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An evaluation of the relative roles of the unstirred water layer and receptor sink in limiting the in-vitro intestinal permeability of drug compounds of varying lipophilicity

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Abstract

The roles of the unstirred water layer (UWL) and receptor sink on the in-vitro transmembrane permeability of an increasingly lipophilic series of compounds (mannitol (MAN), diazepam (DIA) and cinnarizine (CIN)) have been assessed. Altered carbogen bubbling rates were used as a means to change the UWL thickness and polysorbate-80 (PS-80), bovine serum albumin (BSA) and α-1-acid glycoprotein (AAG) were employed to alter sink conditions. After correction for solubilisation, Papp data for MAN, DIA and CIN were consistent across varying donor PS-80 concentrations suggesting that for the drugs examined here, the donor UWL did not limit in-vitro permeability. Similarly, altered bubbling rates and receptor sink conditions had no impact on the permeability of MAN. In contrast, decreasing the size of the receptor UWL or adding solubilising agents to the receptor sink resulted in modest enhancements to the permeability of the more lipophilic probe DIA. For the most lipophilic compound, CIN, very significant changes to measured permeability (>30 fold) were possible, but were most evident only after concomitant changes to both the UWL and sink conditions, suggesting that the effectiveness of enhanced sink conditions were dependent on a decrease in the width of the UWL.

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Introduction

Adequate intestinal permeability is an essential criterion for oral bioavailability. The screening of new chemical entities for their transepithelial transport characteristics is therefore a key aspect of the selection process for potential drug candidates (Bohets et al 2001; Caldwell et al 2001; Stenberg et al 2002; Stoner et al 2004; Di & Kerns 2005). In general, in-vitro permeability data obtained for moderately lipophilic compounds with reasonable aqueous solubility is predictive of in-vivo permeability and therefore useful for assessing potential permeability limitations to oral absorption (Artursson & Karlsson 1991; Rubas et al 1993; Yee 1997; Pade & Stavchansky 1998). In contrast, the predictive ability of in-vitro permeability screens for highly lipophilic poorly water soluble drugs (subsequently referred to only as 'highly lipophilic' for clarity) is typically less robust, and in-vitro permeability estimations tend to underestimate the maximum absorbable dose in-vivo (Hilgers et al 2003). The problem of permeability estimation for highly lipophilic compounds is of particular significance in the current environment where lead molecules emerging from drug discovery programmes are increasingly more lipophilic and less water soluble (Lipinski et al 2001). Furthermore, while solubility and dissolution rate are typically assumed to provide the principal limitations to oral bioavailability for highly lipophilic drugs (since the physicochemical properties that dictate low aqueous solubility often provide for adequate passive membrane permeability), it is becoming increasingly clear that these types of compounds are also substrates for efflux transporters and enterocyte-based metabolic enzymes that may markedly limit effective intestinal permeability. Under these circumstances reliable methods for the in-vitro estimation of intestinal permeability are required.

The sequential processes involved in the transepithelial transport of drug compounds in an in-vitro permeability system are: firstly, diffusion across the unstirred water layer (UWL) on the donor side of the diffusion apparatus; secondly, partition into the (apical or mucosal)

membrane of the epithelial layer; thirdly, diffusion across the epithelial layer; fourthly, partition out of the (basolateral or serosal) membrane; and, finally, diffusion across the UWL on the receptor side of the diffusion apparatus (Tsutsumi et al 2003; Youdim et al 2003). Depending on the in-vitro model, the epithelial layer employed may be either excised intestinal tissue, a cellular monolayer (e.g. Caco-2 cells) or an artificial lipid layer (e.g. PAMPA systems). In-vivo, mixing near the surface of the gastrointestinal epithelium is thought to be sufficient that the role of the UWL on the luminal side of the intestinal epithelium is effectively removed (Lennernas 1998; Youdim et al 2003). Furthermore, the circulation of blood at high flow rates through narrow capillaries on the 'blood side' of the epithelium, coupled with the presence of plasma proteins to expand the effective receptor sink (i.e. the reservoir into which transported drug partitions), also dictates that in-vivo absorption for most compounds appears not to be limited by the serosal UWL or the receptor/transport sink (Lennernas 1998; Youdim et al 2003). As a result, the gastrointestinal epithelium is the primary barrier controlling passive drug permeability from the lumen into the systemic circulation in-vivo.

The potential barriers to permeability provided by the UWL and the receptor sink in-vitro, however, may be more significant and may lead to underestimation of the likely in-vivo permeability, especially for highly lipophilic compounds (Aungst et al 2000; Yamashita et al 2000; Krishna et al 2001; Saha & Kou 2002; Taub et al 2002; Naruhashi et al 2003; Tsutsumi et al 2003; Neuhoff et al 2006). In in-vitro permeability models, the UWL is a static layer of water immediately adjacent to the absorptive membrane, the thickness of which is dependent on the volume of the bulk solution and the stirring rate (Tsutsumi et al 2003; Youdim et al 2003). Although it is not possible to completely eliminate the UWL, the thickness can be reduced by increasing the stirring rate. While the UWL on the donor side of a diffusion cell is likely to affect the rate of tissue uptake, the UWL present on the receptor side is expected to control the partitioning of drug from the tissue (intestinal section, Caco-2 cell, etc.) into the receptor buffer. The conditions present in the receptor chamber also dictate the efficiency of tissue-to-buffer partitioning and this can be encouraged by incorporating 'acceptor moieties', such as micelle forming surfactants (Deferme et al 2002) or serum proteins (Sawada et al 1994, 1999; Aungst et al 2000; Yamashita et al 2000; Krishna et al 2001; Saha & Kou 2002; Taub et al 2002; Neuhoff et al 2006), into the receptor buffer. However, whether the permeability of a drug compound is limited by the UWL or the lack of an effective receptor sink is dependent on the intrinsic permeability and lipophilicity of the drug compound. Thus, the UWL and receptor sink are thought to be increasingly important barriers for highly lipophilic compounds where intrinsic membrane permeability is high and where diffusion across the UWL and tissue-to-buffer partitioning become rate limiting (Lennernas 1998; Tsutsumi et al 2003).

In this study, the relative roles of the UWL and receptor sink in determining in-vitro permeability were studied as a function of the lipophilicity of the probe compounds. Side-by-side diffusion cells were used to examine in-vitro permeability as this enables easy manipulation of the UWL thickness by altering the stirring rate of the bulk solution via adjustment of the gas lift (Hidalgo et al 1991; Tsutsumi et al 2003). Transport studies in the absorptive (i.e. mucosal-to-serosal (M-S)) direction (analogous to apical to basolateral transport in the case of cell monolayers) were conducted using mannitol, diazepam and cinnarizine as representative hydrophilic (cLog $D_{pH7.4}$ −4.7), moderately lipophilic (cLog $D_{pH7.4}$ 2.9) and highly lipophilic (cLog $D_{pH7.4}$ 4.6) model compounds, respectively (ACD Labs-Log D suite, Version 7.05). Polysorbate-80 (PS-80), bovine serum albumin (BSA), and bovine α-1-acid glycoprotein (AAG) were used to manipulate the receptor sink.

Materials and Methods

Materials

^{3}H-diazepam (Specific activity 70 Ci mmol^{-1}) and ^{14}C-mannitol (51 mCi mmol^{-1}) were obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA). Cinnarizine hydrochloride, BSA, AAG and PS-80 were procured from Sigma-Aldrich (St Louis, MO). Water was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA). All other reagents were of analytical grade.

Tissue preparation and mounting

All animal studies were performed in accordance with the guidelines of the Australian and New Zealand Council for the Care of Animals in Research and Teaching and the study protocol was approved by the institutional animal ethics committee. Tissue preparation and mounting was performed as described previously (Johnson et al 2002). Briefly, fasted male Sprague–Dawley rats (300 ± 25 g) were anaesthetised with 5% isoflurane and approximately 15 cm of jejunum was removed. A glass rod was inserted down the length of the segment and the tissue was rapidly stripped of its serosal muscle layers. An incision was made along the length of the mesenteric border and segments of tissue were mounted in side-by-side diffusion chambers (Navicyte, Sparks, NV). Modified Krebs bicarbonate ringer (KBR) buffer (6 mL) was immediately added to both mucosal and serosal chambers (Ungell et al 1992). The modified KBR contained (in mM) 147.2 Na^{+}, 5.1 K^{+}, 1.25 Ca^{2+}, 1.2 Mg^{2+}, 115.2 Cl^{-}, 15 HCO_3^{-}, 0.1 $H_2PO_4^{-}$, 1.8 HPO_4^{2-}, 1.2 SO_4^{2-}, 11.5 D-glucose, 4.9 L-glutamate^{-}, 4.9 pyruvate^{-} and 5.4 fumarate^{2-}. The diffusion chambers were then placed in a manifold and the temperature maintained at 37°C using a circulating water bath. Constant bubbling of oxygen and carbon dioxide (95% O_2–5% CO_2) through the buffer allowed for mixing and pH maintenance at 7.4. Tissues were equilibrated for 30 min before initiation of the transport experiment.

Diazepam and cinnarizine transport studies

UWL and receptor sink studies

Transport studies for diazepam and cinnarizine were conducted in the absorptive (M-S) direction in the absence and presence of PS-80 (0.1, 0.5, 1.0 and 2.0% w/v), BSA (1.0% w/v) or AAG (0.2% w/v) in the receptor chamber

under high (~240 bubbles/min) O_2–CO_2 bubbling rates. M-S transport studies were also conducted under low (~60 bubbles/min) O_2–CO_2 bubbling rates in the absence and presence of PS-80 (1% w/v), BSA (1.0% w/v) or AAG (0.2% w/v) in the receptor chamber. Across all experiments, 1.0% w/v PS-80 was maintained in the donor compartment to prevent precipitation and non-specific adsorption. Differing O_2–CO_2 bubbling rates (controlled via an adjustable air inflow valve) were employed to create different levels of agitation (stirring) in the donor and receptor chambers.

K_a estimation

To determine the micellar association constant (K_a) of diazepam and cinnarizine for PS-80 micelles under the conditions used in the UWL and receptor sink studies, M-S transport studies were conducted at high O_2–CO_2 bubbling rates in the presence of varying donor chamber PS-80 concentrations (0, 0.05, 0.1, 0.5, 1.0 and 2.0% w/v for diazepam; 0.5, 1.0, 1.5 and 2.0% w/v for cinnarizine). A concentration of 1.0% w/v PS-80 was maintained in the receptor chamber under all conditions. K_a and free fraction were subsequently calculated using the reciprocal permeability approach as described previously (Katneni et al 2006).

Sample collection and analysis

For diazepam, permeability studies were initiated by the addition of 0.5 μCi (1.0 nM) of radiolabelled drug to the donor chamber (the mucosal chamber for M-S studies). Initial and final donor samples (200 μL each) were collected at 5 and 180 min. Receptor samples (200 μL, replaced with fresh buffer and later corrected for dilution) were collected at 20-min intervals for 180 min. Samples were collected into 5-mL scintillation vials to which 1 mL scintillation fluid (Starscint, Perkin Elmer, Boston, MA) was added. All samples were briefly vortexed and radioactivity quantified using a Tri-Carb 2800TR liquid scintillation counter (Perkin Elmer, Boston, MA).

For cinnarizine, permeability studies were initiated by the addition of 50 μM cinnarizine to the donor (mucosal) chamber. M-S flux of cinnarizine was assessed by collecting receptor samples (200 μL, replaced with fresh buffer and later corrected for dilution) at 30-min intervals to 210 min. Donor chamber samples were collected at the beginning (5 min after addition of cinnarizine) and end (at 210 min) of the experiment. To 200 μL of receptor and donor samples, 100 μL acetonitrile and 50 μL ethanol was added and the samples were vortex mixed and stored in the refrigerator until analysis. On the day of analysis, both receptor and donor samples were vortex mixed and samples were centrifuged for 5 min at 11 000 *g* (Mini Spin Plus; Eppendorf AG, Hamburg). For receptor samples, the clear supernatant was transferred directly to autosampler vials. In the case of donor samples, 100 μL of the supernatant was diluted to 1000 μL with mobile phase A (see below) and the contents vortex mixed before transferring to autosampler vials. Samples of the receptor (50 μL) and donor (5 μL) were analysed by high-performance liquid chromatography (HPLC). The HPLC system comprised a Waters 712 WISP autosampler and 600E pump (Milford, MA) and a Shimadzu RF-10AXL fluorescence detector (Kyoto, Japan). Chromatograms were recorded using a Shimadzu GR5A integrator (Kyoto, Japan). Samples were eluted through a SymmetryShield RP18 (3.5 μm 4.6 × 100 mm) column (Waters, Milford, MA) using either isocratic or gradient mobile phases as described below. Mobile phase A consisted of 20 mM ammonium acetate buffer (pH 5.0)–methanol–acetonitrile (80:10:10, v/v/v), and mobile phase B was 20 mM ammonium acetate buffer (pH 5.0)–acetonitrile (40:60, v/v). The donor samples were eluted isocratically using a 12:88 v/v mixture of mobile phase A and B. Receptor samples were eluted at 1 mL min^{-1} in gradient mode using an initial ratio of 30:70 v/v mobile phase A:B. After 9 min, the proportion of mobile phase B was increased using a linear gradient profile to 100% over 1 min and maintained for 15 min to wash off late eluting peaks. The proportion of mobile phase B was subsequently returned to 70% over 1 min and the column allowed to equilibrate at 30:70 v/v mobile phase A:B for 14 min before injection of the next sample. Samples were analysed using excitation and emission wavelengths of 249 nm and 311 nm, respectively, and the cinnarizine peak eluted at approximately 5.5 min and 9.0 min using the isocratic and gradient methods, respectively. Calibration standards were prepared separately for receptor and donor samples using KBR buffer collected from mucosal and serosal chambers during sham experiments containing appropriate concentrations of PS-80. Cinnarizine calibration curves were constructed in the range of 1–100 ng mL^{-1} for receptor samples and 3–30 μg mL^{-1} for donor samples. Separate calibration standards were prepared for samples containing PS-80 (where KBR containing 1.0% w/v PS-80 was used for both donor and receptor standards), BSA (where KBR containing 1.0% w/v BSA was used for receptor calibration standards only) and AAG (where KBR containing 0.2% w/v AAG was used for receptor calibration standards only). All the calibration samples were prepared, processed and analysed as described above for experimental samples and sample concentrations calculated by comparison to the calibration curve.

Assay validation was conducted using replicate (n = 4) quality control samples at low, medium and high concentrations and the results were accurate to within ±10% of the nominal concentration and precise to within a CV < 10% over the concentration range examined.

Effect of PS-80, BSA and AAG on epithelial integrity

To assess the possible effects of PS-80, BSA and AAG on the integrity of the intestinal epithelium under the conditions used for the UWL and sink studies, the M-S permeability of the paracellular integrity marker mannitol was examined. M-S transport studies were conducted using radiolabelled mannitol (^{14}C-mannitol, 0.5 μCi) in the presence of varying concentrations of PS-80 (0, 0.01, 1.0 and 2.0% w/v) in the donor chamber using high O_2–CO_2 bubbling rates. An additional set of M-S transport studies for mannitol were conducted in the absence and presence of varying concentrations of PS-80 (0.01, 1.0 and 2.0% w/v), BSA (1.0% w/v) or AAG (0.2% w/v) in the receptor chamber at both low (all conditions) and high (all conditions except for 1.0% w/v BSA where excessive foaming prohibited studies at high bubbling rates) O_2–CO_2

bubbling rates. In all cases, 1% PS-80 was incorporated into the opposite chamber to mirror the conditions used for diazepam and cinnarizine transport studies. Sample collection, processing and quantification were performed as described for diazepam.

Data analysis

Uncorrected apparent permeability coefficients ($Papp_{uncorr}$) were calculated for each compound from the flux data into the receptor chamber using Equation 1:

$$Papp_{uncorr}\ (cm\ s^{-1}) = (dX/dt)/(A \times C_0) \quad (1)$$

where dX/dt is the amount transported into the receptor chamber with respect to time (nmol s^{-1}), C_0 is the initial total concentration of drug in the donor chamber (free plus micelle-associated) and A is the surface area available for diffusion (1.78 cm^2).

Estimation of the micellar association constant (K_a) of diazepam and cinnarizine for PS-80 micelles and correction of permeability data obtained in the presence of surfactant ($Papp_{uncorr}$) to obtain the true permeability ($Papp_{corr}$) was performed using the reciprocal permeability approach as described previously (Katneni et al 2006).

Statistical differences between the data at low and high bubbling rates for each receptor sink condition (i.e. comparison of the impact of bubbling rate) and between the data obtained under varying receptor sink conditions with the corresponding control at each bubbling rate (i.e. comparison of the inclusion of differing receptor sinks) was determined by a one-way analysis of variance with a post-hoc Tukey's multiple comparisons procedure.

Results

Correction of the diazepam and cinnarizine M-S $Papp_{uncorr}$ data for solubilisation

Uncorrected ($Papp_{uncorr}$) M-S Papp data for diazepam obtained in the presence of increasing donor PS-80 concentrations at high bubbling rates were used to estimate the K_a of diazepam for PS-80 micelles using the reciprocal permeability approach (Katneni et al 2006). The reciprocal permeability plot for diazepam was linear across the PS-80 concentration range studied (data not shown) and the K_a, obtained from the slope of the plot, was $0.29 \times 10^{-3}\ \mu M^{-1}$. This value was essentially the same as that obtained previously under low bubbling conditions ($0.28 \times 10^{-3}\ \mu M^{-1}$) (Katneni et al 2006) suggesting, as expected, that the degree of agitation did not affect micellar partitioning of DIA and that PS-80 had no effect on the transcellular integrity of the intestinal epithelium. The permeability of diazepam under surfactant-free conditions ($Papp_{corr}$) at the higher bubbling rate was obtained by extrapolation of the reciprocal permeability plot to zero surfactant concentration and was 72.5×10^{-6} cm s^{-1}. The M-S $Papp_{uncorr}$ data of diazepam obtained under low and high bubbling rates with PS-80 in the donor chamber and under varying sink conditions in the receptor chamber were subsequently corrected for solubilisation using the corresponding K_a values (i.e. 0.28 and $0.29 \times 10^{-3}\ \mu M^{-1}$ for low and high bubbling rates, respectively).

The K_a of cinnarizine for PS-80 micelles could not be accurately obtained under low bubbling conditions since measured permeability was extremely low. However, in light of the similarities in K_a obtained for diazepam under low and high bubbling conditions, all cinnarizine Papp data were corrected for solubilisation using the K_a value previously obtained under high bubbling conditions ($1.14 \times 10^{-3}\ \mu M^{-1}$) (Katneni et al 2006).

Mannitol, diazepam and cinnarizine permeability in the presence of PS-80 in either donor or receptor chambers at high bubbling rates

The M-S Papp data for mannitol obtained in the presence of varying concentrations of PS-80 in the donor or receptor chambers under high O_2–CO_2 bubbling rates is shown in Figure 1. The data indicate the lack of effect of PS-80 on the permeability of mannitol regardless of inclusion in either donor or receptor compartments.

The corresponding data for diazepam and cinnarizine in the presence of increasing concentrations of PS-80 in the donor or receptor chambers are presented in Figure 2. For both diazepam (Figure 2A, open circles) and cinnarizine (Figure 2C, open circles; data reproduced from (Katneni et al 2006)), M-S $Papp_{uncorr}$ decreased with increasing donor PS-80 reflecting increasing solubilisation and a decrease in free drug concentration. Correction of the data for the effects of solubilisation, however, revealed relatively limited changes in permeability with increase in donor chamber PS-80 concentration (Figure 2B – diazepam $Papp_{corr}$ open circles; Figure 2D – cinnarizine $Papp_{corr}$ open

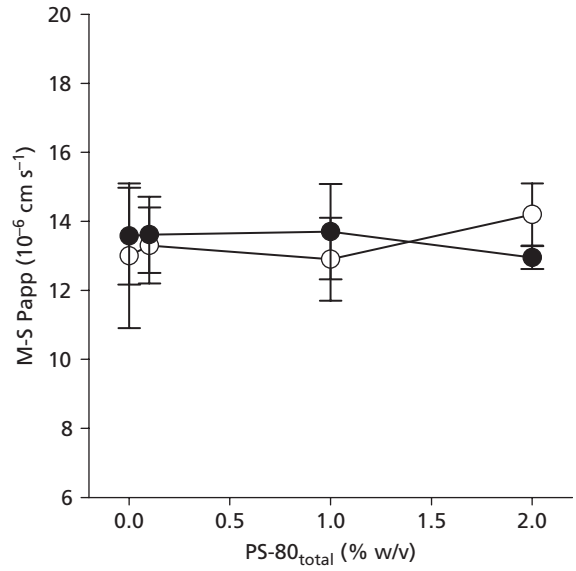

Figure 1 Effect of varying concentrations of donor (open circles) and receptor (filled circles) PS-80 on the M-S Papp of mannitol. Data represent the mean ± s.e.m. for n = 3–4 observations.

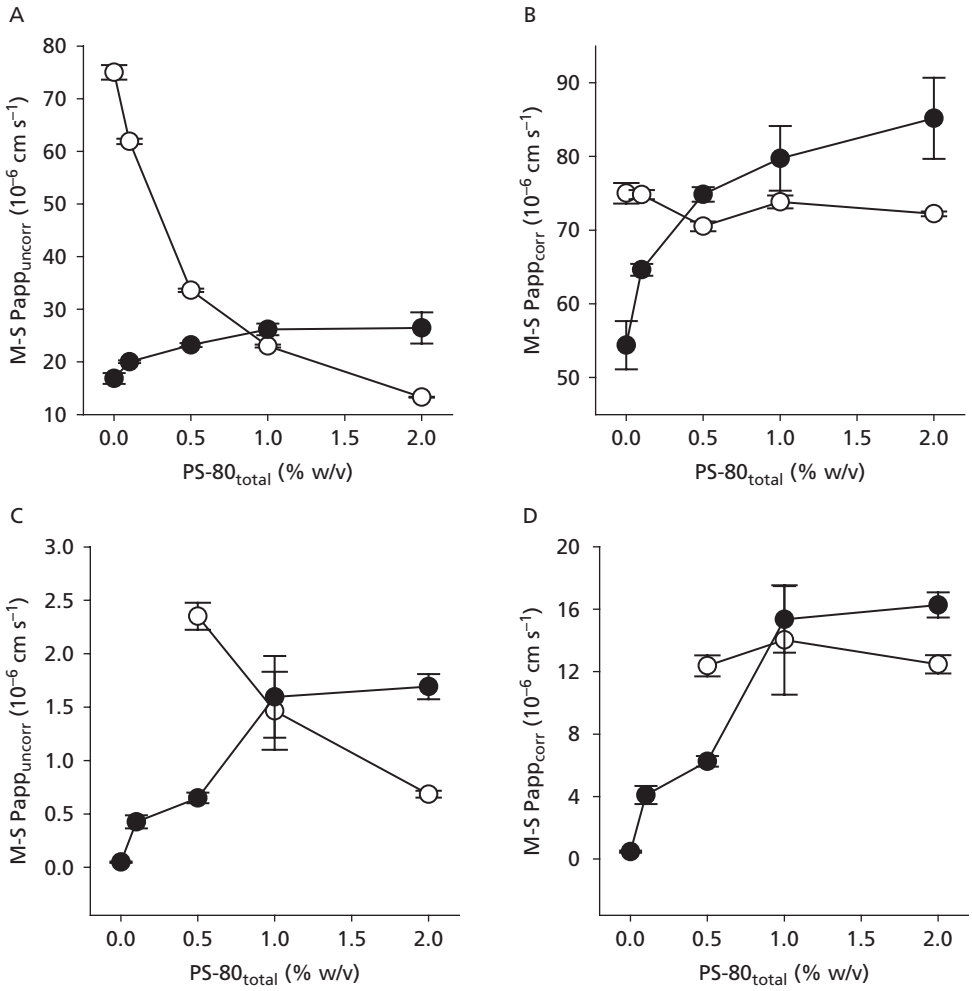

Figure 2 Effect of varying concentrations of donor (open circles) and receptor (filled circles) PS-80 on the uncorrected M-S Papp ($Papp_{uncorr}$) of diazepam (A) and cinnarizine (C). The M-S $Papp_{corr}$ of diazepam and cinnarizine obtained following correction of $Papp_{uncorr}$ for solubilisation is shown in Panel B and Panel D, respectively. All data represent the mean ± s.e.m. for n = 3–6 determinations.

circles), suggesting limited effects on permeability beyond solubilisation.

For experiments undertaken in the presence of increasing concentrations of PS-80 in the receptor chamber (Figure 2, filled circles), data had first to be corrected for the solubilising effect of the 1% w/v PS-80 that was included in the donor chamber as a precautionary measure to avoid potential issues associated with precipitation and non-specific adsorption in the donor chamber. The presence of 1% w/v PS-80 in the donor chamber reduced the free concentration of diazepam by a factor of 0.31 and for cinnarizine by 0.104. Correction for donor solubilisation therefore led to the application of a constant correction factor of 3.23 and 9.62 for diazepam and cinnarizine data, respectively. This is evident by comparison of data (filled circles) in Figure 2A, B (diazepam) and C, D (cinnarizine). In all cases, examination of the impact of increasing concentrations of PS-80 in the receptor (rather than the donor) chamber revealed an increase in measured apparent permeability with increasing concentrations of PS-80. The relative change in permeability was most pronounced for cinnarizine, where an increase in measured $Papp_{corr}$ of >30 fold was evident on comparison of data obtained in the absence of PS-80 and in the presence of 1–2% w/v PS-80 in the receptor chamber.

Effect of changes to bubbling rate and receptor sink on the M-S permeability of mannitol, diazepam and cinnarizine

M-S $Papp_{uncorr}$ data for diazepam and cinnarizine obtained in the presence of differing receptor sink agents (control KBR with no additives, 1.0% w/v PS-80, 0.2% w/v AAG or 1.0% w/v BSA) at low and high bubbling rates were firstly corrected for donor chamber solubilisation (since 1.0% w/v PS-80 was also present in the donor chamber) to obtain corrected Papp data ($Papp_{corr}$). The $Papp_{corr}$ data for diazepam and cinnarizine and the Papp data for mannitol under the same conditions are shown in Figure 3. These data were used to evaluate the impact of the receptor sink and UWL conditions on transmucosal permeability of drugs with varying lipophilicity. Data could not be obtained with 1.0% w/v BSA in the

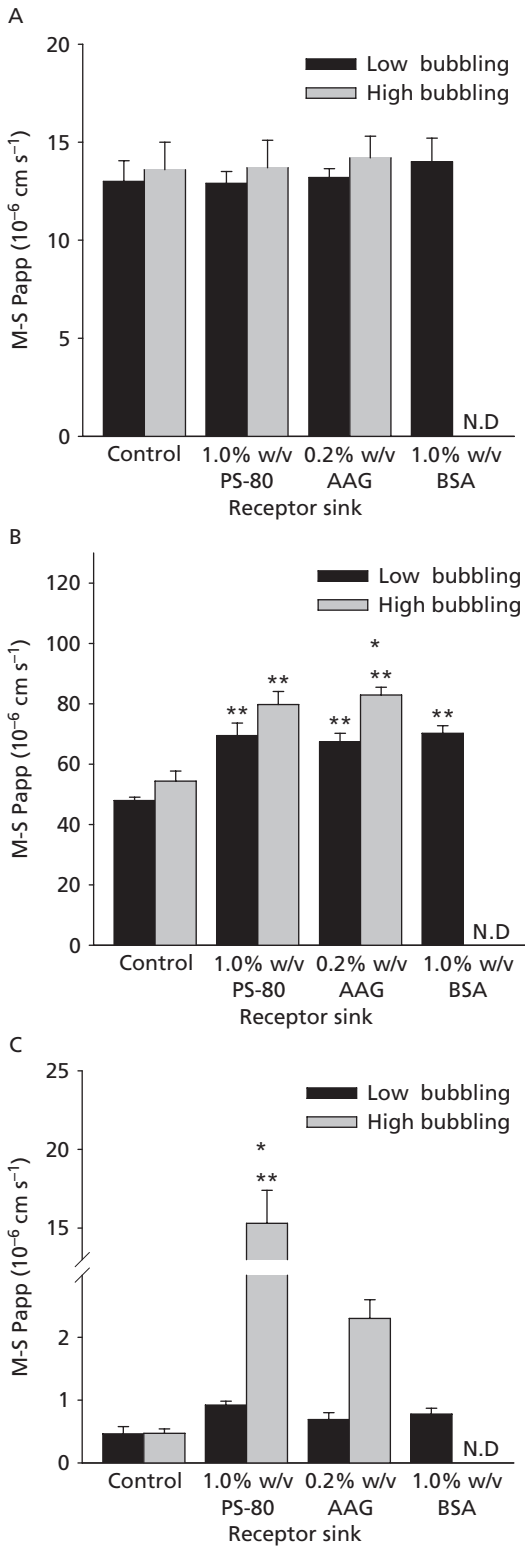

Figure 3 Effect of bubbling rate and varying receptor sink on the M-S permeability of mannitol (A), diazepam (B) and cinnarizine (C). Data represent the mean ± s.e.m. for n = 3–6 determinations. Data with 1.0% w/v BSA under high bubbling rate could not be determined due to excessive frothing. *$P < 0.05$, data at low vs high bubbling rate at a given receptor sink condition; **$P < 0.05$ vs corresponding control obtained at the same bubbling rate.

receptor chamber at high bubbling rates due to excessive frothing leading to loss of buffer from the chamber.

For mannitol, neither increasing the bubbling rate nor altering the receptor sink conditions had any effect on the M-S Papp. In the case of diazepam, increasing the bubbling rate resulted in small increases in M-S $Papp_{corr}$ across all receptor sink conditions. Similarly, altered receptor sink conditions (i.e. addition of 1.0% w/v PS-80, 0.2% w/v AAG or 1.0% w/v BSA) at any given bubbling rate also resulted in small (and similar) increases in the measured M-S $Papp_{corr}$ values. For cinnarizine, alteration in the receptor sink conditions had a moderate (up to 2-fold) impact on M-S permeability at low bubbling rates and the apparent permeability was low in all cases. At higher bubbling rates, however, changes to the nature of the receptor sink had a significant impact on M-S permeability, and this was most evident when 1.0% w/v PS-80 was included in the receptor chamber.

Discussion

For moderately lipophilic drugs (Log P 1–3), good correlations between in-vitro estimates of intestinal permeability and in-vivo intestinal permeability are typically possible (Artursson & Karlsson 1991; Rubas et al 1993; Yee 1997; Pade & Stavchansky 1998; Stoner et al 2004). In contrast, similar predictions are often not possible for highly lipophilic drugs where poor aqueous solubility, adsorption to the walls of the diffusion apparatus and high tissue affinity commonly result in underestimation of in-vitro permeability value (Hilgers et al 2003). The reduced in-vitro permeability is thought to reflect an increasing barrier to permeability provided by the UWL and receptor sink in-vitro, in contrast to the situation in-vivo where the barrier provided by the intestinal membrane dominates (Ingels & Augustijns 2003; Tsutsumi et al 2003; Youdim et al 2003). In this study, experiments were conducted in an attempt to better understand the relative roles of the donor and receptor UWL and the nature of the receptor sink on the transepithelial permeability of drugs with varying lipophilicity. A non-ionic surfactant (PS-80) and two serum proteins (BSA and AAG) were employed to alter the receptor sink and low and high O_2–CO_2 bubbling rates were utilised as a means to alter UWL thickness.

Effect of donor PS-80 on the M-S Papp of mannitol, diazepam and cinnarizine: the potential role of the donor UWL

As shown in Figure 1, the presence of increasing concentrations of PS-80 in the donor chamber did not lead to changes in the M-S permeability of the hydrophilic probe molecule mannitol, suggesting that paracellular integrity of the membrane was not compromised under the conditions employed. In contrast, the presence of PS-80 in the donor chamber led to a decrease in the M-S $Papp_{uncorr}$ of the lipophilic compounds, diazepam and cinnarizine, consistent with a decrease in thermodynamic activity secondary to

micellar solubilisation (Figure 2A, C, open circles). The linearity of the reciprocal permeability plots for both DIA (data not shown) and CIN (Katneni et al 2006) confirmed this hypothesis and correction of the DIA and CIN M-S $Papp_{uncorr}$ data for the free drug concentrations resulted in M-S $Papp_{corr}$ data that were consistent across the donor PS-80 concentration range (Figure 2B, D, open circles). The M-S permeability of cinnarizine and diazepam was therefore seemingly independent of increases in mucosal surfactant concentrations (beyond solubilisation-related changes). In support of these observations, Sawada and co-workers have previously reported a linear correlation between uptake permeability (P_e) and free drug concentration for two lipophilic cytoprotective agents (Sawada et al 1999) and chlorpromazine (Sawada et al 1994) in the presence of BSA.

Interestingly, our data are in apparent conflict with the suggestion that surfactants may reduce the resistance to transport across the UWL (Amidon et al 1982) and might therefore be expected to increase cellular uptake with increasing donor surfactant concentrations. In these studies, however, uptake data has not been obtained specifically and the Papp data described reflect the overall permeability. Previous studies have suggested that uptake kinetics for highly lipophilic drugs may be 2–3 orders of magnitude higher than the kinetics of drug appearance in the receptor chamber, and that the appearance kinetics are kinetically independent of the rate of uptake and essentially rate limiting (Sawada et al 1999). As such, changes in uptake kinetics that may be evident in the presence of donor surfactant are unlikely to be manifest in changes in transmucosal Papp.

Effect of receptor PS-80, BSA and AAG on the M-S Papp of mannitol, diazepam and cinnarizine

The permeability of mannitol was unchanged in the presence of increasing concentrations of PS-80 in the receptor chamber, mirroring the lack of effect seen in the presence of donor chamber surfactant and confirming the integrity of the membrane under the experimental conditions employed (Figure 1, filled circles). In contrast to the effects observed in the presence of increasing donor PS-80 concentrations, however, increasing the concentration of PS-80 in the receptor chamber resulted in increases in the M-S $Papp_{uncorr}$ of both diazepam and cinnarizine (Figure 2A, C, filled circles). These trends were maintained when diazepam and cinnarizine M-S Papp data were corrected for changes in free drug concentration stemming from the presence of 1.0% w/v PS-80 in the donor chamber (Figure 2B, D, filled circles). The enhancement in M-S permeability of cinnarizine evident in the presence of higher receptor surfactant concentrations and a high bubbling rate was much higher (>30 fold) than that observed for diazepam (>2 fold). The greater increase in cinnarizine permeability is consistent with the higher lipophilicity of cinnarizine (cLog $D_{pH7.4}$ 4.6) when compared with diazepam (cLog $D_{pH7.4}$ 2.9), and therefore the greater need for effective sink conditions to promote partitioning out of the tissue into the receptor chamber and prevention of drug adsorption to the walls of the receptor chamber.

To further examine the relative roles of the receptor sink and the UWL in the permeability of mannitol, diazepam and cinnarizine, M-S drug transport was monitored under different receptor sink conditions and at low and high bubbling rates (Figure 3). As expected, the M-S Papp of mannitol, was unchanged regardless of sink conditions or altered bubbling rates, suggesting that diffusion across the UWL and partition into the receptor chamber were not significant limitations to permeability. This is consistent with previous suggestions that the UWL barrier is not expected to be rate limiting for low permeability drugs (Lennernas 1998).

The measured M-S $Papp_{corr}$ of diazepam, a moderately lipophilic compound with Log P of 3.2 (Moffat et al 2004) and cLog $D_{pH7.4}$ of 2.9, increased by up to 50% in the presence of PS-80 or plasma proteins in the receptor chamber (Figure 3B). The increase in cellular permeability for lipophilic drugs in the presence of receptor additives in the our Ussing chamber experiments is consistent with several previous studies using cell culture models (Sawada et al 1994; Aungst et al 2000; Krishna et al 2001; Saha & Kou 2002; Kokate et al 2007). The magnitude of the increase in M-S permeability across the different receptor sink conditions was similar at both low and high bubbling rates suggesting approximately equivalent roles for both the UWL and the nature of the receptor sink in limiting the transmucosal permeability of diazepam.

In contrast to the relatively modest changes in apparent permeability observed for diazepam, changes to the receptor sink and stirring rate had marked and differential effects on the M-S permeability of cinnarizine, a highly lipophilic compound with Log P of 5.8 (Belsner et al 1993) and cLog $D_{pH7.4}$ of 4.6 (ACD Labs-Log D suite, Version 7.05). Under low bubbling conditions, the M-S permeability of cinnarizine was approximately 2-fold higher than the control values in the presence of PS-80 or plasma proteins in the receptor chamber. Under higher bubbling conditions, however, while control Papp (i.e. data in the absence of receptor additives) did not change significantly, increases of 5 and 33 fold above control were apparent in the presence of 0.2% w/v AAG and 1.0% w/v PS-80, respectively. In contrast to diazepam, where the magnitude of the increase in the M-S permeability at high bubbling rates was similar with either 1.0% w/v PS-80 or 0.2% w/v AAG in the receptor (1.2 vs 1.5 fold), the increase in M-S permeability for cinnarizine was much greater in the presence of 1.0% w/v PS-80 when compared with 0.2% w/v AAG (33 fold vs 5 fold). This likely reflects differences in the affinity of cinnarizine and diazepam for PS-80 micelles relative to AAG.

These data suggest that partition out of the epithelial layer and diffusion across the receptor UWL are significant barriers to the transmucosal permeability of highly lipophilic drugs and that for changes to the nature of the receptor sink to be most effective, the barrier associated with the UWL must also be minimised. Equally, changes to the UWL alone were not sufficient to increase effective permeability, and significant changes in permeability were only apparent when the receptor sink was increased along with increases in the stirring rate (and therefore a decrease in the UWL thickness). Together, these results suggest that the transmucosal

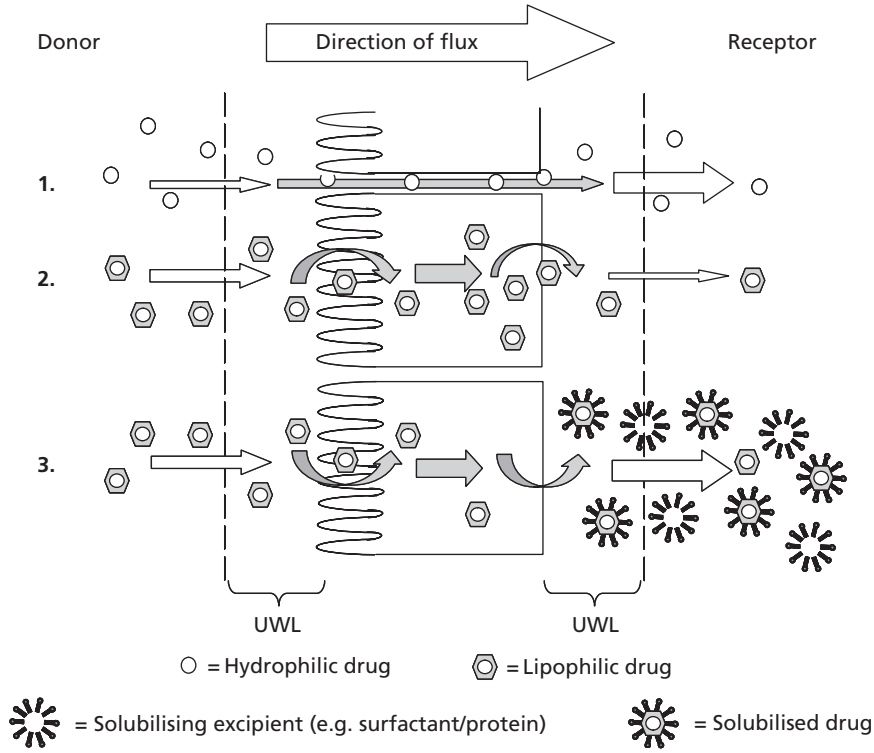

Figure 4 Diagrammatic representation of the differential effects of changes to the donor and receptor UWL and the nature of the receptor sink on in-vitro transepithelial permeability of lipophilic and hydrophilic drug compounds. 1. Highly water soluble hydrophilic compounds, such as mannitol, which have low transcellular membrane permeability, are primarily absorbed via the paracellular route, and have low affinity for solubilising receptor moieties (e.g. plasma proteins and surfactants). Intrinsic membrane permeability will be limiting and therefore changes to the UWL or receptor sink are not expected to impact on apparent permeability. 2. In contrast, for highly lipophilic drug compounds such as cinnarizine, partitioning into the membrane and intrinsic membrane permeability are expected to be high, and the resistance of the UWL and partitioning out of the membrane into the receptor chamber are expected to be rate limiting. 3. Under these circumstances, the presence of PS-80 or plasma proteins in the receptor sink shifts the tissue–receptor partitioning process in favour of the receptor buffer thereby driving the permeability process. However, the effectiveness of changes to receptor sink conditions also depends on concomitant changes to the stirring rate (and therefore the width of the UWL), presumably reflecting a reduction in the barrier to diffusion of micellar-associated or protein-bound drug across the receptor unstirred water layer.

permeability of highly lipophilic drugs under control conditions is typically limited by both diffusion across the UWL and the lack of effective receptor sink conditions (Figure 4). Note that it is not possible in these studies to de-couple the potential for improved receptor sink conditions to enhance measured permeability via enhanced drug partitioning out of the membrane or reduced drug adsorption to the walls of the receptor chamber, and both may play a role. Increasing agitation is also likely to reduce the size of the UWL on the donor side of the diffusion apparatus, a situation previously shown to increase the uptake kinetics of lipophilic compounds, including a range of polychlorinated biphenyls (PCBs) and chlorpromazine, into cellular monolayers (Dulfer et al 1996; Sawada et al 1999). The increase in M-S $Papp_{corr}$ observed for the more lipophilic drug molecules (diazepam, cinnarizine) in these studies at higher stirring rates may therefore reflect increases in both uptake and efflux kinetics. Realisation that the cellular uptake kinetics of lipophilic drugs are typically much higher than cellular efflux, however, suggests that effects in the receptor chamber are likely to dominate transcellular transport.

Conclusions

These studies, using excised rat intestinal tissue in Ussing chambers, confirm previous in-vitro studies using cell monolayers that suggest that the inclusion of solubilising additives, such as plasma proteins or surfactants, in the receptor chamber of in-vitro diffusion experiments may significantly improve in-vitro estimates of the transepithelial permeability of highly lipophilic drugs. These data further highlight the importance of addressing both receptor UWL and receptor sink conditions to optimise permeability estimates for highly lipophilic drug compounds in-vitro. In the case of cinnarizine, permeability data were optimised under conditions of high bubbling rates and in the presence of 1% w/v PS-80 in the receptor chamber; however, the most appropriate choice of receptor chamber conditions will be drug specific and dependent on the physicochemical properties of the drug and the solubiliser. The data also suggest that care should be taken when assessing the permeability of, for example, a series of lipophilic analogues if the receptor sink is enhanced by the addition of plasma proteins or other solubilising agents, since differing affinities of the probe compounds for additives (surfactants,

proteins, etc.) added to enhance the receptor sink may lead to changes in apparent permeability that are unrelated to membrane transport.

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