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The impact of P-glycoprotein efflux on enterocyte residence time and enterocyte-based metabolism of verapamil

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Abstract

P-glycoprotein (P-gp) can limit the intestinal permeability of a number of compounds and may therefore influence their exposure to metabolizing enzymes within the enterocyte (e.g. cytochrome P450 3A, CYP 3A). In this study, the intestinal metabolic profile of verapamil, the influence of P-gp anti-transport on the cellular residence time of verapamil, and the impact of this change in residence time on the extent of enterocyte-based metabolism have been investigated in-vitro, utilizing segments of rat jejunum and side-by-side diffusion chambers. Verapamil exhibited concentration-dependent P-gp efflux and CYP 3A metabolism. The P-gp efflux of verapamil (1 μM) increased the cellular residence time across the intestinal membrane (approximately 3-fold) in the mucosal to serosal (m to s) direction relative to serosal to mucosal (s to m), yielding significantly greater metabolism (approximately 2-fold), presumably as a result of the prolonged exposure to CYP 3A. Intestinal metabolism of verapamil generated not only norverapamil, but resulted also in the formation of an N-dealkylated product (D-617). Norverapamil and D-617 accumulated significantly in mucosal chambers, relative to serosal chambers, over the time course of the experiment. Based on these in-vitro data, it was apparent that P-gp efflux prolonged the cellular residence time of verapamil (m to s) and therefore increased the extent of intestinal metabolism, and also played a role in metabolite secretion from within the enterocyte.

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Introduction

The intestinal P-glycoprotein (P-gp) efflux pump and enterocyte-based metabolism have been proposed to contribute a major barrier to the oral bioavailability of a number of compounds (Wacher et al 1996; Watkins 1997). Differentiation between permeability limitation as a result of either metabolism or efflux (or both) however is complicated in many cases by a large overlap in substrate specificity between P-gp and cytochrome P450 (CYP) 3A (Wacher et al 1995; Wandel et al 1999). The P-gp efflux transporter has been studied extensively in-vitro utilizing excised animal tissues (Saitoh & Aungst 1995; Emi et al 1998; Yumoto et al 1999) and cultured cell lines, such as Caco-2 (Hidalgo & Li 1996; Nerurkar et al 1996; Yumoto et al 1999), where significant polarity in permeability has been observed for a wide range of compounds. The impact of P-gp on drug permeability in-vivo however is less clear and evidence of P-gp attenuated absorption tends to be restricted to substrates with a low dose and/or low intrinsic permeability. In contrast, prehepatic metabolism, primarily by CYP 3A4, has been shown to result in

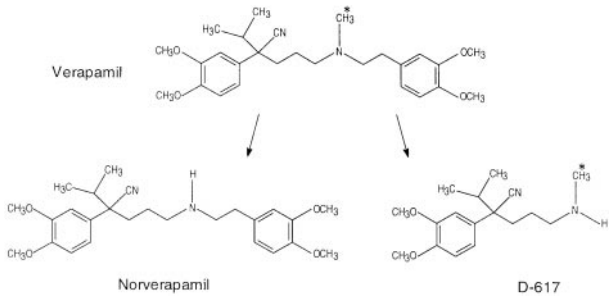

Figure 1 CYP 3A/1A2 mediated metabolic pathway of verapamil. * Indicates the location of tritium in the radiolabelled species [N-methyl-^{3}H]verapamil and [N-methyl-^{3}H]D-617.

significant elimination of many drugs before hepatic and systemic exposure (Benet et al 1996; Thummel et al 1997).

Verapamil is a substrate for both P-gp and CYP 3A, and its efflux has been well characterized in rat intestinal tissue and in cell culture systems (Saitoh & Aungst 1995; Döppenschmitt et al 1999; Pauli-Magnus et al 2000). The ultimate extent of absorption of a high dose/high permeability drug, such as verapamil, however, may not be notably attenuated by intestinal P-gp in-vivo (Sandström et al 1998), as evidenced by the fact that verapamil is almost 100% absorbed from the gastrointestinal tract (Hamann et al 1984). Rather, the more important limiting factor in the transport of intact verapamil across the enterocyte appears to be the extent of enterocyte-based metabolism. The systemic biotransformation of verapamil has been studied previously and has been shown to occur primarily through N-demethylation and N-dealkylation by CYP 3A4, and to a smaller extent by CYP 3A5 and CYP 1A2 (Figure 1) (Kroemer et al 1993; Tracy et al 1999). O-Demethylation of verapamil and its demethylated/dealkylated metabolites by the CYP 2C subfamily may also occur and approximately 7% of an orally administered dose is found in the urine as O-demethylated verapamil (D-703), with less than 5% recovered unchanged (Eichelbaum et al 1979; Busse et al 1995). Surprisingly, although verapamil has a high first-pass effect that includes a significant prehepatic (intestinal) component (Fromm et al 1996, 1998), the intestinal metabolism of verapamil has not been studied in detail and to this point the formation of only norverapamil has been documented (Sandström & Lennernäs 1999; Sandström et al 1999).

Importantly, for high permeability compounds such as verapamil, whilst P-gp may not limit bioavailability through a reduction in intestinal permeability per se, it is possible that the action of the P-gp efflux pump may slow transit across the enterocyte such that exposure to CYP 3A enzymes is increased, thereby enhancing prehepatic extraction. This hypothesis of the interplay between efflux and metabolism has been suggested previously (Benet et al 1996; Watkins 1997). Supporting experimental evidence, however, is scant, and to this point observations have been made only in cell culture models, where the extent of efflux and metabolism is commonly over- and under-estimated, respectively, when compared with tissues in-vivo (Prueksaritanont et al 1996; Schmiedlin-Ren et al 1997; Anderle et al 1998).

In this study, the efflux and metabolism of verapamil has been examined in-vitro using freshly excised rat intestinal tissue where enzyme and transporter levels reflect the in-vivo situation. Using this model, the primary metabolic products of verapamil have been identified and quantified, and an estimation of the likely impact of P-gp on the extent of intestinal metabolism has been examined. These data have extended previous observations and have shown that whilst P-gp may not significantly reduce the extent of intestinal absorption, it is likely that efflux leads to prolonged cellular residence time and thereby enhances the extent of prehepatic metabolism of verapamil.

Materials and Methods

Materials

[N-Methyl-^{3}H]-verapamil HCl was from New England Nuclear (Boston, MA), unlabelled verapamil HCl and norverapamil HCl were from Sigma Chemical Co. (St Louis, MI) and Alltech Associates (NSW, Australia), respectively. Midazolam was used in the form of Hypnovel injection (midazolam (as HCl) 5 mg, NaCl 5 mg, HCl 1 mg, NaOH as required to pH 3.3, water for injections to 1 mL, Roche, NSW, Australia). All other reagents were of analytical grade or higher, and water was obtained from a Milli-Q (Millipore, Milford, MA) water purification system.

Tissue preparation

All animal studies were performed in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. The institutional animal experimentation ethics committee approved the study protocol.

After an overnight fast, male Sprague-Dawley rats (275–325 g) were anaesthetized with 5% isoflurane and approximately 15 cm of jejunum was removed. A glass rod was inserted down the length of the segment and the

tissue was rapidly stripped of its serosal muscle layers. An incision was made along the length of the mesenteric margin, six segments of tissue were mounted in side-by-side diffusion chambers (Navicyte, Sparks, NV), and 6 mL prewarmed buffer was immediately added to the mucosal and serosal chambers. The buffer contained (in mM): Na^+, 141; K^+, 5; Ca^{2+}, 1.2; Mg^{2+}, 1.2; Cl^- 122; HCO_3^-, 25; $H_2PO_4^-$, 0.4; and HPO_4^{2-}, 1.6. Additionally, serosal and mucosal buffers contained 10 mM D-glucose and 10 mM D-mannitol, respectively. Temperature was maintained at 37°C and the pH was approximately 7.4 when buffers were circulated by a gas-lift of carbon dioxide and oxygen (5:95). Tissues were equilibrated for 30 min before the addition of radiolabelled (1 μCi, 3.1 TBq mmol^{-1}) and/or unlabelled (1–100 μM) verapamil.

Metabolite identification and quantification

These studies were conducted in the mucosal to serosal (m to s) direction only. Samples collected for metabolite identification by LC/MS analysis were taken from the donor and receptor chambers after a 2 h exposure to a relatively high concentration (100 μM) of verapamil added to the mucosal (donor) chamber to enhance assay sensitivity. Quantification of labelled and unlabelled metabolites was achieved using HPLC separation and fluorescence detection, or liquid scintillation counting of fractionated eluent. In this case, donor and receptor chamber samples were taken 2 h after incubation with likely sub-K_m concentrations of verapamil and a trace amount of [N-methyl-^{3}H]-verapamil (1 μM and 1 μCi, respectively).

Metabolite identification

Identification of unknown metabolites was achieved via LC/MS analysis using a Waters 2690 separations module and a 996 diode array detector (Waters, Milford, MA) coupled to a Micromass Platform LCZ mass spectrometer (Manchester, UK) utilizing positive-ion electrospray ionization. Chromatographic separation was via a YMC ODS-AM (5 μm, 4.6 × 250 mm) column (Kyoto, Japan) and the mobile phase (1 mL min^{-1}) consisted of acetonitrile and 5 mM potassium phosphate (32.5:67.5) adjusted to pH 3. Identification of molecular mass was performed at a cone voltage of 30 V and confirmation of metabolite structure was achieved by fragmentation at 20, 40 and 60 V. Retention times of D-617, D-703, norverapamil and verapamil were 5.8, 12.5, 16.3 and 17.2 min, respectively.

Metabolite quantification

Unlabelled norverapamil was assayed using identical chromatographic conditions to those described above, with the exception of a Waters 712 WISP autosampler and 600E pump and fluorescence detection (Perkin-Elmer LC 240, UK) with excitation and emission wavelengths of 280 and 310 nm, respectively. Norverapamil was quantified by comparison with an external standard curve and the assay was accurate to within 97 ± 3% of target concentration, precise (CV < 10%), and with an LOQ (limit of quantification) of 20 pmol mL^{-1}. To quantify radiolabelled verapamil and radiolabelled metabolites (for which no external standards were available), eluent from the same assay was collected into 20-s fractions by a FRAC-100 fraction collector (Pharmacia, Uppsala, Sweden). After the addition of 3 mL scintillation fluid (Starscint, Canberra-Packard, Meriden, CT), each fraction was counted using a Tri-carb 2000CA liquid scintillation counter (Packard). The radioactivity, as DPM (disintegrations per minute), collected in each fraction was plotted against time to construct a radiochromatogram where the area under each radiolabelled peak was calculated to quantify [N-methyl-^{3}H]verapamil, [N-methyl-^{3}H]D-617 and [N-methyl-^{3}H]D-703 utilizing the specific activity of the tritiated verapamil (3.1 TBq mmol^{-1}). The appearance (% cm^{-2}) of verapamil and its metabolites in the donor and receptor chambers after 2 h was then calculated using the cross-sectional area available for transport (A) equal to 1.78 cm^2.

Verapamil flux and metabolism studies

Flux and metabolism experiments were conducted in a concentration-dependent manner (1, 20 and 100 μM verapamil) in the mucosal to serosal (m to s) and the serosal to mucosal (s to m) directions. When studies were conducted in the presence of 500 μM midazolam, 196 μL of mucosal and serosal buffer was removed and replaced with an equal volume of Hypnovel injection 5 min before the addition of verapamil. Donor chamber samples (200 μL) were taken immediately after the addition of radiolabelled verapamil. At the conclusion of the incubation (2 h) 200-μL samples were removed from receptor chambers for liquid scintillation counting and HPLC analysis. Initial donor chamber samples were analysed for total radioactivity by liquid scintillation counting as described above. Receptor chamber samples (200 μL) were mixed with 100 μL mobile phase (acetonitrile, 0.05 M phosphoric acid, TEA (40:60:1)) and subjected to HPLC separation to determine the degree of verapamil metabolism as follows. A 100-μL sample

was injected onto a Columbus C18 (5 μm, 4.5 × 250 mm) column (Phenomenex, Torrance, CA) via a 712 WISP autosampler and a Waters 600E pump and mobile phase was delivered at 0.6 mL min^{-1}. The eluent was collected in 1-min fractions and each fraction was then analysed for radioactivity. A second 100-μL sample of each acidified receptor chamber sample was analysed for total radioactivity to determine the recovery of radioactivity off the column. Fluorescence detection was used to confirm the retention time of verapamil (7.4 min). The recovery (mean ± s.d.) of radioactivity injected onto the column was 102 ± 18 % (n = 96 injections) with an LOQ of 0.1 pmol mL^{-1}, where the precision CV was < 10 % and recovery was > 90 %.

Data analysis

Of the total radioactivity sampled from the receptor chamber, the percentage that was associated with intact verapamil (% intact) was determined by equation 1, where RA_V was the verapamil associated radioactivity collected off the column (via radiochromatographic assessment) and RA_T was the total radioactivity collected off the column from receptor chamber samples.

$$\% \text{ intact} = 100 \times (RA_V/RA_T) \quad (1)$$

Flux (J, % cm^{-2} over 2 h) was calculated by equation 2 where V_R and V_D were the concentrations of intact verapamil in the receptor and donor chambers, respectively, at time t. At t = 0 V_D was found to be within 5 % of the manufacturer's specifications.

$$J = ([V_R]_{t=2})/[V_D]_{t=0}) \times (100/A) \quad (2)$$

Data describing the % intact and total drug flux were calculated for m to s (subscript ms) and s to m (subscript sm) experiments. The efflux ratio (ER), residence time ratio (RTR) and % intact ratio (%IR) were calculated by equations 3, 4 and 5, respectively:

$$ER = J_{sm}/J_{ms} \quad (3)$$

$$RTR = 1/ER \quad (4)$$

$$\%IR = \% \text{ intact}_{sm}/\% \text{ intact}_{ms} \quad (5)$$

These ratios aimed to exemplify the differences in flux, residence time and the extent of metabolism between the m to s and s to m directions of transport. For example, an ER of 2 would result from flux in the s to m direction being twice that in the m to s direction, and would correspond to an RTR of 0.5 i.e. the residence time in the tissue would be twice as high m to s than s to m. Similarly, a %IR of 2 would describe an experiment where the amount of drug remaining intact (i.e. non-metabolized) in the m to s direction was half of that observed s to m. Therefore a %IR greater than unity would represent more metabolism occurring in the m to s direction when compared with s to m.

Statistical analysis

Statistical analysis of flux and % intact data was performed by two-way analysis of variance with multiple comparisons (Tukey). All directional ratios were calculated based on comparison of the geometric means of each data set and the 95 % confidence limits were generated from the difference in the means using log transformed data.

Results and Discussion

This study has identified the major metabolic products of verapamil formed in the rat jejunum and supports earlier findings that the intestinal transport of such metabolites is influenced by P-gp (Häußermann et al 1991; Pauli-Magnus et al 2000). The extent of metabolism of verapamil has also been shown to be dependent on the cellular residence time within the tissue, which in turn is modified by the action of the P-gp efflux pump.

Metabolite identification and quantification

Analysis of donor and receptor chambers after 2-h incubation with 100 μM verapamil revealed the presence of only two major metabolites. The N-demethylated product, norverapamil, was formed, as described previously (Sandström & Lennernäs 1999; Sandström et al 1999). The presence of the N-dealkylated product, D-617, was confirmed by mass spectrometry where the following ions were detected at a retention time of 5.8 min: m/z 291, 260 and 151. The fragmentation pattern is described in Figure 2 and this retention time corresponded to a radiolabelled peak that was collected in subsequent experiments and analysed to quantify D-617. D-703 was detected in the donor chamber of one tissue but in amounts below the limit of quantification. The N-dealkylated and demethylated product (D-620)

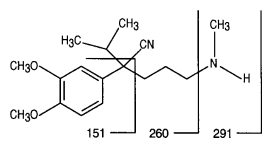

Figure 2 Fragmentation pattern of the N-dealkylated verapamil metabolite D-617 performed with positive-ion electrospray ionization mass spectrometry.

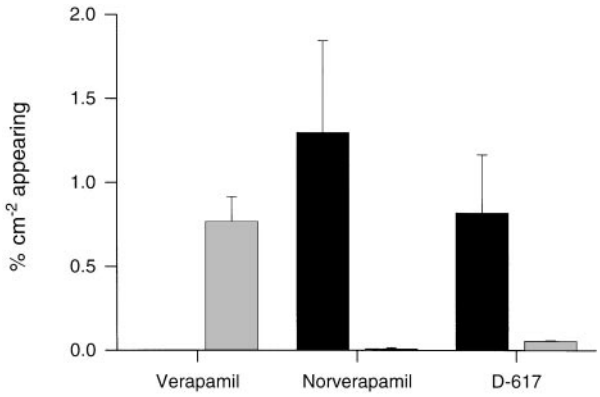

Figure 3 Accumulation (% cm^{-2}) of verapamil, norverapamil and D-617 in receptor (serosal) chambers (grey) and norverapamil and D-617 in donor (mucosal) chambers (black) after 2 h incubation with 1 μM mucosal verapamil in an m to s intestinal permeability experiment across rat jejunal tissue. Mean $\pm$ s.e.m. of n = 3 determinations.

Table 1 Bidirectional concentration-dependent flux of verapamil across rat jejunal tissue over 2 h, and at 1 μM in the presence of 500 μM midazolam.

Verapamil (μM)	m to s flux (% cm^{-2} over 2 h)	s to m flux (% cm^{-2} over 2 h)
1	0.316 ± 0.099^a	0.902 ± 0.343^a
20	0.967 ± 0.186^b	1.282 ± 0.319^b
100	1.580 ± 0.310^c	1.882 ± 0.287^c
1 + midazolam	1.537 ± 0.185^d	1.785 ± 0.317^d

Mean $\pm$ s.d. of n = 6 determinations, statistical differences in flux over the concentration range studied were as follows: [a]compared with 20 μM, 100 μM and 1 μM + midazolam ($P \leqslant 0.05$); [b]compared with 1 μM, 100 μM and 1 μM + midazolam ($P \leqslant 0.05$); [c]compared with 1 μM and 20 μM ($P \leqslant 0.05$); [d]compared with 1 μM and 20 μM ($P \leqslant 0.05$); s to m flux was significantly larger than m to s flux at 1 μM verapamil ($P \leqslant 0.05$).

and other various O-demethylated metabolites were not present in detectable quantities.

Figure 3 shows the accumulation of verapamil, norverapamil, and D-617 in donor and receptor chambers after 2-h exposure to 1 μM verapamil in the donor chamber. Experiments were performed in the m to s direction across rat jejunal tissue. Of the verapamil dose applied to the mucosal (donor) chamber, only 0.77% of the drug in the donor chamber appeared unchanged in the receptor solution after 2 h. Very small quantities of the metabolites norverapamil ($\sim$ 0.01%, below LOQ) and D-617 (0.05%) were found in the receptor chamber; however, significant quantities of both metabolites were detected in the donor chamber (1.3% and 0.82% of the initial verapamil dose, respectively). Stability of verapamil in the donor and receptor chambers (in the absence of intestinal tissue) was confirmed after 2 h incubation of verapamil in donor and receptor chamber buffers that were collected at the conclusion of blank experiments with stripped rat jejunal tissue (data not shown). Importantly, these data showed that the extent of accumulation of verapamil metabolites in the donor chamber at an initial donor chamber concentration of 1 μM verapamil was significantly higher than the amount of intact drug transported across the tissue (0.77%). This confirms the importance of enterocyte-based metabolism in the biodisposition of verapamil. The intestinal formation of the N-dealkyl metabolite D-617 has not been reported previously and it is apparent from these studies that the production of D-617 and norverapamil should be accounted for when studying the intestinal metabolism and permeability of verapamil in the rat.

It has been reported that the metabolites, norverapamil and D-617, can interact with P-gp in cell culture systems (Häußermann et al 1991; Pauli-Magnus et al 2000). Norverapamil was found to be a better inhibitor of P-gp than D-617 in human lymphoma cells, however both compounds have demonstrated significant P-gp efflux in experiments performed in kidney epithelium derived L-MDR1 cells. In both of those previous studies, the metabolites were not intrinsically generated via metabolism of parent verapamil, but rather were applied to the culture medium directly. The data presented here describes the simultaneous formation and transport of the metabolites of verapamil from the enterocyte. It is therefore likely that P-gp was responsible for the apparent preferential secretion of metabolites into the mucosal compartment observed here.

These preliminary experiments demonstrated that P-gp and CYP 3A appeared to have a substantial impact on the intestinal permeability of verapamil. Subsequent experiments therefore examined the concentration-dependency of flux and metabolism of verapamil, and the likely impact of P-gp efflux on the residence time and extent of metabolism.

Verapamil flux

The bidirectional flux of verapamil across excised rat intestinal tissue was determined in a concentration-dependent manner and the results are summarized in Table 1. The flux of verapamil increased in the m to s and s to m directions as the concentration of drug was raised (Table 1). It appeared that the P-gp mediated

Table 2 Bidirectional concentration-dependent metabolism (represented by the percentage of intact verapamil in the receptor chamber (% intact)) across rat jejunal tissue after 2 h and at 1 μM in the presence of 500 μM midazolam.

Verapamil (μM)	m to s (% intact)	s to m (% intact)
1	19.95 ± 2.53^{a}	35.46 ± 5.22^{a}
20	63.75 ± 4.52^{b}	72.80 ± 3.70^{b}
100	86.68 ± 4.21^{c}	88.44 ± 4.91^{c}
1 + midazolam	93.84 ± 4.98^{d}	89.36 ± 4.23^{d}

Mean $\pm$ s.d. of n = 6 determinations, statistical differences in flux over the concentration range studied were as follows: [a]compared with 20 μM, 100 μM and 1 μM + midazolam ($P \leqslant 0.05$); [b]compared with 1 μM, 100 μM and 1 μM + midazolam ($P \leqslant 0.05$); [c]compared with 1 μM and 20 μM ($P \leqslant 0.05$); [d]compared with 1 μM and 20 μM ($P \leqslant 0.05$); % intact s to m was significantly higher than m to s at 1 μM verapamil ($P \leqslant 0.05$).

efflux process was almost totally saturated at 100 μM, and the data was consistent with a published K_m of 30 μM for verapamil in the rat jejunum (Makhey et al 1998). The expected drop in % s to m flux on increasing verapamil concentrations (as P-gp became saturated) was not observed and most likely reflected extensive metabolism of the drug at 1 μM, resulting in diminished transport of intact verapamil. Despite this, the s to m flux was always higher than the m to s flux (although only statistically significant at 1 μM), confirming the drug efflux process, and two-way analysis of variance revealed significant differences in flux between the m to s and s to m directions ($P < 0.001$) and over the four conditions studied ($P < 0.001$). More importantly, midazolam is commonly used as a CYP 3A substrate/inhibitor, and it was clear from the data described here that at 500 μM it was an effective inhibitor of P-gp also, since the directional flux of 1 μM verapamil in the presence of midazolam showed no significant polarity and was not significantly different to the data obtained at 100 μM verapamil concentrations.

Verapamil metabolism

Table 2 summarizes the % intact data for verapamil after passage through a segment of rat jejunum in-vitro. At 1 μM in the m to s direction, HPLC separation of the receptor chamber sample revealed that only 20 % of the eluted radioactivity was associated with intact verapamil, the remainder being associated with the solvent front (most likely consisting of tritiated D-617, formaldehyde, and formic acid (Williams 1995)). This proportion of verapamil remaining intact (i.e. non-metabolized) was almost double when verapamil transport was studied s to m, indicating the influence of the direction of drug transport on the extent of metabolism. As expected, the proportion of radioactivity associated with verapamil in receptor samples steadily increased as the concentration in the donor compartment was raised, to the point where almost total saturation of metabolism was observed at 100 μM. Similar inhibition of metabolism was also achieved with the addition of 500 μM midazolam. Two-way analysis of variance revealed that the extent of metabolism in the m to s direction was greater than that in the s to m direction after accounting for the effect of concentration, and that significant differences existed in the % intact data over the four groups ($P < 0.001$).

Effect of efflux on metabolism

The ability of P-gp to affect the extent of metabolism of drugs has been postulated hypothetically and simulated using theoretical models (Benet et al 1996; Watkins 1997; Ito et al 1999), but studies in this field are still lacking. In Caco-2 cells, the formation of the 1η-hydroxy metabolite of ciclosporin (cyclosporin) by a

Table 3 Efflux, residence time, and metabolism ratios (s to m/m to s) obtained for verapamil on passage across rat jejunal tissue over a 2 h experimental period (calculation methods described in *Data analysis*, 95% confidence limits in parenthesis).

Verapamil (μM)	Efflux ratio	Residence time ratio	% intact ratio
1	2.78 (1.82–4.27)[a]	0.36 (0.23–0.55)[a]	1.75 (1.49–2.12)[a]
20	1.32 (0.99–1.77)	0.76 (0.57–1.01)	1.15 (1.05–1.25)[a]
100	1.20 (0.96–1.50)	0.83 (0.67–1.04)	1.02 (0.95–1.05)
1 + midazolam	1.15 (0.94–1.41)	0.87 (0.71–1.06)	1.05 (0.89–1.02)

[a]Ratios significantly different to unity 1 ($P \leqslant 0.05$).

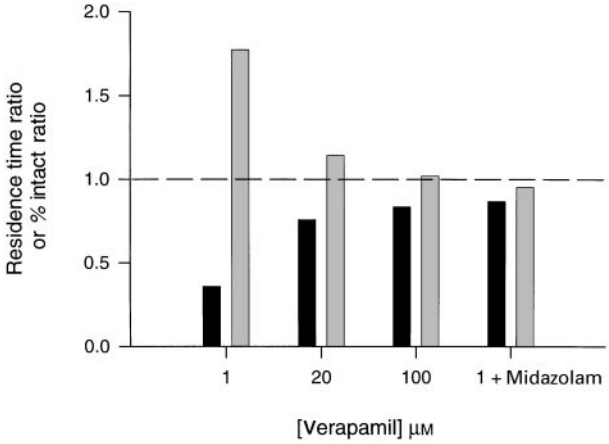

Figure 4 Concentration-dependent residence time ratio (black) and % intact ratio (grey) for verapamil after passage through a segment of rat jejunal tissue. Data obtained after a 2 h incubation time with 1–100 μM verapamil and 1 μM verapamil in the presence of 500 μM midazolam. Ratios were based on geometric means as described in *Data analysis* (95% confidence limits are summarized in Table 3).

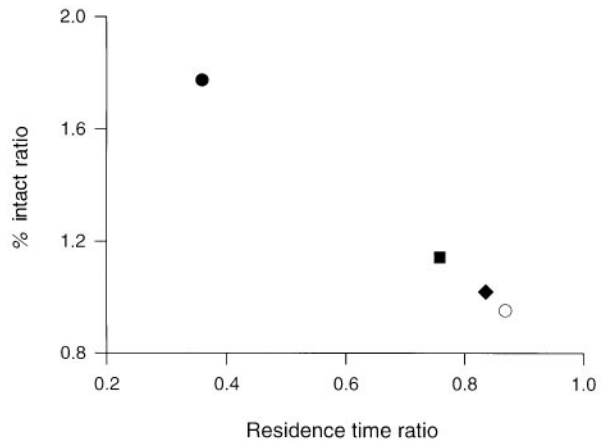

Figure 5 Correlation of residence time ratio and % intact ratio for verapamil across rat jejunal tissue at verapamil concentrations of 1 (●), 20 (■), and 100 μM (◆) and using 1 μM verapamil in the presence of 500 μM midazolam (○).

CYP 3A-like enzyme was significantly larger in the m to s direction than the s to m direction (Gan et al 1996), similar to the findings presented here. Studies with indinavir in Caco-2 cells stimulated to express CYP 3A4 yielded similar results where the amount of metabolite produced per mole of indinavir transported was almost 5-times higher m to s than s to m (Hochman et al 2000). However, the studies described here are the first to utilize animal tissues for such experiments where physiological levels of both metabolizing enzymes and transporters are present.

The s to m/m to s ratios and 95% confidence limits for efflux, residence time and metabolism are summarized in Table 3. Figure 4 depicts the relationship between the residence times (an effect of efflux) and the degree of metabolism (measured as % intact) of verapamil over the concentration range studied. At 1 μM, verapamil flux in the s to m direction was almost 3-times higher than that in the m to s direction, this difference corresponding to a relatively shorter s to m residence time (cf. m to s) and therefore a low RTR of 0.36. A similar pattern, but of diminishing magnitude, was seen over the concentration range studied. As this difference in the directional residence time became smaller (RTR approaching unity), the difference in the extent of metabolism in the two directions was reduced also (%IR approaching unity). This suggested that the extent of metabolism was not only dependent on concentration (as expected) but also on the direction and therefore speed of transport. This relationship between residence time and metabolism is illustrated further in Figure 5, where statistical evaluation revealed significant correlation and regression ($P = 0.001$, $r^2 = 0.9785$) between the RTR and %IR of verapamil. It appears therefore that P-gp prolonged the m to s transport of verapamil, allowing greater exposure of the drug to CYP 3A (through increased residence time), resulting in more effective metabolism. In contrast, in the s to m direction, P-gp facilitated drug transport and diminished the exposure of verapamil to CYP 3A (through decreased residence time), and therefore relatively less metabolism was observed. This model is described schematically in Figure 6. Mechanistically, the proposed relationship between P-gp efflux, apparent residence time, and enterocyte-based metabolism does not preclude an impact on metabolism due to P-gp mediated changes in cellular drug distribution and accumulation patterns, and therefore CYP 3A access within the enterocyte; however, these aspects have not been addressed in this study.

Conclusion

In this in-vitro rat intestinal model it has been shown that verapamil is primarily N-demethylated and N-dealkylated to form norverapamil and D-617, respectively, and that both metabolites appear to be substrates for intestinal P-gp. P-gp efflux led to reduced flux of the parent verapamil and its metabolites across the tissue to the serosal (receptor) compartment and enhanced accumulation in the mucosal (donor) compartment. These studies supported the concept that, in-vitro, P-gp can affect the cellular residence time of its

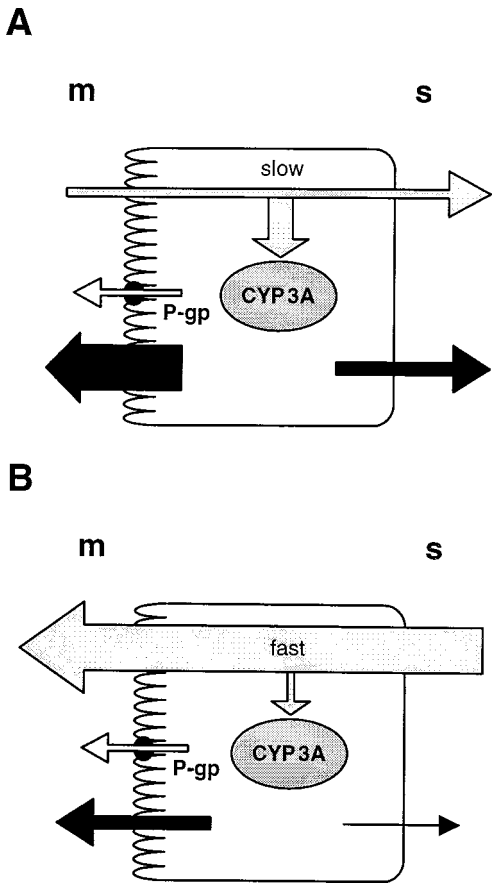

Figure 6 Schematic diagram describing the influence of P-gp on its substrates and exposure to CYP 3A enzymes in the (A) m to s direction where residence time is increased by P-gp and (B) s to m direction where residence time is decreased by P-gp. The grey arrows represent the speed and direction of drug transport and the black arrows represent the amount of metabolite formed and its distribution.

substrates (in this case verapamil) and that this increased residence time across the enterocyte may influence the exposure of the compound to metabolizing enzymes, leading to enhancement of enterocyte-based, prehepatic metabolism. Studies with selective P-gp and CYP 3A inhibitors are required to elucidate and separate the effects of efflux and metabolism on drug permeability in-vitro and in-situ in an attempt to understand the relationship that may exist between these two processes in-vivo.

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