International Journal of Pharmaceutics 409 (2011) 164–168

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The effect of polyoxyethylene polymers on the transport of ranitidine in Caco-2 cell monolayers

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ARTICLE INFO

Article history:
Received 12 November 2010
Received in revised form 25 February 2011
Accepted 25 February 2011
Available online 4 March 2011

Keywords:
Permeability
Caco-2
Excipients
H_2 antagonists
Ranitidine
P-glycoprotein

ABSTRACT

Previous *in vivo* studies using PEG 400 showed an enhancement in the bioavailability of ranitidine. This study investigated the effect of PEG 200, 300 and 400 on ranitidine transport across Caco-2 cells. The effect of PEG polymers (20%, v/v) on the bi-directional flux of ^{3}H-ranitidine across Caco-2 cell monolayers was measured. The concentration dependence of PEG 400 effects on ranitidine transport was also studied. A specific screen for P-glycoprotein (P-gp) activity was used to test for an interaction between PEG and P-gp. In the absence of PEG, ranitidine transport showed over 5-fold greater flux across Caco-2 monolayers in the secretory than the absorptive direction; efflux ratio 5.38. PEG 300 and 400 significantly reduced this efflux ratio ($p < 0.05$), whereas PEG 200 had no effect ($p > 0.05$). In concordance, PEG 300 and 400 showed an interaction with the P-gp transporter, whereas PEG 200 did not. Interestingly, with PEG 400 a non-linear concentration dependence was seen for the inhibition of the efflux ratio of ranitidine, with a maxima at 1%, v/v ($p < 0.05$). The inhibition of ranitidine efflux by PEG 300 and 400 which interact with P-gp provides a mechanism that may account for the observations of ranitidine absorption enhancement by PEG 400 *in vivo*.

1. Introduction

Many drugs undergo absorption in the small intestine and although it has a large surface area of around 120 m^2, the residence time can be short here. A dosage form will spend an average of 3–4 h in the small intestine (Davis et al., 1986), but this can be very variable (Fadda et al., 2009) and as low as 30 min (Davis et al., 1986). Drug absorption can occur through simultaneous passive diffusion and active mechanisms, involving transcellular and paracellular routes. The paracellular route is controlled by tight junctions, and the transcellular route is influenced by cellular machinery (plasma membrane channels, carriers, exchangers and efflux transporters). Ranitidine is an H_2 receptor antagonist that has an absorption window in the small intestine, but poor absorption in the colon (Williams et al., 1992). The bioavailability of ranitidine has been shown to be improved in male subjects by the administration of low dose PEG 400 (Schulze et al., 2003; Ashiru et al., 2008). At high doses, however, the improvement in bioavailability was not observed. It is currently unknown whether the diminished effect at higher doses is due to the tendency of PEG to accelerate small intestinal transit (Basit et al., 2001; Schulze et al., 2003) or absolution of the PEG 400 absorption-enhancing mechanism.

It has been reported that ranitidine is primarily transported across Caco-2 cells via the paracellular route (Gan et al., 1993; Collett et al., 1996). However, more recent studies have suggested that paracellular transport accounts for 60% of the absorptive transport whilst transcellular processes, including transporters such as human organic cation transporter 1 [OCT], account for the other 40% (Bourdet et al., 2006; Bourdet and Thakker, 2006). The absorption of ranitidine is also affected by efflux transporters. P-glycoprotein (P-gp), multidrug resistance-associated protein 1 and 2 (MRP 1, MRP 2) and breast cancer resistance protein (BCRP) expel drug into the lumen of the intestine and many drugs are substrates of these transporters; consequently the bioavailability and pharmacokinetics of these drugs are controlled by the expression of these carriers. The efflux protein P-gp has been implicated in intestinal ranitidine transport (Collett et al., 1999) whilst cimetidine (another H_2 antagonist) has been identified as both a P-gp and BCRP substrate (Collett et al., 1999; Pavek et al., 2005).

PEG 300 and 400 are commonly used pharmaceutical excipients employed to enhance the solubility of drugs and there is evidence that PEG can inhibit efflux transporters (Hugger et al., 2002a). One group have reported a dose-dependent inhibition of P-gp in excised rat intestine in the presence of PEG 400 (Johnson et al., 2002). PEG 300 and PEG 400 have also been shown to inhibit P-gp in Caco 2 cells (Rege et al., 2001). Based upon our own *in vivo* observations on ranitidine bioavailability (Ashiru et al., 2008), we hypothesise that low molecular weight PEGs can improve ranitidine transport

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doi:10.1016/j.ijpharm.2011.02.059

by way of P-gp inhibition in a concentration-dependent manner. Therefore, the aim of this study was to investigate the effect of PEG 400 and its lower molecular weight analogues (PEG 200 and 300) on ranitidine transport using the Caco-2 epithelial cell model.

2. Materials and methods

2.1. Materials

The PREDEASY ATPase Kit containing human P-gp membranes from Sf9 insect cells, was obtained from Tebu-bio (Peterborough, UK). Caco-2 cells (human adenocarcinoma cell line) were obtained from the European Collection of Cell Cultures (ECACC) (Wiltshire, UK). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, non-essential amino acids, L-glutamine, 0.25% trypsin–EDTA, gentamicin (50 mg/ml), Hanks' Balanced Salt Solution (HBSS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), polyoxyethylene glycol (PEG) 200, 300 and 400 were purchased from Sigma–Aldrich (Dorset, UK). Ranitidine (99.9% purity) was obtained from Zhongnuo Pharmaceutical Co., Ltd. (China). Transwell® Corning Costar Corporation (12-well, 1.13 cm^2 surface area, 0.4 μm pore size) and 162 cm^2 flasks were obtained from Fisher (Leicestershire, UK). ^{14}C-mannitol (specific activity = 61 mCi/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). ^{3}H-ranitidine (specific activity = 2.5 Ci/mmol) was purchased from Moravek, CA, USA. Scintillation Cocktail (Emulsifier) was obtained from Perkin Elmer (Buckinghamshire, UK).

2.2. Methods

2.2.1. P-gp ATPase activity

ATPase activity of ranitidine and PEG 400 and its two lower molecular weight analogues (200 and 300) were measured using the PREDEASY ATPase Kit as per manufacturers' instructions. Briefly, the P-gp containing membrane was diluted with assay mix (50 mM Mops–Tris, pH 7.0; 50 mM KCl; 5 mM sodium azide; 2 mM DTT; 0.1 mM EGTA–Tris, pH 7.0; 1 mM ouabainin distilled water). Diluted membrane solution (40 μl) was loaded into the wells of a 96-well plate. Test compounds PEG 200, 300 and 400 were dissolved in DMSO to produce 300 μM solutions. From these solutions 1 μl was taken and added to the membrane suspension. The same volume of DMSO was added to the control wells and the reaction mixtures pre-incubated at 37 °C for 20 min. The reaction was started by the addition of 10 μl ATP (magnesium salt) solution and stopped 10 min later by the addition of 100 μl developer solution. After 2 min 100 μl of blocker solution was added to the wells and then further incubated for 30 min at 37 °C before reading the absorbance at 610 nm in a microplate spectrophotometer. The drug stimulated ATPase activity (nmol/min/mg of protein) was determined as the difference between the amounts of inorganic phosphate released from ATP in the absence and presence of vanadate. Phosphate standards were prepared in each plate and verapamil served as a positive control. Drug-stimulated P-gp ATPase activity was reported as fold-stimulation relative to the basal P-gp ATPase activity in the absence of drug (DMSO control). A compound was classified as an activator if the fold-stimulation was greater than 2-fold over the DMSO control.

2.2.2. Caco-2 cell culture

2.2.2.1. Cell maintenance. Caco-2 cells (passages 25-55) were grown and maintained in culture as previously described (Hidalgo et al., 1989). Briefly, cells were grown in 162 cm^2 cell culture flasks and subcultured weekly on achieving 80–90% confluency. Cell culture growth medium was Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, and 0.1% (v/v) gentamicin (50 mg/ml). Cells were maintained in an incubator at 37 °C with humidified environment of 95% and 5% CO_2. Medium was changed every 2–3 days.

2.2.2.2. Growth of cell monolayers. For the transport studies, cells were seeded at a density of 60,000 cells/cm^2 onto Transwell® polycarbonate membranes with a 12 mm diameter, pore size of 0.4 μm and a surface area of 1.13 cm^2. Cells grown on Transwell® membranes were maintained by providing 0.5 ml of culture medium to the apical (A) compartment and 1.5 ml to the basolateral (B) compartment. Medium was replaced every 2–3 days until the cells were ready for the permeability experiments (days 21–28).

2.2.2.3. Transepithelial electrical readings (TER). The integrity of the cell monolayers during the growth phase was monitored by taking TER readings using an EVOM™ epithelial voltohmmeter (World Precision Instruments, Hertfordshire, UK). The resistance of the monolayer was determined by subtracting the total resistance (membrane support and cell monolayer) from the membrane support resistance. All cells were used at TER greater than 700 Ω cm^2.

2.2.3. Transport studies

All transport studies were performed on Transwell® grown Caco 2 cells maintained in culture for 21–28 days. Before performing the transport studies the TER was measured to ensure cell monolayer integrity. The cell culture medium was then removed and washed three times with pre-warmed transport buffer (HBSS with HEPES, pH 7.4) prior to the start of the experiment. In all bidirectional transport studies, either HBSS or PEG dissolved in HBSS were present on both sides of the Caco-2 cell monolayers. This was done to maintain osmotic pressure for the duration of the study as the PEG solutions are hyperosmotic (Rege et al., 2001; Hugger et al., 2002a). The integrity of the monolayer during the experiment was confirmed by concomitant addition of ^{14}C-mannitol to all the test solutions.

In the absorptive (A-to-B) transport studies, 1.5 ml of HBSS or PEGs dissolved in HBSS at 20% (v/v), was added to each receiver (B) compartment. Into the donor (A) compartment was added 0.5 ml of HBSS or PEGs dissolved in HBSS, spiked with ^{14}C-mannitol and ^{3}H-ranitidine (along with cold ranitidine to a total concentration of 0.1 mM). For the secretory (B-to-A) transport studies, 1.5 ml mixture of radiolabeled mannitol and ranitidine (total concentration 0.1 mM) were added to the basolateral chamber instead. The transport study was performed under stirring conditions at a speed of 50 rpm (Gyrotory Shaker Model G2, New Brunswick Scientific Co., UK). At 30 min intervals (0, 30, 50, 90, 120, 150 and 180 min), 100 μl samples were removed from the receiver compartment and each compartment was appropriately replenished with HBSS or HBSS containing PEGs. The amount of radiolabeled solute transported across the Caco 2 cell monolayers was determined using a Beckman Coulter LS6500 liquid scintillation counter (Buckinghamshire, UK). The apparent permeability coefficients (Papp; cm/s) for the radiolabeled solute were determined in the absorptive and secretory direction using the equation:

$$\text{Papp} = \left(\frac{1}{AC_o}\right)\left(\frac{dQ}{dt}\right) \quad (1)$$

where dQ/dt is the flux across the monolayer, A is the surface area of the Transwell® membrane (1.13 cm^2), and C_o is the original donor concentration of the radiolabeled solute.

The efflux ratio was determined by dividing the Papp in the B-to-A direction by the Papp in the A-to-B direction. An efflux ratio greater than one indicates predominance of secretory transport suggesting the presence of an efflux transporter.

Table 1
The apparent permeability values for ^{14}C mannitol across Caco-2 cell monolayers in the presence of PEG 200, 300 and 400 (20%, v/v).[a]

Excipient (% v/v)	Mannitol Papp (cm/s $\times 10^{-6}$)
0	0.75 ± 0.05
PEG 200	0.86 ± 0.05
PEG 300	0.93 ± 0.01
PEG 400	0.95 ± 0.06

[a] The bidirectional transport of ^{14}C-mannitol (specific activity – 0.61 Ci/mmol) was examined across Caco-2 cell monolayers in the absence (no PEG, only HBSS) and presence of 20% (v/v) PEG 200, 300 or 400 on both sides of the Caco-2 cell monolayers (grown 21–28 days; $n = 3$); experiment performed in triplicate with 3 replicates per variable on each occasion. Samples (100 μl) were taken from the receiver compartments every 30 min for 3 h and each receiver compartment was replenished with the appropriate transport buffer solution (HBSS or PEG in HBSS). The apparent permeability coefficients (Papp) for ^{14}C-mannitol were calculated as described in Section 2.

2.2.4. Statistics

All values were expressed as mean ± SD. Cell culture data are the mean of three separate experiments with replicates of $n = 3$ on each occasion. Statistical evaluation of data was performed with SPSS® (version 15.0, SPSS Inc., Chicago, IL, USA). Data were compared using either *t*-test or one-way analysis of variance (ANOVA). In all cases, a difference was considered significant at $p \leq 0.05$.

3. Results

3.1. Effect of PEG on mannitol flux and transepithelial electrical resistance (TER)

Before investigating the effects of PEG on the transport of ranitidine across Caco-2 cell monolayers, it was important to determine whether PEG affects cell monolayer integrity. In these studies, TER measurements and mannitol transport were used to test cellular integrity in the presence of a 20% (v/v) PEG 200, 300 and 400 over a 180 min period. The results showed that the average transport of mannitol in the control monolayers ($0.75 \pm 0.05 \times 10^{-6}$ cm/s) and in those treated with PEG 200, 300, 400 were not significantly different from each other (Table 1; $p > 0.05$). Changes in TER were not considered significant ($p > 0.05$) compared to control for all PEGs. TER values in the presence of PEG were typically >700 Ω cm^2.

3.2. Effect of ranitidine and PEG on P-gp ATPase activity

The interaction between ranitidine and the PEG analogues on P-gp was investigated using a P-gp ATPase activity kit. Of these, only PEG 200 fell below the ATPase stimulation ratio of 2 (Table 2). The other compounds were shown to stimulate P-gp ATPase activity (ratio above 2).

Table 2
Effect of ranitidine and PEG analogues on ATPase activity; screen for P-gp interaction.[a]

Compound	ATPase assay ratio	ATPase activator/interaction with P-gp (Y/N)
Ranitidine	4.15	Y
PEG 200	0.53	N
PEG 300	3.71	Y
PEG 400	3.06	Y

[a] Drug-stimulated Pgp ATPase activity was reported as fold-stimulation relative to the basal Pgp ATPase activity in the absence of drug (DMSO control). A compound is classified as an activator if the fold-stimulation was greater than 2-fold over the DMSO control (Polli et al., 2001).

Table 3
Effects of PEG 200, 300 and 400 (20%, v/v) on ^{3}H-ranitidine transport across Caco-2 cell monolayers.[a]

Excipient	Papp (cm/s $\times 10^{-6}$) A-to-B	Papp (cm/s $\times 10^{-6}$) B-to-A	Papp(B-to-A)/Papp(A-to-B)
0	1.06 ± 0.01	5.72 ± 0.2	5.38
PEG 200	1.07 ± 0.08	5.40 ± 0.65	5.05
PEG 300	2.26 ± 0.09	5.63 ± 0.70	2.49
PEG 400	1.51 ± 0.05	5.91 ± 0.1	3.9

[a] The bidirectional transport of ^{3}H-ranitidine (concentration 0.1 mM; specific activity – 2.5 Ci/mmol) was examined across Caco-2 cell monolayers in the absence (no PEG, only HBSS) and presence of 20% (v/v) PEG 200, 300 or 400 on both sides of the Caco-2 cell monolayers (grown 21–28 days; $n = 3$); experiment performed in triplicate with 3 replicates per variable on each occasion. Samples (100 μl) were taken from the receiver compartments every 30 min for 3 h and each receiver compartment was replenished with the appropriate transport buffer solution (HBSS or PEG in HBSS). The apparent permeability coefficients (Papp) for ^{3}H-ranitidine were calculated as described in Section 2.

3.3. Effect of PEG analogues on the bidirectional transport of ranitidine

The Papp values for the absorptive and secretory transport of ^{3}H-ranitidine across Caco-2 cell monolayers in the absence and presence of 20% (v/v) of PEG 200, 300 and 400 are shown in Table 3. The results show that in the absence of PEG (control monolayers), ranitidine exhibited polarised secretory transport (an efflux ratio significantly above 1). In the presence of PEG 300 and 400 (but not PEG 200), the efflux ratio decreased compared to control, although not to a level where efflux is totally abolished, i.e. a ratio of 1. The lowest efflux ratio value was 2.49 for PEG 300; there was an increase in absorptive transport of ranitidine in the presence of PEG 300 and 400, whilst secretory transport remained largely unaffected.

3.4. Effect of PEG 400 concentration on the transport of ranitidine

The Papp for the permeation of ^{3}H-ranitidine across Caco-2 cell monolayers in the absorptive and secretory directions in the presence of various concentrations of PEG 400 are shown in Fig. 1. In the presence of PEG 400 there is predominance of secretory transport of PEG 400 at all concentrations, except for 1% (v/v) where the efflux ratio was at its lowest value of 1.2 (Fig. 2). At concentrations up to 1% (v/v) there was a progressive reduction in secretory and concomitant increase in absorptive transport of ranitidine. At PEG 400 concentrations between 1% (v/v) and 20% (v/v), the inhibition of secretory transport became progressively weaker. All the concentrations of PEG 400 tested had a significant effect on the efflux ratio compared to control (ANOVA, $p < 0.05$).

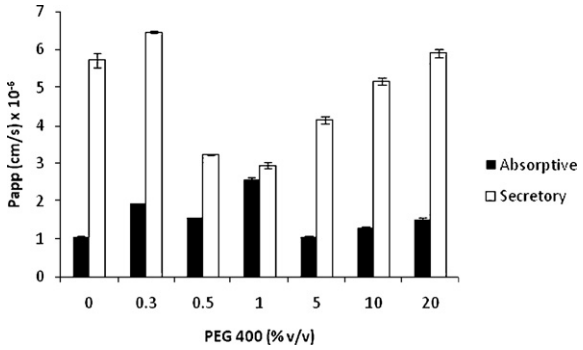

Fig. 1. Effects of different concentrations of PEG 400 on the bidirectional transport of ^{3}H-ranitidine across Caco-2 cell monolayers (mean ± SD, $n = 3$). Open bars indicate transport in the secretory direction, closed bars indicate transport in the absorptive direction.

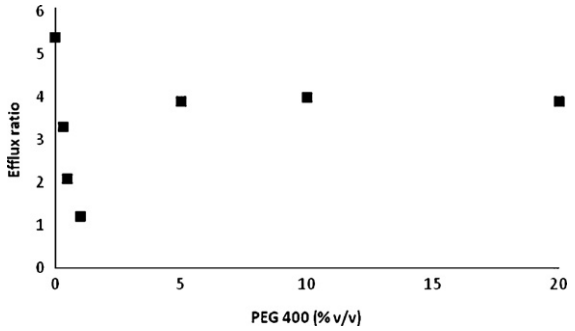

Fig. 2. Effects of different concentrations of PEG 400 on the efflux ratio (Papp secretory/Papp absorptive) of ^{3}H-ranitidine across Caco-2 cell monolayers.

4. Discussion

The bioavailability of ranitidine in male subjects is improved by the administration of low dose – 1% PEG 400 (Schulze et al., 2003; Ashiru et al., 2008). The mechanism for this effect is unknown and the present study investigated whether PEG of different molecular weights produces similar effects *in vitro*, and if so how these are mediated.

PEGs are amphiphilic, non-micelle forming hydrophilic polymers that are considered inert and safe (up to 40%, v/v) for use as pharmaceutical excipients. In this study we verified that PEG does not influence paracellular transport by demonstrating that the permeability of mannitol, a hydrophilic paracellular marker, was unchanged in the presence or absence of PEG 200, 300 or 400 at 20% (v/v). The mannitol Papp $\sim 1 \times 10^{-6}$ cm/s in the presence or absence of PEG was similar to that observed by Rege et al. (2001). As PEG solutions increase osmolality in comparison to standard Caco-2 assay media, the potential to affect drug flux by movement of water across the cell layer was negated by placing PEG in both donor and receiver chambers of the diffusion apparatus to avoid generating a hyperosmotic gradient. The mannitol Papp and TER data confirm that the osmotic pressure did not affect Caco-2 monolayer integrity (Inokuchi et al., 2009).

PEG 300 has no influence on the passive transport of drugs *in vitro* (Hugger et al., 2002a), but there are reports that certain PEG analogues such as PEG 400, PEG 2000 and D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) can affect the P-gp transporter (Johnson et al., 2002; Hugger et al., 2002a,b; Shen et al., 2006; Yamagata et al., 2007; Mudra and Borchardt, 2010) although the precise mechanisms by which this occurs remain elusive. Ranitidine is generally regarded to be a substrate for P-gp (Gan et al., 1993; Takamatsu et al., 2001; Bourdet et al., 2006), although Polli et al. (2001) classified ranitidine as a non-substrate. This disparity in defining a compound as a P-gp substrate, or an inhibitor, is not uncommon and can result from different assay sensitivities or inter-laboratory variation in the assays for P-gp transporter–drug interaction. Our data for Caco-2 cell monolayer efflux and P-gp ATPase activity indicate that ranitidine is a P-gp substrate. However, it is noted that the P-gp ATPase assay does not distinguish P-gp substrates from inhibitors and does not measure transport directly. In our studies PEG 300 and 400 stimulated P-gp ATPase and had the ability to inhibit ranitidine efflux in Caco-2 cells, whereas PEG 200 had no effect in either assay. PEG 300 and 400 (20%, v/v) reduced the ranitidine efflux ratio of ~5.5, principally through an increase in absorptive flux. PEG of similar and larger molecular weight (PEG 400, 2000 and 20,000) have been reported to inhibit the polarised efflux of rhodamine 123 when used at concentrations between 0.1 and 20% (v/v or w/v) (Shen et al., 2006).

The effect of PEG 400 concentration on ranitidine efflux ratio was parabolic with a maximum effect of complete inhibition of efflux at 1% (v/v). The reason for the reduced effectiveness at concentrations greater than 1% (v/v) PEG 400 is unclear, but interestingly the concentration effect of PEG 400 on ranitidine transport *in vitro* was similar to the concentration-dependency observed previously for the enhancement of bioavailability of ranitidine *in vivo* (Ashiru et al., 2008). At higher concentrations of PEG 400 there may be competition for the paracellular route between ranitidine and PEG itself. The paracellular route has been reported to contribute 60% of ranitidine flux under certain conditions (Bourdet et al., 2006) and the existence of a saturable paracellular transport pathway has been postulated. PEG has been used as a marker of paracellular permeability (Kim, 1996; Watson et al., 2001; Linnankoski et al., 2010), albeit some reports question its suitability as a paracellular marker as it exhibits higher permeability compared to other markers of comparable molecular weight (Artursson et al., 1993; Iqbal et al., 1993). In this study we did not monitor PEG transport.

The mechanism by which PEG reduces Pgp ATPase activity may involve blocking the binding site or direct interaction of PEG with allosteric sites in the P-gp pump, which have been shown to be present (Dey et al., 1997; Maki et al., 2003). PEG 300 has been reported to inhibit P-gp by alteration of the polar head group regions thus altering membrane fluidity and affecting P-gp activity (Hugger et al., 2002a). Altered membrane fluidity as a result of osmotically-driven water transfer across the mucosa was also suggested to explain the concentration dependent reduction in digoxin efflux in the rat intestine by PEG 400 (Johnson et al., 2002).

5. Conclusion

These *in vitro* data correspond to results from the *in vivo* study in showing that PEG 400 at lower doses enhances the transport of ranitidine. The observation that both ranitidine and PEG interact with P-gp and the efflux of ranitidine in Caco-2 cells is inhibited by PEG 400 suggest that transporter inhibition may be the absorption-enhancing mechanism. Although the mechanism of action for the unusual PEG 400 concentration effect on ranitidine transport was not elucidated conclusively, the effect of PEG on drug transport at concentrations relevant for drug formulation was demonstrated.

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