An In Vitro Examination of the Impact of Polyethylene Glycol 400, Pluronic P85, and Vitamin E d- α -Tocopheryl Polyethylene Glycol 1000 Succinate on P-Glycoprotein Efflux and Enterocyte-Based Metabolism in Excised Rat Intestine

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ABSTRACT The potential inhibitory effects of 3 excipients (polyethylene glycol [PEG] 400, Pluronic P85, and vitamin E d- α -tocopheryl polyethylene glycol 1000 succinate [TPGS]) on the P-glycoprotein (P-gp) -mediated efflux of digoxin (DIG) and cytochrome P450 3A (CYP3A) -mediated metabolism of verapamil (VRP) have been examined in an in vitro permeability model.

Experiments were conducted utilizing rat jejunal tissue mounted in diffusion chambers and included assessment of the serosal to mucosal (s to m) transport of DIG and the formation of norverapamil (NOR) during the mucosal to serosal transport of VRP, as measures of P-gp efflux and CYP3A metabolism, respectively.

The presence of PEG at 1%, 5%, and 20% (wt/vol) reduced both the s to m flux of DIG (by 47%, 57%, and 64%, respectively, when compared to control) and the metabolism of VRP (by 54%, 78%, and 100%) in a concentration-dependent manner. P85 (0.1% wt/vol) significantly reduced s to m DIG flux by 47% and inhibited VRP metabolism by 42%. TPGS had insignificant effects on both metabolism and efflux at a concentration of 0.01% (wt/vol). The P-gp inhibitory effects of PEG and P85 were evident regardless of whether the excipient was added to the mucosal side, the serosal side, or both sides of the tissue.

The current data suggest that inclusion of PEG and P85 as solubilizing agents during in vitro permeability assessment may have a significant impact on both drug metabolism and efflux processes. These compounds

appear to exert their effects on P-gp primarily via direct transporter inhibition or indirectly, through effects on buffer osmolarity, membrane fluidity, and/or mitochondrial toxicity and subsequent adenosine triphosphate (ATP) depletion.

KEYWORDS: P-glycoprotein, CYP3A, intestinal, metabolism, permeability, PEG.

INTRODUCTION Adequate intestinal permeability is a prerequisite for oral bioavailability, and therefore intestinal permeability assessment has become a key process in the development of new chemical entities intended for oral delivery. Permeability assessment is often carried out in vitro, utilizing models designed to recreate, or at least reflect, the absorptive processes of the small intestine. Commonly employed biological systems include cell culture models (eg, Caco-2 cells [1]) and excised intestinal tissues (either animal or human) mounted in side-by-side diffusion chambers. In the absence of substantive solubility, stability, or metabolism issues, drug permeability (as defined by the permeability coefficient) has been shown to correlate well with the (oral) fraction absorbed for a range of drug molecules^{2,3}.

Information gained from routine permeability assessment can also aid in the understanding of the role of membrane transporters and antitransporters (eg, P-glycoprotein [P-gp]) and enterocyte-based metabolism (via enzymes such as the cytochrome P450 3A [CYP3A] isozymes) in the intestinal transport of probe compounds. In the enterocyte, P-gp is an apical transmembrane protein responsible for the efflux (secretion) of a number of structurally diverse compounds and is also widely expressed in other human tissues, including the liver, kidneys, placenta, and blood-brain barrier⁴. P-gp is therefore a potential determinant of not only the extent of oral absorption but also the pattern of tissue distribution and elimination of its substrates. In vivo, P-gp has been shown to provide a substantial barrier to the effective oral bioavailability of drug molecules, including digoxin, talinolol, grepafloxacin, and HIV-1 protease inhibitors⁵⁻⁸. CYP3A, the most abundant CYP enzyme, is present in both the liver and the intestine in humans and rats⁹, and CYP3A-mediated metabolism within the enterocyte has been

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demonstrated for a number of compounds¹⁰⁻¹². Previous studies have suggested that P-gp efflux and CYP3A metabolism in the enterocyte act in concert to protect the body from absorption and subsequent exposure to potentially toxic xenobiotics and therefore may also reduce the passage of intact drug across the intestinal mucosa¹³.

Substrates for P-gp efflux and CYP3A metabolism are commonly, but not exclusively, lipophilic and as a result are typically poorly soluble in the aqueous transport buffers generally employed in permeability and/or metabolism experiments¹⁴. This frequently necessitates the inclusion of solubilizing agents or excipients, such as cosolvents or surfactants, in order to enhance drug solubility, and therefore the apparent concentration gradient, to promote drug flux to the point where analytical sensitivity is not limiting. Although data in this regard are scarce, it has previously been shown that some surfactants can inhibit P-gp efflux, and, more recently, inhibition of CYP3A metabolism by certain solubilizing agents has also been suggested^{15, 16}. A limitation of these previous studies is that they have been performed using cell monolayers and cDNA expression systems, which may not accurately reflect the normal biochemistry of the small intestine. In this regard, many cell culture models exhibit variable and/or quantitatively different P-gp and CYP3A expression levels¹⁷⁻²⁰. The conduct of intestinal permeability experiments using excised intestinal tissues mounted in Ussing chambers is therefore well suited to the investigation of the impact of P-gp and CYP3A on drug permeability, since transporters such as P-gp and metabolic enzymes including CYP3A are present at physiological levels (at least for the species under examination). Accurate estimation of the interaction of cosolvents and surfactants with P-gp and CYP3A is important, as permeability assessment conducted in their presence (to aid solubility of a probe molecule) may obscure potential interactions of the probe compound with both intestinal P-gp and CYP3A.

To this end, the studies described in this paper have focused on 3 excipients, Pluronic P85 (P85), a block copolymer made up of 2 equal-length polyoxyethylene chains separated by a polyoxypropylene (PO) chain (molecular weight 4600 d, 42% PO), polyethylene glycol (PEG) 400, and vitamin E α -tocopheryl polyethylene glycol 1000 succinate (TPGS), a surfactant consisting of a lipophilic tocopheryl moiety connected by a succinate bridge to a PEG 1000 chain. The concentrations of the excipients employed in these experiments reflect a conservative range that could potentially be utilized to aid the solubility of probe compounds in permeability and metabolism experiments, and are also consistent with concentrations of the excipients utilized in previous *in vitro* studies^{21, 22}. The impact of these excipients on intestinal P-gp efflux and CYP3A metabolism *in vitro* was studied utilizing modified Ussing chambers and excised rat jejunal tissue. The serosal to mucosal (s to m) transport (efflux) of the P-gp substrate digoxin (DIG) was used as a marker of intestinal P-gp mediated efflux

(secretion), and the formation of norverapamil (NOR) from the CYP3A substrate verapamil (VRP) was used as a marker of CYP3A activity. VRP is also a P-gp substrate, a common property of many compounds metabolized by CYP3A²³ but was used in these studies because of its common application in permeability assessment. The data show that even relatively small amounts of PEG 400 and Pluronic P85 were capable of inhibition of both P-gp and CYP3A, whereas TPGS had little effect on either process in this model. A preliminary investigation of the mechanism of P-gp/CYP3A inhibition was also conducted.

MATERIALS AND METHODS

Materials

Verapamil HCl and norverapamil HCl were supplied by Sigma Chemical Co (St Louis, MO) and Alltech Associates (Baulkham Hills, Australia), respectively. [³H]-digoxin (584.66 GBq.mmol⁻¹) and [³H]-verapamil (3.1 TBq.mmol⁻¹) were obtained from New England Nuclear (Boston, MA), and unlabeled digoxin was used in the form of Lanoxin Adult injection (digoxin 500 µg, ethanol 160 mg, propylene glycol 830 mg, citric acid [anhydrous] 1.38 mg, sodium phosphate 3.58 mg, water for injection to 2 mL, Glaxo Wellcome, Boronia, Australia). Ketoconazole was from ICN Biomedicals (Aurora, OH), PSC833 was a kind gift from Novartis (Basel, Switzerland), and midazolam was used in the form of Hypnovel injection (midazolam [as HCl] 5 mg, NaCl 5 mg, HCl 1 mg, NaOH to pH 3.3, water for injection to 1 mL, Roche, Dee Why, Australia). Vitamin E TPGS was obtained from Kodak Eastman (Kingsport, TN), polyethylene glycol 400 was supplied by BDH Laboratory Supplies (Poole, England), and Pluronic P85 was from BASF (Noble Park, Australia). All other reagents were of analytical grade or higher, and water was obtained from a Milli-Q (Millipore, Bedford, 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, and the study protocol was approved by the Victorian College of Pharmacy Animal Experimentation Ethics Committee. As described previously²⁴, male Sprague-Dawley rats were anaesthetized with 5% isoflurane, and a ~15 cm segment of jejunum was removed. The tissue was rapidly stripped of its serosal muscle layers and mounted in side-by-side diffusion chambers (Navicyte, Sparks, NV). Then 6 mL of room temperature buffer was immediately added to the mucosal and serosal chambers. The control buffer contained (in mM): Na⁺, 147.2; K⁺, 5.1; Ca²⁺, 1.25; Mg²⁺, 1.2; Cl⁻, 115.2; HCO₃⁻, 15; H₂PO₄⁻, 0.4; HPO₄²⁻, 1.8; SO₄²⁻, 1.2; D-glucose, 11.5; L-glutamate-, 4.9; pyruvate-, 4.9; and fumarate²⁻, 5.4. Temperature was maintained at 37°C, and pH was ~7.4 when buffers were circulated by a gas lift of carbon

dioxide and oxygen (5:95). Buffers containing excipients were prepared from control buffer by the addition of PEG (0.1%, 1%, 5%, and 20% wt/vol), P85 (0.01% and 0.1% wt/vol), and TPGS (0.01% wt/vol). The compounds ketoconazole (KC, 50 mM), PSC833 (PSC, 20 μ M), or midazolam (MDZ, 20 μ M) were employed as nonselective P-gp/CYP3A, selective P-gp, and selective CYP3A inhibitors, respectively. The concentrations of the inhibitors employed were iteratively determined to be the most effective and selective for the probe compounds under study via dose-ranging studies conducted in an identical in vitro system (data not shown). Excipient or inhibitor buffer was employed in both the mucosal and serosal chambers in appropriate experiments unless specified. Tissues were allowed to equilibrate in the presence of control, inhibitor, or excipient buffer for 30 minutes before the addition of DIG or VRP for the assessment of efflux or metabolism, respectively.

DIG efflux studies

Efflux inhibition was monitored by assessment of the steady-state s to m flux of DIG over 2 hours in the presence and absence of excipients or inhibitors. After the equilibration period, 18.74 μ L of Lanoxin Adult injection was added to the mucosal and serosal chambers (which contained 6000 μ L of buffer) to give a final concentration of 1mM DIG, immediately followed by the addition of 1 μ Ci of [3 H]-DIG into the serosal (donor) chamber only. After 1 minute, an initial 200 μ L sample was withdrawn from the donor chamber, and subsequent 200 μ L samples were removed from the receptor chamber every 15 minutes for 2 hours. The receptor chamber samples were replaced with an equal volume of appropriate buffer (1 μ M DIG in control, excipient, or inhibitor buffer), and the dilution of the radiolabeled marker was taken into account when calculating DIG flux. After the addition of 1 mL of scintillation cocktail (Starscint, Packard Bioscience, Meriden, CT), all samples were briefly vortexed and analyzed for radioactivity by a Tricarb 2000CA liquid scintillation counter (Packard Bioscience, Meriden, CT).

Additional studies were conducted with PEG and P85 to explore the mechanism of the interaction between the excipient and P-gp efflux. First, studies were conducted with the excipient placed in either the mucosal or the serosal chamber only. Second, the effect of PEG on passive DIG permeability was investigated by flux determinations measured in the presence of total P-gp inhibition (achieved via the addition of 50 μ M KC). Finally, the effect of elevated osmotic pressure on P-gp activity was addressed by the addition of mannitol (125 mM chamber concentration) to the mucosal chamber, the serosal chamber, or both chambers.

VRP metabolism studies

In studies investigating the effect of the excipients and inhibitors on enterocyte-based metabolism, VRP was added to the mucosal chamber (donor) to achieve a final

concentration of 20 μ M. An initial 200 μ L sample was withdrawn 1 minute after the addition of VRP, and further 200 μ L samples were withdrawn from both donor and receptor chambers at 1 and 2 hours. Donor chamber samples were diluted with an equal volume of fresh buffer to bring them into the linear range of the high performance liquid chromatography (HPLC) assay; 200 aliquots of these diluted donor chamber samples and all 200 μ L receptor chamber samples were then acidified by the addition of 100 μ L of mobile phase (acetonitrile and 25 mM potassium phosphate pH 3, 30:70) prior to the quantification of VRP and NOR using a validated HPLC assay (accuracy 97% $\pm$ 3% of target concentration, coefficient of variation (CV) <10%, limit of quantitation (LOQ) 20 pmol/mL, linear range 0.02-10 μ M). The HPLC consisted of a Waters 712 WISP autosampler and 600E pump (Milford, MA), a Perkin-Elmer LC 240 fluorescence detector (Beaconsfield, England) with excitation and emission wavelengths set to 280 and 310 nm respectively, and a Shimadzu GR5A integrator (Kyoto, Japan). A 100 μ L portion of acidified sample was injected onto an Xterra MS C₁₈ 5 μ M 3.9 $\times$ 150 mm column (Waters, Milford, MA), and mobile phase was delivered at 1 mL/min.

Data and statistical analysis

The mean DIG steady-state flux (%/cm²/hr) for each group (control, excipient, or inhibitor) was determined by regression of the linear region of the cumulative DIG transported versus time plot, and statistical comparisons were made between control and excipient or inhibitor data sets by Student t test or one-way ANOVA with multiple comparisons (Tukey).

No significant adsorption of VRP or NOR to the diffusion chambers was observed in prestudy validation experiments (data not shown). Similarly, mass balance (recovery) across the buffer and tissue compartments for verapamil and metabolites was determined over 2 hours and found to be effectively complete (94% $\pm$ 5%, mean $\pm$ SD, n = 6). The percent of VRP disappearing from the donor chamber was therefore subsequently taken as a measure of tissue uptake. The percent of VRP appearing in the receptor chamber, and the mole percent of NOR appearing in the donor and receptor chambers after 2 hours were calculated for each experiment. Because of interrater variability in the extent of metabolism of VRP, the percent change in the formation of NOR in the presence of the excipients/inhibitors was compared to control data collected in the same experiment from the same animal and was subsequently averaged across 3 separate experimental runs. Confidence intervals (95%) were generated to determine the statistical significance of the ratio between the presence and absence of excipients/inhibitors and were deemed significant when the 95% confidence interval did not include 0%. Two further mole ratios were calculated to relate the total amount of NOR formed and the total amount of VRP transported across the tissue to the

Table 1. Steady-State S to M DIG Flux (%/cm²/hr) Across Stripped Rat Jejunal Tissue in the Presence and Absence of 5% PEG, 5% PEG Plus 50 μ M KC, 0.1% P85, and 125 mM Mannitol*

Condition	Control	Additive Placement		
		Mucosal	Serosal	Both
PEG 5%	0.626 $\pm$ 0.114	0.394 $\pm$ 0.047 [†]	0.253 $\pm$ 0.082 ^{†‡}	0.323 $\pm$ 0.065 [†]
PEG 5% + KC [§]	0.298 $\pm$ 0.025	0.334 $\pm$ 0.043	0.235 $\pm$ 0.029 ^{†‡}	0.232 $\pm$ 0.020 ^{†‡}
P85 0.1%	0.597 $\pm$ 0.095	0.348 $\pm$ 0.091 [†]	0.329 $\pm$ 0.062 [†]	0.308 $\pm$ 0.045 [†]
Mannitol 125mM	0.628 $\pm$ 0.064	0.397 $\pm$ 0.060 [†]	0.508 $\pm$ 0.089	0.347 $\pm$ 0.020 [†]

*Mean $\pm$ SD from n = 6 experiments from 3 different rats. S to M indicates serosal to mucosal; DIG, digoxin; PEG, polyethylene glycol; KC, ketoconazole.

[†]Significantly lower than control ($P < .05$).

[‡]Significantly lower than mucosal addition ($P < .05$).

[§]KC was present in both chambers at 50 μ M in all experiments (including control) regardless of the site of application of PEG.

amount of VRP taken up into the tissue (disappearance) (Equations 1 and 2 respectively).

$$(1) \text{ NOR formed/VRP taken up} \\ (\text{mol/mol}) = \frac{[NOR]_D + [NOR]_R}{[VRP_{(0)}]_D - [VRP]_D}$$

$$(2) \text{ VRP transported/VRP taken up} \\ (\text{mol/mol}) = \frac{[VRP]_R}{[VRP_{(0)}]_D - [VRP]_D}$$

Concentrations of NOR and VRP are in μ M in the donor (D) or receptor chambers (R) after 2 hours, and $VRP_{(0)}$ is the initial VRP concentration in the donor chamber. Ratios were calculated for each diffusion chamber, and statistical comparisons of the above ratios (mean ratio $\pm$ SD) were made between control and excipient or inhibitor experiments by using Student t test or one-way ANOVA with multiple comparisons (Tukey).

Octanol/buffer distribution coefficients

Distribution coefficients (D') of DIG and VRP between 1-octanol and control buffer or buffer containing the various excipients (pH 7.4, 37°C) were determined using the shake-flask method. Briefly, in duplicate experiments, presaturated octanol and excipient buffers (2 mL) were each spiked with VRP or DIG to give a final concentration of 1 μ M, which contained an accurate trace of the tritiated species (0.25 μ Ci), and vortexed. The 2 phases were then combined in a 10 mL glass tube, mixed using a rotary inverter for 5 minutes, and incubated at 37°C for 24 hours with gentle agitation. After 24 hours the phases were separated by centrifugation (3000g), and duplicate 200 μ L aliquots of each phase were analyzed for radioactivity as described. The apparent distribution coefficient (D') was then calculated from the ratio of the measured disintegrations per minute (DPM) in the octanol phase and the buffer phase.

DIG solubility in Pluronic P85 buffer

DIG solubility in P85 buffer (37°C, pH 7.4) was determined, in duplicate, by dissolving 0.37 mg of DIG with a radiolabeled trace (1.0 μ Ci) in pyridine (100 μ L) in a 10 mL glass tube, evaporating the vehicle, and adding 3 mL of control or P85 buffer (0%-10% wt/vol). Samples (200 μ L) were withdrawn from the tubes and centrifuged, and the supernatant was analyzed for radioactivity until equilibrium solubility was reached (72-96 hours). Equilibrium solubility was defined as the point at which the solubility assessed in subsequent samples changed by <5%. The total amount of DIG (labeled and unlabeled) in solution was then determined and expressed in both μ g/mL and μ M.

RESULTS

DIG efflux studies

The steady-state s to m flux of DIG determined in the presence and absence of the excipients and inhibitors (present in both the mucosal and serosal buffers) is shown in **Figure 1**. P85 and PEG significantly reduced the efflux of DIG in a concentration-dependent manner. TPGS (at 0.01%) had no significant effect on the transport of DIG. Since these studies were conducted with the excipient present in both the mucosal and serosal buffers, additional studies with P85 0.1% and PEG 5% were conducted with the excipients added to either the mucosal or serosal chambers only. Interestingly, both P85 and PEG demonstrated significant inhibition of efflux regardless of the side of application (**Table 1**).

The effect of PEG on the passive permeability of DIG was assessed by studies conducted in the presence of 50 μ M KC and in the presence and absence of 5% PEG. PEG resulted in a 21% or 22% reduction in the passive s to m transport of DIG after serosal addition or addition to both chambers, respectively (**Table 1**). Mucosal application of 5% PEG, however, had no significant

Figure 1. Steady state s to m flux of DIG (%/cm²/hr) across stripped rat jejunal tissue in the presence of the excipients PEG (0.01%-20%), P85 (0.01%-0.1%), and TPGS (0.01%) and the inhibitors MDZ (20 µM), PSC (20 µM), and KC (50 µM). Excipients or inhibitors were placed on both sides of the tissue and statistical comparisons were made relative to control data collected within the same experiment. For the purpose of graphical representation, the overall mean obtained for pooled control data across all experiments is depicted in this figure. Mean $\pm$ SD from n³6 tissues from 3 different rats. *Statistically significant difference compared to respective control data ($P < .05$).

Table 2. VRP Disappearance (Percent Loss) From the Donor Chamber and Appearance (as Percent of Initial Concentration) in the Receptor Chamber After 2 Hours From VRP (20 µM) M to S Transport Studies Across Stripped Rat Jejunal Tissue*

Condition	Disappearance (% Loss)		Appearance (% Transport)	
	Control	Excipient/Inhibitor	Control	Excipient/Inhibitor
PEG 0.1%	23.45 $\pm$ 3.38	17.58 $\pm$ 2.44	1.87 $\pm$ 0.42	1.46 $\pm$ 0.15
PEG 1%	23.45 $\pm$ 3.38	21.49 $\pm$ 0.55	1.87 $\pm$ 0.42	2.26 $\pm$ 0.62
PEG 5%	23.45 $\pm$ 3.38	11.36 $\pm$ 5.08	1.87 $\pm$ 0.42	2.06 $\pm$ 0.69
PEG 20%	23.45 $\pm$ 3.38	5.07 $\pm$ 3.88 [†]	1.87 $\pm$ 0.42	2.68 $\pm$ 0.69
P85 0.01%	19.41 $\pm$ 2.19	16.05 $\pm$ 0.65	1.62 $\pm$ 0.27	1.72 $\pm$ 0.45
P85 0.1%	19.41 $\pm$ 2.19	15.75 $\pm$ 0.70	1.62 $\pm$ 0.27	2.08 $\pm$ 0.43 [†]
TPGS 0.01%	17.60 $\pm$ 6.97	16.00 $\pm$ 3.06	1.86 $\pm$ 0.34	1.12 $\pm$ 0.26
MDZ 20 µM	19.14 $\pm$ 6.83	12.34 $\pm$ 4.43	1.39 $\pm$ 0.15	1.48 $\pm$ 0.63
PSC 20 µM	16.75 $\pm$ 1.95	19.22 $\pm$ 1.13	1.53 $\pm$ 0.40	1.56 $\pm$ 0.14
KC 50 µM	20.11 $\pm$ 2.87	13.55 $\pm$ 1.81 [†]	1.74 $\pm$ 0.28	2.69 $\pm$ 0.44 [†]

*Studies were conducted in the presence and absence of PEG (0.1%-20%), P85 (0.01%-0.1%), TPGS (0.01%), MDZ (20 µM), PSC (20 µM), and KC (50 µM). Control data gathered within each set of experiments are shown and the appropriate statistical comparisons made. Data represent the mean $\pm$ SD from n $\geq$ 6 tissues from 3 different rats. VRP indicates verapamil; M to S, mucosal to serosal; PEG, polyethylene glycol; TPGS, vitamin E d- α -tocopheryl polyethylene glycol 1000 succinate; MDZ, midazolam; PSC, PSC833; KC, ketoconazole.

[†]Statistically significant difference to respective control ($P < .05$).

Figure 2. Reduction in NOR appearance (total of donor and receptor chambers) over 2 hours during VRP (20 µM) m to s transport studies across stripped rat jejunal tissue in the presence of the excipients PEG (0.01%-20%), P85 (0.01%-0.1%), and TPGS (0.01%) and the inhibitors MDZ (20 µM), PSC (20 µM), and KC (50 µM). Excipients or inhibitors were placed on both sides of the tissue, and statistical comparisons were made relative to control data collected within the same experiment. Mean $\pm$ SD from $n=6$ tissues from 3 different rats. Error bars are not present for KC or 20% PEG, as no NOR was formed in either experiment. *Statistically significant reduction ($P < 0.05$).

impact on the passive permeability of DIG when compared to control.

Increasing buffer osmotic pressure by the addition of mannitol (125 mM) resulted in significant reductions in DIG s to m transport when the hyperosmolar buffer was present in the mucosal chamber (38% reduction) or both chambers (37% reduction). In contrast, serosally applied mannitol resulted in no significant changes to DIG transport (**Table 1**).

Metabolism and transport of VRP

In control experiments, between 1.39% and 1.87% of the mucosally applied VRP dose appeared in the serosal (receptor) chamber as unchanged VRP over 2 hours (**Table 2**). Over the same time period, a similar quantity of NOR (1.9 ± 0.9 mole percent) was found in the mucosal (donor) chamber, indicating that the extent of metabolism of VRP was relatively high when compared to the total amount of VRP transported. The appearance of NOR in the receptor chamber was relatively low (0.059 ± 0.049 mole percent) and often below the LOQ even after 2 hours. A significant amount of VRP was lost from donor chambers (17.60%-23.45%, **Table 2**) and attributed to tissue uptake, as no significant adsorption of VRP to the diffusion chamber apparatus was observed.

The effect of PEG, P85, and TPGS on the total amount of NOR appearing in both the mucosal and serosal chambers over the period of a 2-hour transport study with VRP 20 µM added to the mucosal chamber is shown in **Figure 2**. Both P85 and PEG had significant inhibitory effects on the extent of NOR formation in a concentration-dependent manner, and in the presence of 20% PEG, no metabolite was detected in either the donor or the receptor buffer. The effect of TPGS was insignificant at 0.01%. As expected, the selective CYP3A inhibitor MDZ and the nonselective P-gp/CYP3A inhibitor KC both significantly reduced metabolism—by 82% and 100% respectively. The P-gp inhibitor, PSC, had insignificant effects on the appearance of NOR.

The extent of VRP transport and uptake (disappearance from the donor chamber) was also determined; these results are summarized in **Table 2**. VRP appearance in the receptor chamber was in agreement with previous findings²⁵ but was significantly increased by only 0.1% P85 and 50 µM KC (**Table 2**). TPGS, PEG, and the P-gp and CYP3A inhibitors PSC and MDZ had no significant impact on VRP transport. PEG markedly reduced VRP loss from the donor chamber (or tissue uptake) in a concentration-dependent manner, although the effect reached statistical significance with only 20% PEG (**Table 2**).

In an attempt to obviate changes in NOR formation and VRP transport resulting from reduced VRP uptake (and

Figure 3. Ratios of total mole amount of NOR formed (sum of donor and receptor chambers) per mole of VRP lost from the donor chamber (blue bars) and mole VRP transported per mole VRP lost from the donor chamber (green bars) after 2 hours during VRP (20 μ M) m to s transport studies across stripped rat jejunal tissue. Studies were conducted in the presence and absence of PEG (0.1%-20%, Panel A), P85 (0.01%-0.1%, Panel B), and TPGS (0.01%, Panel C) where excipients were added to both chambers. Mean $\pm$ SEM from $n > 6$ tissues from 3 different rats where control data were collected within the same set of experiments.

Table 3. Octanol/Buffer Distribution Coefficients Determined for VRP (1 μ M) and DIG (1 μ M) in the Presence and Absence of PEG (0.1%-20%), P85 (0.01%-0.1%), and TPGS (0.01%) and Equilibrium DIG Solubility Determined in the Presence of P85 (0%-10%) in Buffer*

Condition	Distribution Coefficient		DIG Solubility		
	VRP	DIG	% P85	μ g/mL	μ M
Control buffer	11.68	21.53	0	30.85	39.50
PEG 0.1%	10.54	22.81	0.1	42.94	54.98
PEG 1%	11.10	22.78	1	44.17	56.56
PEG 5%	11.60	19.95	10	68.87	88.19
PEG 20%	10.92	16.26			
P85 0.01%	10.82	21.18			
P85 0.1%	11.01	22.26			
TPGS 0.01%	10.94	21.91			

*Both parameters were determined at pH 7.4 (37°C), and data are the mean of duplicate determinations.

VRP indicates verapamil; DIG, digoxin; PEG, polyethylene glycol; TPGS, vitamin E d- α -tocopheryl polyethylene glycol 1000 succinate.

not due to inhibition of P-gp or CYP3A activity), ratios describing the mole amount of NOR formed, and VRP transported, per mole of VRP taken up into the tissue were calculated and are summarized in **Figure 3**. This type of ratio analysis compounds the errors associated with both VRP uptake and extent of NOR formation, and therefore no statistically significant differences were observed. However, it is evident that PEG, even after correcting for its effect on reducing VRP uptake, decreased VRP metabolism and increased transport of intact VRP across the tissue. The effects of P85 and TPGS on these 2 ratios were less remarkable.

Octanol/buffer distribution coefficients

Octanol/buffer distribution coefficients for VRP and DIG are summarized in **Table 3**. The excipients had little impact on the distribution coefficients of VRP and DIG, although there was evidence of decreased DIG partitioning at 5% and 20% PEG.

Digoxin solubility in Pluronic P85 buffer

To determine if significant loss of DIG thermodynamic activity occurred at the supermicellar concentration of 0.1% P85 (critical micelle concentration [CMC] = 0.03%)²⁶, DIG solubility in 0% to 10% P85 in buffer was determined (**Table 3**). The solubility of DIG in buffer (31 μ g/mL) is in accordance with previously reported solubility measurements of 27 and 40 μ g/mL^{27, 28}. Only small increases in solubility were evident over the working concentration range in the permeability experiments (0.01%-0.1%). Solubility was approximately double that of control at very high (10%) concentrations of P85, suggesting limited solubilization of DIG at lower (<0.1%) concentrations of P85.

DISCUSSION

The studies presented here have addressed the impact of excipients on efflux and metabolism within intact intestinal segments, and the results have direct applicability to the widespread use of excipients and cosolvents to increase the solubility of probe compounds during in vitro drug permeability assessment. To probe

the impact of common additives on P-gp efflux and CYP3A metabolism, these studies have specifically assessed the impact of P85, TPGS, and PEG on the s to m flux of DIG and on the transport and metabolism of the P-gp/CYP3A substrate VRP. Reduction in the s to m flux of DIG and a change in the appearance of NOR were used as primary measures of inhibition of intestinal P-gp-mediated efflux and CYP3A metabolism respectively. DIG and VRP have not been shown to interact significantly with MRP1 or 2²⁹⁻³¹, and CYP3A is the primary enzyme responsible for the intestinal metabolism of VRP³²; therefore, the observations made in these studies most likely reflect alterations in P-gp- and CYP3A-mediated events (although this does not preclude the possibility that other unidentified transporters and enzymes may also have been inhibited by the excipients and/or inhibitors employed in these studies).

In this experimental system, P85 and PEG had significant effects on both DIG efflux and VRP metabolism. TPGS, however, had little impact on either process at a concentration of 0.01% (unfortunately, experiments utilizing higher concentrations of TPGS were not possible since considerable foaming of the transport buffers occurred in the presence of higher TPGS concentrations, resulting in significant buffer loss from the reservoirs). In contrast, previous studies have shown that TPGS significantly inhibits the P-gp efflux of amprenavir across Caco-2 cells at much lower concentrations than those used here (<0.002%)²². This discrepancy in the effect of TPGS across these 2 models warrants further investigation and may involve differences in enzyme expression across the 2 systems or the ability of the TPGS to effectively access P-gp in the Ussing chamber model.

P85 is a block copolymer that has been shown previously to inhibit P-gp, and this inhibitory activity has been demonstrated in monolayers of bovine brain microvessel endothelial cells, Caco-2 cells, and porcine kidney epithelial cells in vitro and in mice in vivo^{21, 33}. The current data are in general agreement with these previous studies, although in contrast to the results reported here, Batrakova et al.²¹ found that basolateral

Figure 4. Comparison of inhibitory selectivity of excipients and inhibitors for DIG efflux (blue bars) and VRP metabolism based on total NOR formation (green bars) across stripped rat jejunal tissue. The reduction in DIG efflux was proportionally scaled such that 50 µM KC represents 100% inhibition of efflux.

application of 0.01% P85 did not significantly reduce the basolateral to apical flux of DIG across MDR1-transfected kidney epithelial cells. In our studies, 0.1% P85 resulted in significant reduction in s to m flux of DIG regardless of the site of application (mucosal or serosal). The P-gp inhibitory activity of P85 in the serosal compartment could not be attributed to a reduction in thermodynamic activity since only small changes in the distribution coefficient and buffer solubility of DIG were seen in the presence of 0.1% P85. This is consistent with the experimental concentration of P85 (0.1%) being only slightly above the reported CMC for P85 (0.03%)²⁶. Of significance to the conduct of in vitro permeability studies is the observation that in addition to effects on P-gp efflux, the addition of 0.1% P85 to the buffer bathing both sides of the excised tissue also resulted in significant reduction (46%) in the appearance of NOR, the principle metabolite of VRP. The magnitude of this reduction in appearance of NOR could not be attributed to a reduction in uptake of VRP into the tissue (and therefore a reduction in available substrate) (Table 2) and is thus likely to reflect a reduction in CYP3A-mediated metabolism.

As VRP is a dual P-gp/CYP3A substrate, assessment of the impact of excipients on enterocyte-based metabolism is complicated by the possibility that inhibition of P-gp may lead to indirect changes to the extent of enterocyte-based metabolism. It has been shown previously by ourselves²⁴ and others¹³ that P-gp can influence the extent of enterocyte-based metabolism

of drugs, including VRP, and that NOR is a substrate for P-gp³⁴. However, it is unlikely that P-gp inhibition alone could result in the large reductions in NOR appearance observed here. In support of this assumption, the presence of the selective P-gp inhibitor PSC833 resulted in substantial inhibition of s to m DIG transport but had no significant impact on the appearance of NOR. Cummins et al¹³ also recently examined the interplay of P-gp and CYP3A in Caco-2 cells and suggested that cellular accumulation of parent and metabolite should be accounted for in order to define an accurate extraction ratio on drug passage across the enterocyte. Metabolite levels in the tissue were not examined in the studies described here, and the data should therefore be viewed with the caveat that the total extent of metabolism (in both control and experimental conditions) may be underestimated. The objective of the current studies, however, was not to accurately define the extraction ratio but rather to gain an indication of the relative inhibitory activity of different cosolvents and surfactants for P-gp and CYP3A when compared to a control condition. Under these circumstances, assessment of the degree of metabolism via the appearance of metabolites in the donor and receptor chambers appears justified.

The mechanism by which P85 inhibits VRP metabolism and reduces P-gp activity remains to be elucidated, but recent studies suggest that P85 can result in depletion of cellular adenosine triphosphate (ATP) and may decrease the microviscosity of the apical membrane of

cultured endothelial cells, resulting in substantial loss of P-gp function^{35, 36}. It is possible, therefore, that loss of CYP3A activity may also occur via ATP depletion. The impact of P85 on cellular ATP levels has been suggested to result from interaction with the mitochondria and uncoupling of oxidative phosphorylation through effects on electron transport^{35, 37}.

P85 demonstrated a modest (~2-fold) degree of inhibitory selectivity for P-gp efflux over CYP3A metabolism (**Figure 4**) when compared to the selective P-gp (PSC) and CYP3A (MDZ) inhibitors utilized here. This observation may reflect differences in access to the target enzyme and possibly differing mechanisms of inhibition. The data, however, demonstrate that the use of P85 to increase the solubility of a probe compound in *in vitro* permeability studies may be complicated by interaction of the surfactant with both P-gp and CYP3A. The significance of these effects would need to be assessed on a compound-by-compound basis.

In these studies, the most extensive investigations were carried out in the presence of PEG, where significant effects on both efflux and metabolism were observed. When PEG was placed on both sides of the tissue at 0.1%, 1%, 5%, and 20%, significant reductions in the *s* to *m* transport of DIG were observed (**Figure 1**). Further studies undertaken using a single PEG concentration (5%) indicated that inhibition of transport was greatest when the excipient was included in the serosal buffer (ie, in experiments using PEG in serosal buffer or in both the serosal and mucosal buffer) (**Table 1**), whereas when PEG was placed on the mucosal side only (where greatest access to P-gp was expected) there was only a modest reduction in transport (**Table 1**). These data are seemingly at odds with the suggestion that PEG may reduce P-gp activity by interfering with the structure of the apical membrane and thereby either directly or indirectly affecting the function of the transporter. Rather, the data show that the more significant effects are seen with PEG in the serosal chamber (and therefore most distant from the apical membrane). One possible explanation for this unusual observation is that the cosolvency provided by PEG in the donor (serosal) chamber may alter the partitioning of the drug between the buffer and the membrane, thereby reducing uptake into the tissue. **Table 3**, however, shows that while there is some reduction in the octanol/buffer distribution coefficient (*D'*) on inclusion of 5% and 20% PEG, these small reductions in *D'* are not large enough to explain the observed reduction in DIG transport.

The potential impact of PEG on the partitioning and passive transport of DIG is clarified by the data obtained in the presence of the known P-gp inhibitor KC (50 μ M). **Table 1** shows that in the presence of KC, inclusion of 5% PEG in either the serosal or both the serosal and mucosal chambers reduced the passive flux of DIG and that this reduction was of the same order as the reduction in *D'* described above (ie, ~10%). In combination, these data suggest that the primary mechanism for the reduction in *s* to *m* transport of DIG is

not a change in partitioning or passive transport but rather a reduction in P-gp efflux and that this occurs regardless of whether the excipient is present on the mucosal or serosal sides of the tissue.

PEG (5% wt/vol) contributes an additional 125 mOsm of osmotic pressure to the buffer. Since the function of P-gp appears to depend on the fluidity of the membrane in which it resides³⁸⁻⁴⁰, changes in water transfer across the apical membrane resulting from imbalances in osmotic pressure may result in alterations to membrane fluidity and possible attenuation of P-gp function. To examine the impact of changes in osmotic pressure on P-gp function, experiments were conducted with mannitol (125 mM, equiosmolar to 5% wt/vol PEG) on the serosal side, the mucosal side, or both sides of the tissue. The data in **Table 1** show that a significant reduction in DIG efflux was produced by changes in osmotic pressure. The effects, however, were limited to the generation of osmotic gradients across the mucosal membrane and were insensitive to differences in osmotic pressure generated in the serosal chamber alone. These data indicate that, as suggested previously, P-gp is sensitive to changes in the membrane environment and that creation of a hyperosmotic concentration gradient across the apical membrane may lead to structural changes to the membrane that in turn result in attenuation of P-gp efflux. Importantly, this observation is counter to any potential "water drag" effect where an increase in *s* to *m* flux might be expected in the presence of a hyperosmotic mucosal buffer⁴¹.

The inhibitory effect of mucosally applied PEG on P-gp may therefore result from disturbances in osmotic pressure across the apical membrane. In contrast, serosally applied PEG, although effective in reducing *s* to *m* flux of DIG, does not appear to be capable of producing a substantial osmotic gradient across the apical membrane and only slightly reduces the partition coefficient between the serosal buffer and the tissue and the passive permeability of DIG. The mechanism by which serosal PEG alters P-gp, therefore, remains to be elucidated but may involve a nonspecific "toxicity" such as that previously described for P85—that is, via a reduction in mitochondrial function and cellular ATP levels. This relatively nonspecific mechanism of transporter inhibition is an attractive explanation in this case since it does not rely on specific access of PEG to the P-gp transporter on the apical membrane and is consistent with the parallel inhibition of VRP metabolism discussed below.

The presence of 1%, 5%, and 20% PEG in transport buffer resulted in a significant reduction in NOR appearance in both mucosal and serosal chambers, with 20% PEG completely abolishing NOR formation. The cosolvent effects of PEG in the donor chamber may lead to reduced VRP uptake into the tissue (and therefore reduced production of NOR), and although the *D'* of VRP between PEG buffer and octanol was essentially identical to control (**Table 3**), significant changes in the extent of VRP lost from the donor chamber were evident at 20% PEG. PEG reduced VRP disappearance in a

concentration-dependent manner, and it is likely that a proportion of the excipient's inhibitory effect on NOR formation was due to reduced VRP access to the tissue and subsequently to the CYP3A enzyme. To correct for the reduction in substrate (VRP) access to the tissue, the NOR formation data were corrected for the reduction in VRP uptake. **Figure 3** however, demonstrates that despite this attenuated uptake, the presence of 1%, 5%, and 20% PEG reduced NOR formation by the enterocyte.

The relatively high concentration range of PEG used in these studies reflects the low inhibitory potency of PEG for either efflux or metabolism but was chosen to reflect the likely concentration range that may be employed in *in vitro* studies. Similarly, while P85 displayed greater potency when compared to PEG, the concentrations utilized and the inhibitory effects observed are still significant in the context of the likely concentrations utilized *in vitro* to increase the solubility of probe compounds.

CONCLUSION

This study shows that P85 and PEG may inhibit drug metabolism and drug efflux processes in rat intestinal tissue in modified Ussing chambers. PEG and P85 possess both CYP3A and P-gp inhibitory effects, although PEG lacks the potency of traditional small molecule inhibitors. TPGS had little effect on either process in this setting, despite literature evidence suggesting P-gp inhibitory activity in Caco-2 cells. Further work with TPGS, possibly in different models of P-gp efflux (eg, isolated membrane systems), may be required to help researchers understand this discrepancy. The mechanism by which these compounds exert their observed inhibitory effects appears to be mixed and may involve reduced substrate cellular uptake, direct inhibition of the target enzyme or transporter, and indirect actions on normal cellular function, such as alterations in membrane fluidity and mitochondrial toxicity and subsequent ATP depletion. In summary, this study highlights the possible complications of utilizing solubilizers, such as PEG and P85, to increase the solubility of probe compounds during *in vitro* permeability and metabolism assessment. The data further suggest that while traditional integrity markers such as mannitol may indicate that excipients have a limited impact on passive paracellular permeability, more detailed studies may reveal substantial changes to transcellular events such as drug metabolism and transport processes. It should be noted that the current studies were not designed to evaluate the *in vivo* utility of these solubilizing agents to increase the permeability of P-gp and CYP3A substrates after oral delivery, where dilution in the gastrointestinal tract and improved drug trafficking through the tissue into the portal blood may attenuate the inhibitory activity of these agents.

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NOTE

Since the time of submission of the manuscript, Hugger et al (J Pharm Sci, 2002;91:1980-1990) reported a similar inhibitory effect of PEG 300 on P-gp transport in Caco-2 cell monolayers.

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