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Investigation of regional mechanisms responsible for poor oral absorption in man of a modified release preparation of the α-adrenoreceptor antagonist, UK-338,003: the rational use of *ex vivo* intestine to predict *in vivo* absorption.

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Abstract

Modified release (MR) formulations are used to enhance the safety and compliance of existing drugs by improving their pharmacokinetics. Predicting the likely success of MR formulations is often difficult prior to clinical studies. A systematic *in vitro* approach using mouse and human tissues was adopted to rationalise the *in vivo* pharmacokinetics of 9hr and 15hr MR formulations of an α-adrenoreceptor antagonist, UK-338,003. Immediate release UK-338,003 was well absorbed in man consistent with moderate Caco-2 permeability. In contrast, 9hr and 15hr release formulations showed marked reductions in C_{max} (47.1% and 68.9%) and AUC_{0-72} (32.6% and 54.0%). Colonic intubation resulted in an 81.3% and 73.8% reduction in C_{max} and AUC_{0-72}. Mechanistic studies in isolated mouse tissues showed colonic UK-338,003 permeability (Papp $<0.5 \times 10^{-6}$ cm/sec) was at least 40 times lower than ileum with marked asymmetry. UK-338,003 was found to be a substrate for PGP with a weaker interaction for MRP-type transporters in mouse intestine. PGP inhibition dramatically increased colonic UK-338,003 permeability to the levels observed in ileum. Low UK-338,003 A-B permeability was also observed in *ex vivo* human distal intestine but both the asymmetry and increase in permeability following PGP inhibition were significantly lower. In conclusion, the poor absorption of MR UK-338,003 in man can be explained by a combination of PGP-dependent efflux and low intrinsic permeability in the lower bowel. Regional permeability studies in *ex vivo* tissues employed during drug development can highlight absorption problems in the distal bowel and assess the feasibility of developing successful MR formulations.

Introduction

Oral modified release (MR) formulation can improve a drug's pharmacokinetic and pharmacodynamic properties, and thus lead to both improved patient compliance and safety (Conley et al '06). However, predicting the success of MR formulations for a given drug has proved difficult, particularly for drugs with moderate intestinal permeability and often the success or failure may only become apparent during clinical trials. One of the problems associated with prediction of absorption from a typical 8-24hr MR dosage form is that, unlike immediate release (IR), the drug is released throughout the GI tract with a significant proportion delivered to the distal intestine. As a result, regional differences and, in particular, the efficiency of colonic absorption will be a critical consideration. For a variety of reasons, drug absorption in the colon appears to be more challenging than for the proximal bowel. In physical terms, it is a viscous, low mixing environment with a lower surface area for absorption compared to the proximal bowel. Both paracellular and transcellular permeability appear lower in the colon, although this is highly compound-specific with some incidences of transcellular compounds showing higher permeability in colon than the upper small intestine at least in animal models (Ungell et al., 1998). Using *ex vivo* tissues from different intestinal regions in the mdr1a(-/-) (PGP-KO) mouse, Stephens and co-workers demonstrated that PGP expression increased from proximal to distal bowel, accounting for the low digoxin permeability in the colon (Stephens et al., 2002). There is also direct evidence that active efflux can limit the absorption of drugs in the colon of rats (Abushammala et al., 2006) however, data showing that this occurs in man is equivocal (Makhey et al., 1998). The lower bowel is also known to express several other drug efflux transporters (Zimmerman et al., 2005) although their potential impact on the performance of MR formulations has not been considered. However, there is a significant potential for xenobiotic transporters to affect the absorption of MR formulations because they are delivered to the lower intestine where there is significant transporter expression. In addition the drug is released more slowly and so its

concentration at the site of absorption is lower than for IR formulations thus reducing the potential to saturate transporters.

More effective *in vitro* prediction of the feasibility of developing successful MR formulations would allow MR to be considered at a much earlier stage in exploratory drug development, which might help to reduce the number of failures (Thombre, 2005, Eichenbaum et al., 2006). Basic permeability screens such as Caco-2 may be used to provide an initial assessment of the suitability of a drug candidate for MR development (Thombre, 2005) but there remains some uncertainty about their validity as a model of colonic absorption. Indeed, almost all the extensive literature on the suitability of Caco-2 for prediction of oral absorption in man deal with IR oral administration (for a recent review see Shah et al., 2006), exemplified by Lennernas, (1997) who demonstrated a good correlation between Caco-2 and human jejunal absorption. However, one study has proposed Caco-2 as a reasonable model of colonic permeability (Rubas et al., 1995). In terms of specifically predicting the role of transporters in limiting drug absorption in different regions of the intestine the relevance of Caco-2 is unclear. For example, out of three recent studies comparing patterns of transporter expression in Caco-2 with those in different regions of the intestinal tract, two conclude that Caco-2 resemble the small intestine (Englund et al., 2006, Seithel et al., 2006) while a third suggested that Caco-2 is a more appropriate model of colonic transporters (Calcagno et al., 2006). Such uncertainties highlight the potential limitations of Caco-2 and similar cell models for predicting successful MR candidates and suggest the need for additional *in vitro* models that in terms of inherent permeability and transporter profile may more closely mimic the distal gut *in vivo*. Here we describe the use of alternative *in vitro* models applied to UK-338,003, an orally active α_1-adrenoceptor antagonist which are a class of drugs often developed as modified release (MR) formulations to improve cardiovascular safety (van Kerrebroeck, 2001; Kirby et al., 2005).

The aim of the present study was two-fold; firstly to investigate the *in vivo* performance of MR dosage forms of the development compound UK-338,003, reporting human pharmacokinetic data which compares absorption of the compound from the upper and lower GI tract. Secondly, to investigate the use of *ex vivo* human and mouse intestine, including tissues from PGP-KO mice to determine the relative impact of inherent permeability and interaction with efflux transporters on the absorption of UK-338,003 in different regions of the GI tract providing a rationalisation of the *in vivo* data based on these mechanistic observations.

Materials and Methods

Materials

UK 338,003 (Figure 1) has a molecular weight of 517Da and was synthesised at Pfizer Global Research Ltd. (Sandwich, Kent, UK), GF 120918 (9,10-dihydro-5-methoxy-9-oxo-N-[4-[2-(1,2,3,4-tetrahydro-6,7dimethoxy-2-isoquilonyl)ethyl-4-phenyl]-4-acridinecarboxamide) was a gift from GlaxoSmithKline, Stevenage, UK. [^{3}H]-theophylline and [^{14}C]-mannitol were from NEN Life Science Products (Hounslow, UK). MK571 (*(E)*-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid) was from Calbiochem (CA. USA). All other compounds were obtained from either BDH (Dorset, UK) or Sigma-Aldrich Chemical Co. Ltd. (Poole, UK). Wild-type FVB [mdr1a (+/+)] mice were obtained from local barrier maintained stock. Mdr1a (-/-) (PGP-KO) mice that had been back-crossed for at least 7 generations onto the FVB background were obtained from Taconic Farms (Germantown, NY).

Human pharmacokinetic studies

Two open randomised studies were performed to investigate the pharmacokinetics of UK-338,003 in healthy male subjects. The studies were conducted according to the Association of the British Pharmaceutical Industry guidelines, to the revised Declaration of Helsinki and were approved by a local ethics review committee. Study #1 compared the PK of an oral solution with that of two MR formulations after an overnight fast, using a randomised crossover design. Written informed consent was obtained from 18 healthy male subjects aged 21 to 44 years and weighing between 62 to 100kg, with a body mass index of between 18 and 28 using Quetelet's index. Two subjects withdrew their consent for personal reasons, so 16 subjects received all 3 treatments. The oral solution consisted of 1mg of

UK-338,003 dissolved in 50ml of water with 0.1 M hydrochloric acid. The modified release formulations contained 3mg UK-338,003 in a modified release tablet with either a 9h or 15h release profile.

Study #2 compared oral administration with colonic intubation, using an open randomised design in 9 healthy male subjects. The oral solution formulation utilised in Study #1 was administered and compared with colonic administration of UK-338,003 (1mg) dissolved in 50ml of 0.9% NaCl containing 0.01M hydrochloric acid (infused at a flow rate of 10ml/min via an enteric tube with its tip placed in the terminal ileum). Written informed consent was obtained from 9 healthy male subjects aged 19 to 42 years and weighing between 67 to 94kg, with a body mass index of between 18 and 28 using Quetelet's index. A triple lumen tube for drug administration was supplied by Dentsleeve Pty Ltd (external diameter 3.5mm), with a latex balloon at its tip. The tube was positioned in the duodenum using a flexible fibreoptic paediatric endoscope, approximately 24h prior to administration of UK-338,003. The balloon was inflated with 10ml saline and a continuous infusion of saline (10ml/h) maintained the patency of the tube. On two occasions, the positioning of the tube was visualised by installation of contrast material (Omnipaque®, 2-3ml), via one of the infusion channels. Once the tube was correctly positioned in the terminal ileum, the balloon was deflated to avoid further progression.

In both studies, blood samples (5ml) were collected in heparinised tubes before dosing and at time periods up to 72 h post-dose. Samples were centrifuged (approximately 1500*g* at 4°C for 10 min) within 60 min of sample collection and the plasma removed and stored at -20°C. A minimum of seven days washout was applied between dosing periods.

Animal Tissues

Intestinal tissues were removed from non-fasting male PGP-KO mice (10-16 wks, 20-36g) or age matched FVB mice killed by cervical dislocation. The ileum (the segment stretching from 1 cm to 13 cm proximal to the ileo-cecal junction) or colon was immediately removed and flushed with ice cold, bicarbonate-buffered Ringer solution containing (in $mmol.l^{-1}$) Na^+ 146, K^+ 4.2, Ca^{++} 1.2, Mg^{2+} 1.2, Cl^- 126, HCO_3^- 27, HPO_4^- 1.4 and D-glucose 10 mM (MBR), which had been equilibrated to pH 7.4 by bubbling with 5% CO_2 / 95% O_2. Tissues were mounted intact in modified Ussing chambers (0.52 cm^2 cross-sectional area) without removal of the serosal muscle layer as described previously (Stephen et al., 2002a & 2002b). Mounting was completed within 30 min of removal from the animal. All procedures involving animals conformed to current UK Home Office regulations.

Human Tissues

Human distal colon or terminal ileum was obtained with informed consent, following both local ethical committee approval and the Declaration of Helsinki guidelines, from patients undergoing surgery for benign or malignant tumors. Immediately after resection, macroscopically normal tissues, at least 5 cm from the tumor margin, were used for drug permeability studies. After removal from the abdomen, tissue specimens were immediately immersed in ice cold oxygenated human colonic bicarbonate-buffered ringer solution (HCR) (in $mmol.l^{-1}$) Na^+ 135, K^+ 4.0, Ca^{2+} 1.2, Mg^{2+} 1.2, Cl^- 105, SO_4^- 5, HCO_3^- 26, HPO_4^- 1.2, $H_2PO_4^-$ 0.2 and D-glucose 10 mM which had been equilibrated to pH 7.4 by bubbling with 5% CO_2 / 95% O_2. Following removal of the underlying muscle layers by blunt dissection, mucosal pieces (1 cm^2) were mounted in Ussing chambers within 40 minutes of the tissue being removed from the patient.

Caco-2 studies

Caco-2 cells (American Type Culture Collection, Manassas, Virginia USA) were seeded in 24-well Falcon multiwell plates (polyethylene terephthalate membranes, pore size 1.0µM) at 4.0×10^4 cells/well. The cells were grown in Minimum Essential Medium containing 20% fetal bovine serum, 1% non-essential amino acids, 2mM L-glutamine and 1mM sodium pyruvate. The culture medium was replaced three times every week and the cells were maintained at 37 ºC, with 5% CO_2 and 90% relative humidity. Permeability studies were conducted when the monolayers were 24 days old and passage 36-43.

UK-338003 was prepared at 10µM in Hank's Balanced Salt Solution containing 20mM HEPES to maintain the pH at 7.4. Transport studies were performed in triplicate in both the apical to basolateral (A-B) and basolateral to apical (B-A) directions. After 2 hours incubation at 37 ºC, samples were removed and concentrations determined by LC-mass spectrometry and apparent permeability (P_{app}) values calculated. Monolayer integrity was determined by examination of the flux of [^{14}C] mannitol with a cut-off of 1.5×10^{-6} cm/sec.

Permeability studies

Drug transport across intestinal tissues was measured as described previously (Stephens et al., 2002a & 2002b). Intestinal mucosa was bathed on the mucosal (apical) and serosal (basolateral) surfaces with 5 ml MBR (mouse tissues) or HCR (human tissues), pH 7.4 at 37^{o}C. Spontaneous tissue open-circuit potential difference (P.D.), short-circuit current (I_{SC}) and transepithelial electrical resistance (R_T) were monitored periodically throughout the experiment, otherwise tissues were maintained under open circuit conditions. A 20 min equilibration period allowed for stabilisation of electrical parameters. Human and mouse tissues were excluded in cases where PD values fell below 2 mV at any point during the experiment. Asymmetric permeability of 20µM UK-338,003 was measured in either the apical to basolateral (A-B) or basolateral to apical direction (B-A) in different tissue segments. GF120918 (20µM) was used as a PGP inhibitor and MK571 (20µM) as an

inhibitor of MRP-family transporters where indicated. For permeability studies in the mouse, 1 ml samples were removed from the receiver chamber at t=0 and after each of six 30 min flux periods; in each case this volume was replaced with fresh MBR. In human tissues, drug permeability was measured over two 60 min flux periods. The mean rate of drug appearance over the time course of the experiment was used to calculate the P_{app} value in each case. Some human studies were performed with the addition of the paracellular permeability marker Mannitol, as a further check (in addition to monitoring of electrical parameters) of tissue integrity. In these cases, ^{14}C mannitol (~3 kBq/ml) was added to the donor chamber with unlabeled mannitol (100μM) added to both donor and receiver chambers. Permeability of the high permeability transcellular marker theophylline, was investigated in human and mouse tissue by addition of 20 μM spiked with ~6 kBq/ml of ^{3}H-theophylline. For both mannitol and theophylline studies additional 1ml samples were removed from the receiver chamber at each time point for liquid scintillation counting and replaced with fresh MBR or HCR as appropriate. Where transport inhibitors were used, inhibitors were added to donor and receiver chambers, at the indicated concentration, 60 min after the start of the first flux period.

In all experiments, 100 μl aliquots were taken from the donor chamber at the beginning of the first period and at the end of the final flux period experiment to monitor any changes in donor drug concentrations during the experiment and ensure mass balance. Drugs were added as stock solutions in DMSO giving a final solvent concentration of 0.01-0.3 %.

Values of unidirectional transepithelial apparent permeability (P_{app}) in cm/sec were calculated by :

$$P_{app} = \frac{(dQ/dt)}{C.A} \quad (1)$$

where dQ/dt is the rate of appearance of compound in the receiver chamber, C is the substrate concentration in the donor chamber and A is the cross-sectional area of the tissue (0.52 cm^2 in mouse and 1 cm^2 in human tissues). Values of P_{app} were averaged for control flux periods (before the addition of the inhibitor) and for inhibitor flux periods (after addition of the inhibitor) to yield baseline and post-inhibition values. For analysis, the resulting P_{app} data from several experiments were then pooled (see "statistical analysis" below).

P_{app} values shown are either unidirectional: apical to basolateral (A-B) or basolateral to apical (B-A) or net P_{app} ($P_{app\ B\text{-}A} - P_{app\ A\text{-}B}$). Where values for net P_{app} are shown, positive values represent net secretion in the B-A direction (ie. B-A > A–B) while negative values represent net absorption in the A-B direction (ie. B-A < A-B).

Analysis of UK-338,003

An API 2000 LC/MS/MS (Applies Biosystems, CA, USA) fitted with a turbospray ion source was used for the analysis of UK-338,003, with an HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler and column oven. The compound was retained on a C_{18} analytical column. The analysis was performed using a gradient elution profile as shown in Table 1 at a flow rate of 1.0ml/min and an injection volume of 100µl. Under these conditions UK-338,003 has a retention time of approximately 5.8 mins.

Electrospray ionization was performed in the positive ion mode at unit resolution. Nitrogen was used as the auxilliary, nebulizer, curtain and CAD gas, and was set at 35, 60, 35 and 2 respectively. The source temperature was kept at 400°C. The pause time was 5ms and the dwell time 500ms. The detection and quantitation of UK-338,003 was performed in the multiple reaction monitoring (MRM) mode. The ion transition monitored was *m/z* 507 → 130. The transition ion was selected on the basis of the predominant fragmentation pathway.

The operations of mass spectrometer, LC system, data acquisition and data analysis were carried out using Analyst v1.4 (Applied Biosystems).

Kinetic and Statistical Analysis

Plasma pharmacokinetic parameters for UK-338003 were calculated using standard non-compartmental techniques (WinNonlin version 4, Pharsight Corporation, Mountain View, CA). Kinetic and statistical analyses for the *in vitro* experiments were carried out using PRISM 2.01 (GraphPad Software Inc., San Diego, CA).

Kinetic values (half-maximal effective concentration (EC_{50}) and maximal net flux rate (J_{max}) for substrate dose-effect relationships were calculated by non-linear regression with the method of least squares, fitting for a logistic sigmoid using the Hill equation:

$$J = \frac{J_{min} + (J_{max} - J_{min})}{1 + (C/EC_{50})^{-P})} \qquad (2)$$

where J is net flux, C is donor concentration, P is a constant (the Hill slope), and EC_{50} is the concentration at which half-maximal flux was achieved. The error values associated with the kinetic parameters are asymptotic standard errors returned by the regression routine and are a measure of the certainty of the best fit value. To take account of this, differences in kinetic parameters returned by the regression routine were compared using unpaired Student's *t* test but with a higher threshold for significance of 0.01. Statistical comparisons of all other data (i.e., effects of inhibitors on substrate fluxes) were determined using unpaired Student's *t* test with a significance level of 0.05.

Results

UK-338,003 exhibits poor absorption from MR preparations and following colonic administration of a solution in man: The mean plasma concentration profiles from a randomised crossover study comparing the pharmacokinetics of a 1mg solution of UK-338,003 with 3mg 9 hr and 15 hr modified release formulation in fasted males are shown in Fig 2 and the main pharmacokinetic parameters summarised in Table 2. Compared to the solution, there was a significant reduction in C_{max} and AUC for both 9hr and 15 hr formulations after dose normalisation (Table 2) the greatest reduction was observed in the 15 hr formulation (AUC was 46% and C_{max} 31.1% of that observed for IR). The T_{max} occurred much later for the MR formulations (4.4, 4.7 hr) compared to the oral solution (1.7 hr) with no difference between the 9hr and 15hr preparations. These data would suggest that UK-338,003 is well absorbed in the upper GI tract but that its absorption is significantly restricted as the drug becomes exposed to the lower bowel, particularly the colon. To explore this further, a second crossover study investigated the pharmacokinetics of a 1mg solution of UK-338,003 delivered directly to the distal bowel compared to oral administration (Fig 3). Absorption was markedly impaired following colonic administration with C_{max} and AUC_t being 18.7% and 26.2% compared to oral administration (Table 3). The observed increase in T_{max} from 1.2hr to 3.1 hr is consistent with a slower rate of absorption from the colon. Colonic administration was also associated with a greater inter-subject variability (coefficient of variation for AUC_t. was 84.5% following colonic administration compared to 31.4% after oral dosing). These observations show that UK-338,003, despite being well absorbed orally, was not a suitable candidate for administration as a once a day MR dosage form due primarily to low colonic absorption in man. Further *in vitro* studies were undertaken to understand, at a mechanistic level the reasons for the low absorption of UK-338,003 and to

determine the usefulness of *in vitro* models for rationalising and predicting its MR pharmacokinetics.

UK-338,003 exhibits marked regional differences in permeability in mouse intestine *in vitro* and is a substrate for efflux transporters: Analysis of UK-338,003 permeability at a concentration of 20µM in gut tissues isolated from FVB mice and mounted in Ussing chambers show a moderate permeability in the A – B direction across proximal ileum (Papp 4.2 ±1.3 x 10^{-6} cm/sec) but minimal permeability (<0.5 x 10^{-6} cm/sec ie. below the limit of detection) across colonic tissues (Fig 4). In both regions, there was a marked asymmetry in UK-338,003 permeability with B-A:A-B ratios of 8.5 and >70 in ileum and colon respectively indicating that the drug is a substrate for efflux transporters. The transport inhibitor, GF120918 (20µM), which is a potent inhibitor of PGP at this concentration, reduced but did not abolish the net efflux and increased the A-B permeability of UK-338,003 in both regions although to a different extent. In ileum, GF120918 induced a ~2.7 fold increase in A-B permeability (to 11.3 ± 2.5 x 10^{-6} cm/sec) while, in mouse colon, the inhibitor dramatically increased A-B permeability by at least 14 fold to a level similar to that observed in the ileum (7.3 ± 1.4 x 10^{-6} cm/sec). These data suggest that drug efflux, most likely mediated by PGP, is the primary reason for the low permeability of UK-338,003 observed particularly in colon. However, the residual efflux ratio of 1.8 and 2.9 in ileum and colon respectively, which were consistently present after incubation with 20µM-GF120918, raise the possibility that other non-PGP transporters may also influence UK-388,003 permeability. In Caco-2 monolayers, values for UK-338,003 (10µM) bi-directional permeability appeared more consistent with mouse ileum than colon. P_{app} in the A-B and B-A directions were 7.0±0.2 cm/sec and 33 ± 1.9 giving a B-A:A-B ratio of 4.8; n=3.

Both active efflux and lower intrinsic permeability limit UK-338,003 absorption across human distal intestine *in vitro*: The permeability characteristics of UK-338,003 across *ex vivo* tissues from human distal intestine are shown in Figure 5. Due to the relatively small number of tissues available, combined data from terminal ileum and colon are shown. The A-B permeability of UK-338,003 in these tissues was low with an A-B P_{app} of 0.38±0.16 x 10^{-6} cm/sec and there was clear evidence of drug efflux with a B-A:A-B ratio of 15.3. It is notable that B-A permeability in the human tissues was ~80% lower than observed in the mouse colon suggesting that UK-338,003 may have a lower intrinsic permeability in man. This was borne out by the effects of inhibiting transport with 20μM-GF120918, which significantly increased A-B P_{app} in human tissue (to 2.8±1.0 x 10^{-6} cm/sec) but to a lesser extent than in mouse colon where UK-338,003 permeability increased to 7.3 ± 1.4 x 10^{-6} cm/sec on addition of GF120918 (Fig 5). In line with mouse tissues, there was a residual efflux ratio of ~3.0 after addition of GF120918. These data imply that both active transport processes and low intrinsic permeability to UK-338,003 are likely factors in the poor absorption of UK-338,003 in human distal gut.

Multiple transporters limit intestinal permeability of UK-338,003 in mouse intestine: As reported above there was a residual B-A:A-B ratio for UK-338,003 in both mouse and human tissues, in the presence of the PGP inhibitor GF120918, raising the question as to whether other transporters are involved in limiting UK-338,003 permeability across the distal gut. To investigate this further, UK-338,003 permeability was analysed in ileum isolated from mdr1a(-/-) (PGP-KO) mice that express no functional PGP in the bowel (Fig 6) (Schinkel et al., 1994). The A-B permeability of UK-338,003 in ileum from PGP-KO mice was almost 2-fold higher (7.1±1.5 x 10^{-6} cm/sec) than ileum from wild-type controls (Figs 4 and 6) consistent with a role for PGP as described above. Interestingly, however, despite the lack of

functional PGP, a significant efflux of UK-338,003 (efflux ratio ~3.5) remained in PGP-KO tissues. This was abolished by addition of MK571 (20μM), a selective inhibitor of MRP-type transporters (Gekeler et al., 1995) resulting in a further increase in UK-338,003 A-B permeability to 12.6±1.5 x 10^{-6} cm/sec. These data are consistent with UK-338,003 permeability being limited at least in part by interaction with multiple efflux transporters. The use of ileum from PGP-KO mice allowed a detailed study of the kinetics with which UK-338,003 interacts with PGP and MRP in the mouse gut to be undertaken. Figure 7 shows the net efflux flux of UK-338,003 versus concentration in the ileum isolated from wild-type or PGP-KO mice. The efflux of the compound in wild-type mice, which is dominated by PGP showed an EC_{50} of 31.5 ± 3.8 μM with a J_{max} of 10.4 ± 0.8 nmol.h^{-1}.cm^{-2}. A similar analysis of the residual efflux in PGP-KO mice, likely to be due to an MRP transporter, showed a markedly higher EC_{50} (97.3 ± 13.3 μM) and lower J_{max} (5.9 ± 0.6 nmol.h^{-1}.cm^{-2}) implying that the MRP transporter saturates at much higher concentration of UK-338,003 than does PGP and has a ~50% lower transport capacity.

Theophylline permeability is high in all regions of wild type mouse and human tissues: As a further test of the validity of *ex vivo* human and mouse intestine for predicting the likely performance of candidates for MR, the permeability of theophylline, a compound which is well absorbed as a MR formulation in man (Gonzalez and Straughan, 1994) was tested. This phosphodiesterase inhibitor, is a high permeability, transcellular compound whose absorption is not limited by PGP or other efflux transporters (Saitoh and Aungst, 1995). Theophylline exhibited high permeability across mouse ileum and colon with no difference between the two regions (Papp 30 ± 10 and 28 ± 2.9 x 10^{-6} cm/sec, for ileum and colon respectively). Theophylline also exhibited significant permeability across human intestine although values (14.8 ± 1.8 x 10^{-6} cm/sec) were approximately 50% lower than mouse. Theophylline A-B

permeability was markedly higher than UK-338,003 in both species even after inhibition of efflux of UK-338,003 with GF120918 (Table 4). These data indicate that *ex vivo* preparations of human and mouse intestine show discrimination between compounds that have had differing success as MR formulations.

Discussion

Prediction of the likely oral pharmacokinetics of drugs in MR formulation is more difficult than IR given our poor understanding of the factors determining absorption in the distal bowel. We have employed a systematic *in vitro* approach, based on the use of human and mouse tissues, to investigate the impact of colonic permeability and efflux transporters on the *in vivo* performance of a MR formulation of the α-adrenoreceptor antagonist UK-338,003 in man. *In vivo* data shows that UK-338,003, although well absorbed orally from IR preparations, exhibits poor absorption when administered as an MR formulation with a 9 or 15 hour release profile. T_{max} values for 9hr and 15hr formulations of 4.4hr and 4.7hr respectively in fasted individuals are similar to the small intestinal transit time (Davis et al., 1986; Wilding et al., 2001). This suggests that, even with the 15hr matrix, the small intestine is responsible for a significant proportion of UK-338,003 absorption, despite the fact that it will be present in the colon for long periods. Intubation studies confirmed the poor colonic absorption of UK-338,003 with an 81% and 71% reduction in C_{max} and AUC_{0-72}, compared to oral IR administration. This appeared to result from poor colonic permeability as there was no evidence of significant solubility issues at the concentrations used. On a mechanistic level, there are likely to be two major influences on UK-338,003 colonic absorption, the intrinsic permeability of the colonic membrane and interaction with drug efflux transporters or a combination of these.

In normal mouse tissues, UK-338,003 shows considerable differences in regional permeability with colonic A-B permeability being at least 10 fold lower than proximal ileum but with both tissues showing marked asymmetry indicative of active efflux. GF120918 dramatically increased colonic permeability of UK-338,003 suggesting that its absorption in the colon is severely restricted by PGP, although this compound can also inhibit the transporter BCRP (Tan et al., 2000). GF120918 also raised the A-B permeability of UK-

338,003 in the mouse ileum to a level similar to that observed in the colon, however the greater fold increase in A-B flux elicited by GF120918 in the colon appears consistent with previous evidence that the large bowel has a higher functional level of PGP (Stephens et al, 2002a). Interestingly, even at the relatively high concentration of 20μM, GF-120918 did not completely abolish net secretion of UK-338,003 in either colon or ileum. This suggests that UK-338,003 may be interacting with other intestinal transporters. PGP-KO mouse tissues showed definitively that while the majority of UK-338,003 efflux is accounted for by PGP, a significant non-PGP component is present that was abolished by MK571, a selective inhibitor of MRP-type transporters. A similar approach has been used to identify etoposide as a mixed PGP/MRP substrate (Stephens et al., 2002b). Comparative studies of the dose-dependence of UK-338,003 secretion in normal and PGP-KO ileum indicate that the kinetics of UK-338,003 interaction with PGP and MRP are quite different. The interaction with PGP occurs at lower concentrations (EC_{50} 31.5 ± 3.8μM) than for MRP (EC_{50} 97.3 ± 13.3μM) with PGP also having approximately twice the transport capacity for UK-338,003. These data are consistent with PGP being the primary transporter responsible for UK-338,003 secretion but suggest that as this transporter becomes saturated by higher drug concentrations, an MRP-type transporter takes over as a secondary barrier to UK-338,003 absorption. Evidence suggests that the expression pattern of intestinal MRP transporters is complimentary to that of PGP (Zimmermann et al., 2005). The present data is consistent with these transporters acting in concert to limit the absorption of UK-338,003 in the distal gut, resulting in an adverse *in vivo* kinetic profile for MR formulations.

Although access to tissues from the human terminal ileum and colon was limited, in general terms these show similar absorption characteristics for UK-338,003 to mouse intestine with low A-B permeability and a high efflux ratio (15.3), indicative of active transport. However, even though PGP inhibition by GF120918 markedly increased UK-338,003 permeability in

human tissues by over 7-fold, it remained significantly lower than that observed in mouse colon after GF120918 addition. A likely explanation is that UK-338,003 permeability in human distal intestine is limited by a combination of active PGP-mediated efflux and lower intrinsic permeability. In contrast, mouse has higher intrinsic permeability for UK-338,003 with active efflux being the major factor limiting absorption. The much lower B-A permeability of UK-338,003 in human compared to mouse is consistent with this interpretation. Additional efflux transporters, such as MRP, may be involved in limiting human UK-338,003 permeability and further studies will be needed to address this. However, the relatively low B-A permeability of UK-338,003 in human tissues suggests this is unlikely to be a major factor. Our conclusion is that while PGP inhibition would likely increase UK-388,033 absorption in human colon, the effect may be relatively small when compared to the mouse, due primarily to the overriding effect of a lower intrinsic UK-338,003 permeability in human. Comparative studies of etoposide efflux in rat and human intestine by Makhey and co-workers lend some support to this view. The B-A etoposide permeability was reported as ~5 times higher than the A-B permeability in rat colon, while in the human colon, there was no significant difference between the A-B and B-A directions. Despite the apparent lack of efflux in human colon, etoposide permeability remained low (Makhey et al., 1998).

Where they occur, interactions with colonic transporters may have adverse effects on the PK of MR-formulation drugs including non-linear absorption (Tubic et al., 2006), food interactions (Wagner et al., 2001), co-administered drugs (Lin and Yamazaki, 2003) and other xenobiotics (Abu-Qare et al., 2003). Interactions with colonic transporters may also increase inter-individual variation in PK due to different levels of transporter expression and the impact of transporter polymorphisms (Dey, 2006). In this respect, it is interesting that intubation of UK-338,003 into the distal bowel resulted in a greater inter-individual variation in PK compared to oral administration in the same cohort.

The present study raises interesting questions about how *in vitro* models can be used to assess absorption and the influence of transporters in the lower bowel, and thereby predict a compound's likely suitability as a MR formulation. Caco-2 monolayers are widely used to screen the absorption potential of new drugs and their possible interaction with PGP and other transporters (Bohets et al., 2001). The data for UK-338,003 illustrates problems with the direct extrapolation of data from Caco-2 to different regions of the human GI tract. UK-338,003 exhibited moderate permeability and a modest efflux ratio in Caco-2 monolayers consistent with the favourable oral PK observed in man. However, UK-338,003 permeability in Caco-2 was significantly higher than observed in human or mouse colonic tissues *in vitro*, which were consistent with the low absorption seen on colonic intubation003 and the poor PK properties of the MR formulation in man. In the case of this compound therefore, *ex vivo* colon from both human and mouse appear to be a useful predictor of the drug's performance in the lower bowel. However, while the overall UK-338,003 profile of low permeability and significant interaction with transporters is similar in human and mouse colon, there appear to be important mechanistic differences. In mouse colon, the low permeability of UK-338,003 is due almost entirely to its interaction with transporters. However, while the transporter interaction in human colon appears similar to mouse, the much lower intrinsic membrane permeability of UK-338,003 in human colon is at least as important in determining the level of absorption. The differences between the *in vitro* models are much less apparent with high permeability, non-effluxed compounds such as theophylline which would have been confirmed as a suitable candidate for MR formulation (Gonzalez and Straughan, 1994) using data from either Caco-2, or *ex vivo* mouse or human tissues. Lower colonic permeability in man compared to rats has been noted previously (Masaoka et al., 2006) and may typify a more generalised permeability difference between human and rodents (Kim et al., 2006). This may have a physiological basis in that the longer colonic transit time in man (~24 hours

compared to ~1 hour in mouse) provides greater exposure to potentially toxic substances, and thus a significantly lower intrinsic permeability coupled to high levels of efflux transporters is likely to be beneficial.

Other factors such as gut wall metabolism could theoretically contribute to the poor absorption of UK-388,003 but this seems unlikely in this case. UK-388,003 is metabolised almost exclusively by CYP3A in man (Betts *et al.*, 2007), which is localised primarily in the upper GI tract with much lower levels in distal gut (McKinnon *et al.*, 1994). In rodents, UK-388,003 is metabolised exclusively by CYP2C which has overlapping substrate specificity with CYP3A4 (Betts *et al.*, 2007). Given the evidence for good permeability in the upper intestine it seems unlikely that gut wall metabolism will be an important factor in reducing UK-388,003 permeability in the distal gut.

In conclusion, we have shown, using the example of an α-adrenoreceptor antagonist, UK-338,003, that a complementary approach in which initial assessment of absorption potential in Caco-2 or similar *in vitro* model is combined with permeability profiling in *ex vivo* human and rodent tissues can be more effective in predicting the suitability of compounds for MR development and rationalise PK findings in man. *Ex vivo* tissues from normal and PGP-KO mice demonstrated the interaction between UK-338,003 and colonic transporters and their role in limiting UK-338,003 absorption in this region. Complimentary studies in *ex vivo* human tissues confirmed poor UK-338,003 permeability in human colon resulting from a combination of low inherent permeability and active efflux. Such approaches could be employed relatively early in the drug development process to highlight potential absorption problems in the distal bowel and assess the feasibility of developing a successful MR formulation. This illustrates the usefulness of *ex vivo* human tissues as a tool to elucidate the regional and species specific factors that determine drug permeability along the gut.

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FIGURE LEGENDS

Figure 1: Chemical structure of 4-Amino-6,7-dimethoxy-2-(5-methanesulfonamido-1,2,3,4 tetrahydroisoquinol-2-yl)-5-(2-pyridyl)quinazoline (UK-338,003)

Figure 2: Mean plasma concentration of UK-338,003 following oral administration of a 1mg solution or 3 mg 9 hour and 15 hr controlled release (MR) matrix tablets in man. The plasma concentration is corrected to a 1mg dose and is mean data for the same cohort of 16-18 individuals for each dosage form.

Figure 3: Mean plasma concentration of UK-338,003 after oral or colonic administration in man. A 1mg solution of UK-388,003 was administered either orally or via an enteric tube to the terminal ileum. Mean data is shown for the same 9 individuals in each group.

Figure 4: *In vitro* permeability of UK-338,003 across wild-type FVB mouse ileum and colon. A-B and B-A permeability of 20µM UK-338,003 across mouse tissues mounted in Ussing chambers as described in Materials and Methods. P_{app} is shown in the absence (control) or presence of 20µM GF120918. *p,0.05 B-A P_{app} significantly greater than A-B P_{app} either for control or after addition of GF120918; $^{\#}$ $p< 0.05$ A-B P_{app} with GF120918 compared to A-B P_{app} in control. The A-B flux across the colon was below the limit of detection for UK-338,003 ($<0.5 \times 10^{-6}$ cm/sec). Data is shown as mean ± S.E.M for n = 3-6 experiments in each group.

Figure 5: *In vitro* permeability of UK-338,003 across human distal intestine. A-B and B-A P_{app} of 20µM UK-338,003 across human distal intestine (a combined group of terminal ileum

and colonic tissues) measured either in the absence (control) or in the presence of 20μM GF120918. *p<0.05 Control B-A P_{app} significantly greater than A-B P_{app}; # p<0.05 A-B P_{app} in presence of GF120918 significantly greater than control A-B P_{app}. The dotted line indicates the limit of detection for UK-338,003 (<0.5 x 10^{-6} cm/sec). Data is mean ± SEM for n=6 pieces of intestine from 4 individuals.

Figure 6: *In vitro* permeability of UK-338,003 across PGP-KO mouse ileum. Data shows A-B P_{app} and B-A P_{app} of 20μM UK-338,003 in mdr1a(-/-) mouse ileum either in the absence or presence of 20μM MK571. * p<0.05 B-A P_{app} significantly greater than A-B P_{app}. Data is mean ± SEM for n=4 in each group.

Figure 7: Concentration dependence of net efflux of UK-338,003 in ileum from wild type FVB or mdr1a(-/-) (PGP-KO) mice. Values show mean ± SEM n=3–5 observations at each concentration. Calculated EC_{50} and J_{max} values are 31.5 ± 3.8 mM and 10.4 ± 0.8 $nmol.h^{-1}.cm^{-2}$ respectively for wild-type control tissues and 97.3 ± 13.3 mM and 5.9 ± 0.6 $nmol.h^{-1}.cm^{-2}$ respectively for PGP-KO tissues.

Table 1

Composition of mobile phase and gradient conditions

Time (min)	A (%)	B (%)
0	100	0
2	100	0
4	0	100
5	0	100
5.1	100	0
8	100	0

A: HPLC eluant mix MF3 comprising 2mM ammonium acetate, 0.027% formic acid in 90% Water:10% Methanol (Romil, Cambridge, UK)

B: HPLC eluant mix MF2 comprising 2mM ammonium acetate, 0.027% formic acid in 10% Water:90% Methanol (Romil, Cambridge, UK)

	1mg IR	3 mg Matrix Tablet fasted			
	sol fasted	9 hr	Ratio/difference	15 hr	Ratio/difference
C_{max} (ng/ml)	3.89	2.17	55.6 (42.9, 65.1)	1.21	31.1 (42.9, 65.1)
AUC_{∞}(ng.h/ml)	55.8	38.3	67.4 (53.5, 84.8)	25.6	46 (36.6, 58)
T_{max} (h)	1.7	4.4	2.8 (1.7, 3.8)	4.7	3.0 (2.0, 4.1)
$T_{1/2}$ (h)	14.8	14.2	-0.6 (-1.3, 0.4)	14.7	-0.1 (-1.1, 0.5)

Table 2: UK-338,003 plasma pharmacokinetics following IR or modified released formulations in fasted individuals. All calculation for the 3mg 9hr and 15hr formulations are dose adjusted to 1mg. Comparisons of each MR formulation against the IR formulation for C_{max} and AUC_{∞} were quantified as the ratio of the means expressed as %, and for T_{max} and $T_{1/2}$ as the difference in means. The 90% confidence limit is shown in brackets

	Adjusted means		Ratio/difference
	Colonic administration	Oral administration	(90% CI)
C_{max} (ng/ml)	0.8	4.3	18.7% (9.4%, 37.3%)
T_{max} (h)	3.1	1.2	1.89 (0.75, 3.02)
AUC_{last} (ng.h/ml)	14.2	54.1	26.2% (14.6%, 47.2%)
$T_{1/2}$ (h)	15.2	12.1	3.1 (1.33, 4.90)

Table 3: Comparison of UK-338,003 plasma pharmacokinetics following colonic and oral administration. Comparisons C_{max} and AUC_{last} were quantified as the ratio of the means expressed as %, and for T_{max} and $T_{1/2}$ as the difference in means. The 90% confidence limit is shown in brackets

Compound	Caco-2	Mouse Ileum	Mouse Colon	Mouse Ileum +20μM GF	Mouse Colon +20μM GF	Human Distal Intestine	Human Distal Intestine +20μM GF
UK-338,003							
A-B	7.0±0.2	4.2 ± 1.3	<0.5	11.3 ± 2.5	7.3 ± 1.4	<0.5	2.8 ± 1.0
B-A	33±19	35 ± 3.1	37.4 ±3.7	20.1±1.5	21.0±3.4	5.8±1.1	8.5±1.5
Theophylline							
A-B	26	30 ± 10*	28 ± 2.9*	ND	ND	14.8 ± 1.9*	ND

Table 4: *In vitro* permeability of UK-338,003 (20μM) and theophylline (20μM) in human and mouse intestine. Data is from Caco-2 monolayers, wild-type mouse ileum or colon and distal human intestine. The latter are a combined group of colon and terminal ileum tissues). Values are given as mean ± SEM for P_{app} (x 10^{-6} cm.sec $^{-1}$) in the A-B and B-A directions for UK-338,003 and for A-B only with theophylline. ND = not determined. * $p< 0.05$ signifies that theophylline P_{app} is significantly higher than UK-338,003 P_{app} in corresponding tissues.

Figure 1

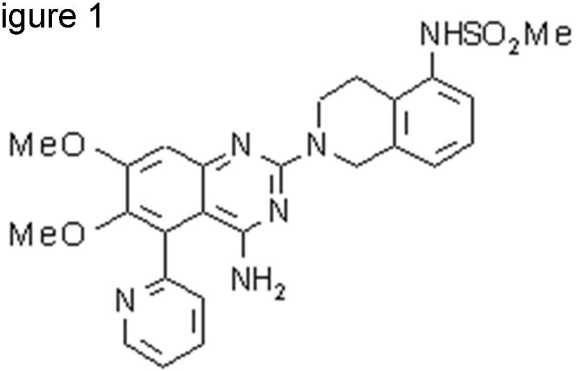

Figure 2

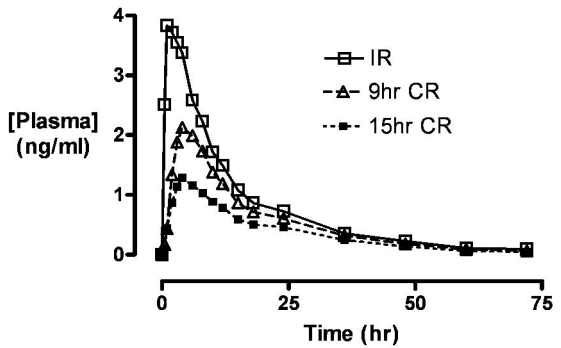

Figure 3

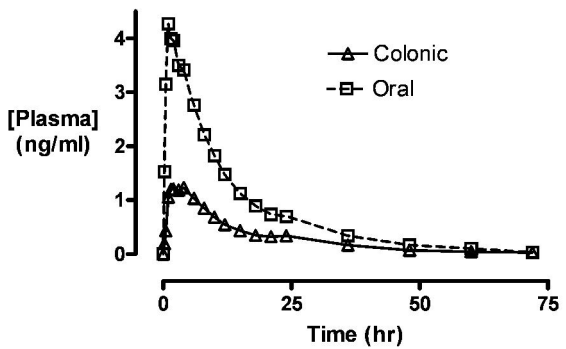

Figure 4

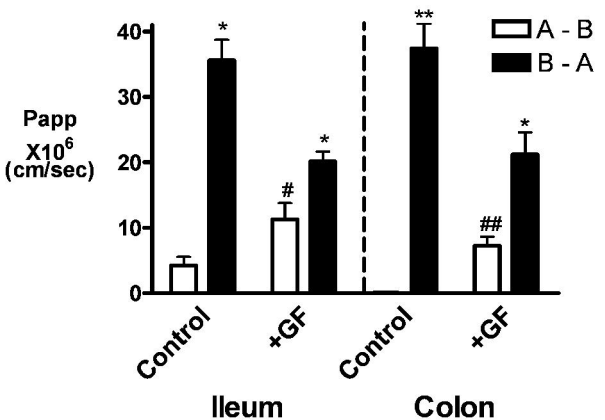
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Figure 5

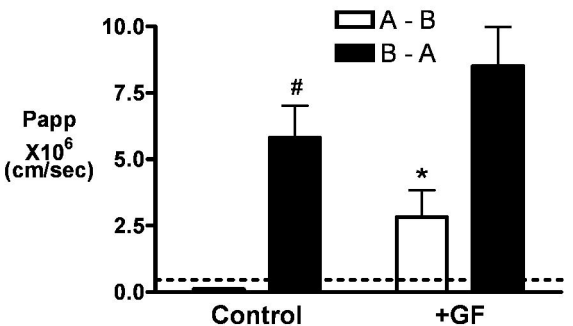


Figure 6

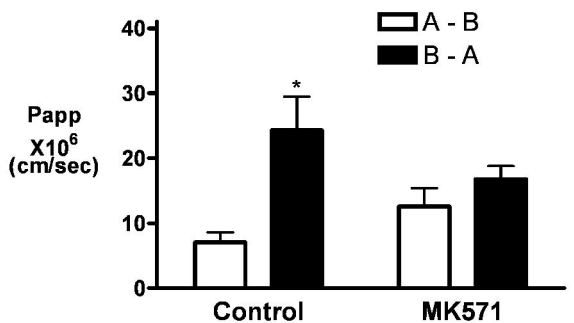
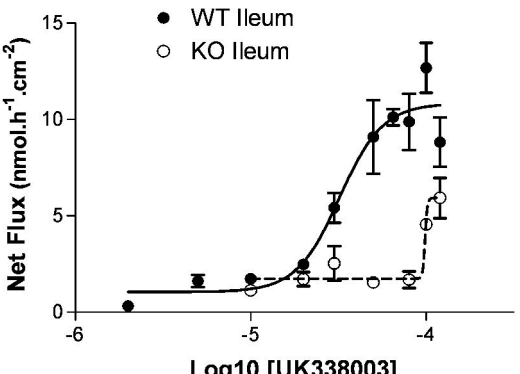


Figure 7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