the cells after a 3-h

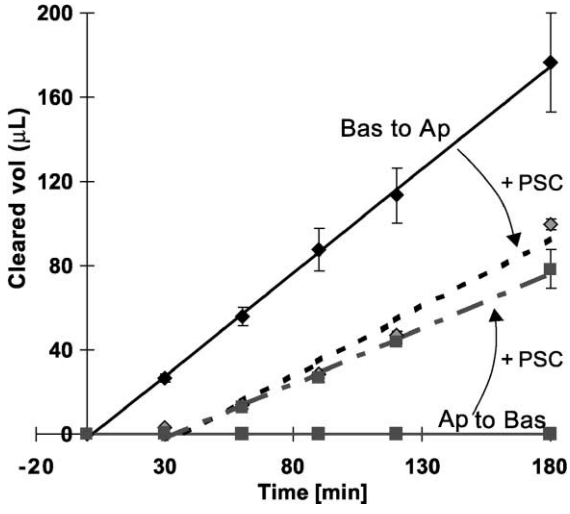

Fig. 6. Transport of 10 μM paclitaxel through 3-week-old Caco-2 cells grown on filter inserts in the presence or absence of 4 μM PSC-833 on both sides of the cell monolayer. Transport is expressed as a concentration independent value (cleared volume). Results are the mean of three to six samples ± S.E.M.

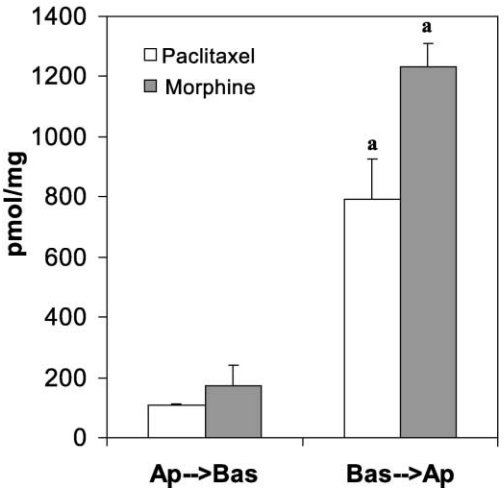

Fig. 7. Drug concentrations associated with the cells after 180 min transport studies conducted with 10 μM concentration of either morphine (solid) or paclitaxel (striped). Transport studies were conducted in either the apical to basolateral or reverse directions. Results are the mean of triplicate samples ± S.E.M. Significant differences between transport directions are shown. [a]$P < 0.005$.

incubation. Almost eight times the amount of paclitaxel and six times the amount of morphine was associated with the cells after the basolateral to apical direction studies as compared to the opposite direction (Fig. 7). Whether there is some type of intracellular packaging of paclitaxel and morphine occurring is unknown and would require further study. It is often noted in our laboratory that compounds with greater net basolateral to apical flux also have greater concentrations associated with the cell monolayers (data not shown).

3.4. Drug efflux

Accumulation studies were repeated for 20 μM morphine over 120 min. Instead of removing the cells, fresh medium was added and aliquots were taken every few min for 60 min to develop a profile of efflux rates for morphine, with or without the presence of inhibitors. The results show that PSC-833 significantly reduced the percentage of morphine effluxed from 85% to less than 70% of the total available pool of morphine accumulated in the cells (Fig. 8A). Surprisingly, verapamil had no effect on the percentage of morphine effluxed considering the very significant reduction of efflux shown for paclitaxel (Fig. 8B). Probenecid significantly reduced efflux of morphine suggesting that morphine transport out of organ systems is more complex than a single defined efflux mechanism. Probenecid also significantly reduced paclitaxel efflux, yet other MRP inhibitors such as indomethacin or genestein did not have any effect (Fig. 8B). The effect of probenecid was difficult to characterise as paclitaxel is not a good MRP substrate. Uptake studies (Fig. 4) confirmed that probenecid, when co-incubated for accumulation studies only, did not lead to greatly increased concentrations of paclitaxel into the cell, yet appeared to be involved in reducing efflux to some extent.

Very little paclitaxel was absorbed into the cells under normal conditions, which made measuring the drug escaping the cytoplasm very difficult. Therefore, the cells incubated with paclitaxel had their associated inhibitors present during both accumulation and efflux stages to increase initial intracellular paclitaxel in order to provide measurable kinetics for efflux studies. PSC-833 caused the rate of efflux for paclitaxel to be reduced with PSC-833 inhibiting a large number of P-glycoprotein sites, resulting in a time to half maximal transport 5 min greater than normal (Table 2), however, this increase failed to reach significance. Conversely, both PSC-833 and probenecid had quicker times to half maximal transport (Table 2) for morphine efflux even though the total amount effluxed was lower over 60 min, only becoming significant for probenecid ($p = 0.02$). This was likely due to the inhibitors not being present until the beginning of efflux studies. It may have taken some time for the inhibitors to effectively bind to their associated receptors, which would have allowed normal efflux in the initial stages before efflux

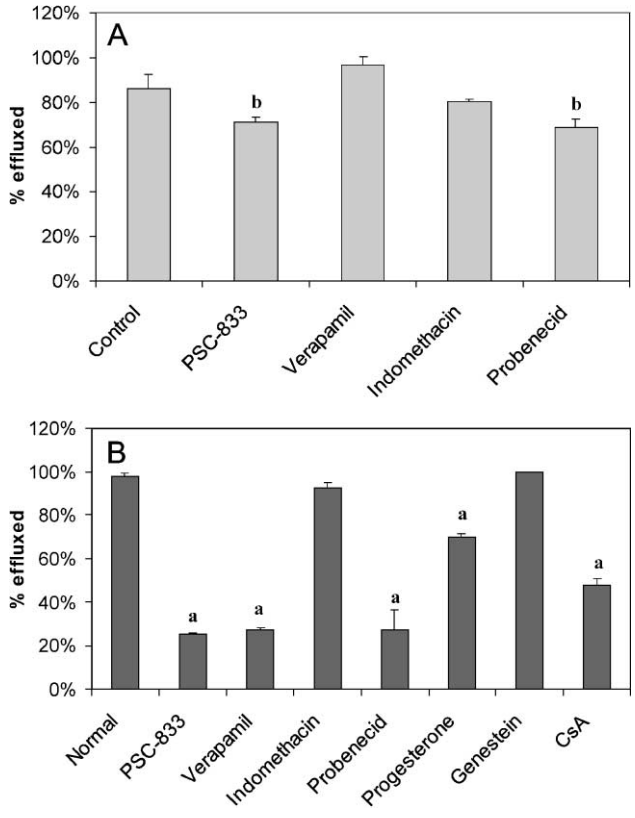

Fig. 8. (A) Morphine and (B) paclitaxel efflux from Caco-2 cells. The percentage of drug effluxed into the medium during 60 min after 2 h of uptake from 20 μM morphine or 50 μM paclitaxel. Results are the mean of triplicate samples ± S.E.M. [b]$P < 0.05$, [a]$P < 0.005$.

Table 2
Rates of efflux into the surrounding medium for 20 μM morphine and 50 μM paclitaxel after a 2-h initial incubation

Inhibitor	Max amount effluxed		Time to 1/2 maximal efflux	
	Morphine (pmol/mg)	Paclitaxel (pmol/mg)	Morphine (min)	Paclitaxel (min)
None	1198 ± 173	45 ± 14	19.8 ± 2.1	13.6 ± 8.2
PSC 833[a]	806 ± 228	720 ± 203*	7.3 ± 5.1	18.2 ± 3.2
Verapamil[a]	1278 ± 269	90 ± 41	10.9 ± 5.0	16.4 ± 10.5
Indomethacin[a]	1162 ± 134	71 ± 57	16.0 ± 4.4	13.1 ± 15
Probenecid[a]	726 ±54 *	21 ± 9	8.3 ± 2.3*	14.1 ± 10.2

Efflux parameters were calculated over a 60-min period. As uptake of paclitaxel was almost negligible under normal conditions, inhibitors were present during the uptake and efflux stages of this study for this compound. Results show the mean of three to four samples ± S.E.M.

[a] PSC-833 used at 4 μM, verapamil used at 100 μM, indomethacin used at 75 μM and probenecid was used at 500 μM.

* $P < 0.05$.

tapered off due to reduced numbers of available transport proteins.

4. Discussion

Previous efflux results have shown our Caco-2 cell line to possess strong P-glycoprotein activity (Crowe and Lemaire, 1998). Transport of MRP substrates has also been shown in our Caco-2 cells and other laboratories have confirmed that MRP is also present in Caco-2 cell monolayers although with lower activity than P-glycoprotein (Gutmann et al., 1999; Makhey et al., 1998). Hence, it was of interest to explore both MRP and P-glycoprotein inhibition in this study to understand morphine transport into and through tight cell monolayers.

Initially, cell uptake experiments indicated that morphine may not have been a substrate for P-glycoprotein as the addition of potent inhibitors of P-glycoprotein activity did not increase morphine accumulation, yet were clearly effective at increasing paclitaxel accumulation. However, a higher flux in the basolateral to apical direction was observed than in the other direction suggesting the involvement of an effluxing transport system for morphine. Additionally, it was possible to saturate this efflux mechanism, allowing equilibrium transport, with high μM concentrations of morphine and the addition of PSC-833, a highly efficient P-glycoprotein inhibitor. This confirmed our Caco-2 model as ideal for determining P-glycoprotein mediated efflux without requiring drug induced or otherwise modified cell lines.

In this study, PSC-833, cyclosporin A, and even progesterone were more effective at allowing accumulation of paclitaxel than verapamil, illustrating differences in activity between typical P-glycoprotein substrates. Yet this order of effectiveness was not consistent when evaluating inhibitors that allowed efflux once cellular levels had increased. In this case, verapamil had a greater ability than progesterone to prevent efflux once accumulation had occurred. A recent report evaluating morphine transport in a rat small intestine model described a slow efflux mechanism for morphine, yet with no inhibitory action of verapamil, concluded that P-glycoprotein was not involved (Doherty and Pang, 2000). Use of multiple P-glycoprotein inhibitors may have altered this conclusion, as this Caco-2 study showed a greater effect of PSC-833 than verapamil upon cellular morphine levels.

A recent study examining morphine bi-directional transport in a primary BBB model suggested that morphine was a weak P-glycoprotein substrate with transport being around 50% greater in the basolateral to apical direction (Letrent et al., 1999b). However, BBB models are renowned for their leakiness in culture, often with trans-epithelial electrical resistance values around 100–150 Ω cm^2. This is much lower than the 2000 Ω cm^2 of an intact BBB. Under these conditions, paracellular transport could hide any differentiation that may have been observed if tighter junctions on the membranes were present. Additionally, high μM concentrations that could start to saturate the efflux pump and possible low P-glycoprotein protein levels would further reduce the ability to observe true efflux characteristics.

The results here show efflux at twice the rate shown previously. Lower concentrations of morphine coupled with the higher trans-epithelial electrical resistance of Caco-2 monolayers has most likely resulted in a greater difference between apically and basolaterally directed transport than that reported earlier in bovine brain endothelial cells (Letrent et al., 1999b).

Following our results reinforcing the role of P-glycoprotein in morphine bi-directional transport, it was expected that accumulation would have been increased with PSC-833 exposure, especially as the concentrations used for transcellular transport were similar to those used in accumulation studies, yet this did not occur here. A previous study using colchicine resistant Chinese hamster ovary cells showed a threefold decrease in morphine accumulation over sensitive cells (Callaghan and Riordan, 1993). Verapamil and vinblastine increased [^{3}H]morphine accumulation in the drug resistant cell line, suggesting the effect was P-glycoprotein related. Unfortunately, it is unclear what concentration of morphine was used in that study. In addition, as with many radiolabelled drug studies, it can be difficult to ascertain whether results are indicative of morphine or a metabolite without prior HPLC separation. Another potential problem involves using drug induced multidrug resistance characteristics, which have been shown by many groups to lead to a raft of changes within the cell line, not only related to P-glycoprotein which can complicate results observed (Ambudkar et al., 1999; Tessner and Stenson, 2000). Nevertheless, their result did suggest some efflux activity against morphine in this cell line regardless of the ultimate cause. Letrent et al. (1999a) have also shown in a rat model, that morphine's antinociceptive effects can be subdued through i.v. administration of a potent P-glycoprotein inhibitor.

The mass of circumstantial evidence that P-glycoprotein is partially responsible for reduced influx of morphine into the brain interstitium is slowly increasing. There are only a limited number of publications addressing the issue of morphine uptake rates into the brain being lower than its log *P* of 0.89 and low molecular weight would suggest (Avdeef et al., 1996). Much of the data that indicates the involvement of P-glycoprotein has come from rat or mice studies, with few publications in the in vitro arena, with these being usually bovine or murine in origin. Our study used a human cell line.

Whole human data is very scarce. One publication that specifically examined the effect of P-glycoprotein inhibitors on brain concentrations concluded that no differences were observed between control and P-glycoprotein inhibited patients. However, a recent clinical paper showed the duration of action of morphine was increased significantly when verapamil was added to the branchial plexus (Reuben and Reuben, 2000). Although these authors did not consider inhibition of active efflux proteins, their data is consistent with inhibition of P-glycoprotein efflux by verapamil. This human data increases the likelihood that the efflux conditions shown in our current work using Caco-2 cells can be analogous to that of the human BBB. It is clear from the inhibition of paclitaxel in this study that verapamil is not as effective as other P-glycoprotein inhibitors. Therefore, if verapamil is having an effect on the activity of morphine in patients then more potent P-glycoprotein inhibitors would be expected to further enhance the action of morphine in the body. Further evidence in this field comes from a recent report finding diltiazem increases morphine accumulation into the brains of rhesus monkeys (Kishioka et al., 2000). Diltiazem had previously been shown to increase the immunosuppressive action of cyclosporin A through P-glycoprotein pump inhibition (Lo and Burckart, 1999). Now that diltiazem has been shown to increase morphine analgesic action it is becoming clear that P-glycoprotein has a role in morphine pharmacodynamics. Hence, monitoring the efficacy of morphine when co-administered with P-glycoprotein substrates such as immunosuppressants, Ca^{2+} channel blockers and cytotoxic drugs may be warranted. Tolerance to morphine is another physiological condition that has been partially attributed to P-glycoprotein, at least in animal studies so far (Aquilante et al., 2000). Emetic effects through the chemoreceptor trigger zone and other localised peripheral effects do not develop tolerance. Only behind the blood–brain barrier does tolerance become an issue in long term use, where P-glycoprotein mediated efflux of morphine may give the appearance of tolerance to the drug.

Although much is now known of the affinity of paclitaxel for P-glycoprotein, the paclitaxel results here were of interest beyond that of a positive control, as they indicated the aggressive nature of P-glycoprotein activity in this Caco-2 cell line.

Current in vitro studies of P-glycoprotein mediated morphine efflux have been conducted with primary cultures of bovine brain microcapillary endothelial cells (Letrent et al., 1999b). There are many factors influencing comparisons from in vitro to in vivo environments. Two of these are limited tight junction formation, which the Caco-2 cell line addresses better than current brain endothelial cell models, and dysfunction, low expression or switching to an alternate form of P-glycoprotein. This Caco-2 cell line exhibits very high P-glycoprotein activity, and being human there are no alternate forms of MDR1 to become upregulated in preference to the in vivo form (Hafny et al., 1997; Regina et al., 1998; Schinkel, 1999). It has been shown that some substrates are effluxed at different rates by mdr1a and mdr1b proteins in murine cells. It is unknown whether this is the case for morphine. Other problems with in vitro/in vivo comparisons are lack of flow, protein binding interference and lack of sink conditions which remains true regardless of the cell type used. Nevertheless, the results presented here clearly show very strong P-glycoprotein activity with paclitaxel. Morphine efflux, although less than paclitaxel is clearly an issue that requires attention as this drug is routinely used in cocktails that could potentially result in increased central nervous system exposure to morphine.

Caco-2 cells possess MRP activity in addition to that of P-glycoprotein, although the P-glycoprotein pump is still the predominant multidrug efflux pump in these cells (Gutmann et al., 1999; Makhey et al., 1998). The MRP inhibitor, probenecid, was shown in this study to reduce the efflux of morphine from Caco-2 cells. Additionally, increased accumulation of morphine into Caco-2 cells was found when intracellular glutathione concentrations were depleted. Phase II metabolism of morphine occurs mainly via glucuronide conjugation (Glare and Walsh, 1991), but glutathione has been shown to be involved (Yamano et al., 1997). Morphine can be metabolised to morphinone, and this can be conjugated with glutathione as a secondary metabolite (Yamano et al., 1997). MRP transports glutathione conjugates out of cells, but is also thought to be involved in co-transport of unconjugated compounds along with glutathione. Although not conclusive for MRP involvement, the complex nature of transport studies shown here with morphine indicates that components of P-glycoprotein and at least one other efflux system has a role to play in accumulation of this drug. This is the first time an MRP-mediated efflux system has been suggested for morphine.

In summary, extensive paclitaxel efflux by our Caco-2 model suggests strong P-glycoprotein effluxing potential for this cell line. The traditional inhibitor, verapamil, used in many previous studies is not as effective at inhibiting P-glycoprotein substrates as PSC-833 or cyclosporin A and depending on the concentration used may not show clear P-glycoprotein inhibition. Thus, the use of multiple P-glycoprotein inhibitors should be used to decide whether or not a test compound is likely to be a P-glycoprotein substrate. However, it is likely that morphine is also effluxed by supplementary mechanisms such as MRP. Preliminary work also suggests P-glycoprotein is upregulated in morphine

dependence (Aquilante et al., 2000). A concerted effort is required to understand the implications of active efflux involvement when providing doses of morphine in cocktail applications that require BBB transport. This Caco-2 model, although epithelial, can provide an insight into active efflux events that are occurring at the BBB until a high trans-epithelial electrical resistance brain endothelial cell line is developed.

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