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Research Article

# Dual modes of 5-(N-ethyl-N-isopropyl)amiloride modulation of apical dipeptide uptake in the human small intestinal epithelial cell line Caco-2

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**Abstract.** Selective pharmacological $Na^+/H^+$ exchange (NHE) inhibitors were used to identify functional NHE isoforms in human small intestinal enterocytes (Caco-2) and to distinguish between direct and indirect effects on transport via the intestinal di/tripeptide transporter hPepT1. The relative potencies of these inhibitors to inhibit $^{22}Na^+$ influx identifies NHE3 and NHE1 as the apical and basolateral NHE isoforms. The $Na^+$-dependent (NHE3-sensitive) component of apical dipeptide ([$^{14}$C] Gly-Sar) uptake was inhibited by the selective NHE inhibitors with the same order of potency observed for inhibition of apical $^{22}Na^+$ uptake. However, 5-(N-ethyl-N-isopropyl)amiloride (EIPA) also reduced [$^{14}$C]Gly-Sar uptake in the absence of $Na^+$ and this inhibition was concentration and pH (maximal at pH 5.5) dependent. NHE3 inhibition by S1611 and S3226 modulates dipeptide uptake indirectly by reducing the transapical driving force ($H^+$ electrochemical gradient). EIPA (at 100 µM) has similar effects, but at higher concentrations (>200 µM) also has direct inhibitory effects on hPepT1.

**Key words.** $H^+$-coupled transport; dipeptide transport; intestinal absorption; PepT1; $Na^+/H^+$ exchange; NHE3; EIPA; amiloride.

The human intestinal di/tripeptide transporter hPepT1 is localised to the brush-border membrane of the human small intestinal epithelium [1]. hPepT1 acts as an absorptive portal and plays an essential role in the absorption of nutrients in the form of small di- and tripeptides [2] and in the high oral bioavailability of a variety of orally active peptidomimetic drug compounds [3–8]. PepT1 mRNA is expressed in the absorptive cells (enterocytes) lining the villi along the full length of the small intestine [9].

Most studies aimed at identifying the substrate specificity and transport characteristics of peptide absorption across the human small intestine have utilised the human intestinal cell line Caco-2 grown as confluent monolayers of polarised cells. Early studies demonstrated the presence of a pH-dependent transporter for the aminocephalosporin antibiotics cephalexin [10] and cephradine [11] and the dipeptide Gly-Sar [12] at the brush-border membrane of Caco-2 cell monolayers. The related cDNA (PepT1) was originally cloned from rabbit small intestine [13] and later isolated and named hPepT1 from both human small intestine [14] and Caco-2 cells [1]. The substrate specificity of hPepT1 (expressed heterologously in *Xenopus laevis* oocytes or mammalian cell lines) is identical to the substrate specificity of di/tripeptide transport at the brush-border membrane of Caco-2 cell monolayers [15]. However, there are differences in the ionic dependence of peptide transport when hPepT1 activity is compared in isolation and in the endogenous situation where hPepT1 is colocalised in the specialised brush-border membrane with a large number and variety of transport proteins involved in transmembrane solute and ion movement. Following heterologous expression in *X. laevis* oocytes or

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HeLa cells, PepT1 functions as an $H^+$-coupled, pH-dependent, $Na^+$-independent transporter [13, 14, 16], whereas in intact intestinal tissue preparations dipeptide uptake is also dependent on extracellular $Na^+$ [16–21]. The apparent $Na^+$ dependence of dipeptide uptake is the net result of a reciprocal functional relationship between PepT1 and an apically localised $Na^+/H^+$ exchanger [20, 22, 23]. This $Na^+/H^+$ exchanger is NHE3, the third member of solute carrier family 9 [24]. NHE3 was isolated from human small intestine [25] and is expressed in the small intestine exclusively at the apical membrane of mammalian intestinal enterocytes [26, 27]. In Caco-2 cell monolayers, intracellular acidification resulting from apical $H^+$/dipeptide symport, via hPepT1, activates NHE3 without effect on basolateral $Na^+/H^+$ exchange (NHE1) (suggesting that PepT1 activity leads to a localised change in $pH_i$ adjacent to the apical membrane) [23]. NHE3 activity ($H^+$ efflux in exchange for $Na^+$ influx) thus maintains the transapical driving force (the $H^+$ electrochemical gradient) required for optimal $H^+$/dipeptide symport (via hPepT1). Thus inhibition of NHE3 by removal of extracellular $Na^+$ indirectly inhibits hPepT1. This requirement for cooperative functional activity with NHE3 is not limited to hPepT1 as, in intact epithelial preparations, NHE3 activity is also required for optimal transport via the $H^+$-coupled amino acid transporter hPAT1 [28, 29]. Up- or down-regulation of NHE3 activity by physiological, pathophysiological or pharmacological means will, therefore, also indirectly control the intestinal absorptive capacity for transport via $H^+$-coupled transporters such as hPepT1 and hPAT1. NHE3 is known to be down-regulated by activation of the protein kinase A (PKA) pathway. We have been able to mimic this response pharmacologically and have demonstrated that incubation of Caco-2 cell monolayers with forskolin, 8-Br-cAMP or activators of the basolateral $VPAC_1$ receptor leads to an inhibition in NHE3 activity and a concomitant reduction in apical Gly-Sar uptake via hPepT1 [20, 21]. Activation of the PKA pathway has no effect on Gly-Sar uptake under conditions in which NHE3 is inactive, e.g. $Na^+$-free conditions or apical pH 5.5. Clearly, pharmacological modulation of hPepT1 either directly or indirectly will have effects on both nutrient and drug absorption, with the small intestinal epithelium as a potential site of drug-drug interaction. Although many drug compounds interact with hPepT1 most appear to be transported substrates.

In 1967, Cragoe et al. [30] described the synthesis of the pyrazinoylguanidine derivative amiloride and a number of amiloride analogues that inhibit the $Na^+$ channel in urinary epithelia. Amiloride is administered clinically as a $K^+$-sparing diuretic [31, 32]. It was the first drug described as an inhibitor of $Na^+/H^+$ exchange [33]. In studies where the amiloride sensitivity of NHE1 and NHE3 have been compared directly, either following expression in a variety of cell types [PS120, AP-1, LAP(-) or LAP1] [34–37] or in the endogenous situation (Caco-2 cells) [38], amiloride inhibits NHE1 with an $IC_{50}$ of 1.6–10.7 μM while NHE3 is much less sensitive with half-maximal inhibition at 100–438 μM [34–38]. A concentration of 1 mM is generally used to inhibit NHE3 completely [38, 39]. One or two substitutions in the 5-amino nitrogen of amiloride, e.g. 5-(N,N-dimethyl) (DMA), 5-(N-methyl-N-isopropyl) (MIA) and 5-(N-ethyl-N-isopropyl) (EIPA) improve the potency as inhibitors of $Na^+/H^+$ exchange by 12- to 349-fold [40]. EIPA is more potent and NHE isoform selective than amiloride, inhibiting NHE1 with an $IC_{50}$ of 15–25 nM and NHE3 with an $IC_{50}$ of 2–3 μM [34, 35]. Therefore, amiloride analogues with a 5-amino nitrogen substitution, particularly EIPA, are used in many studies as inhibitors of $Na^+/H^+$ exchange [23, 34]. In addition, a number of more selective compounds with higher affinity for $Na^+/H^+$ exchange have been developed. Of these, a benzoylguanidine compound HOE642 (cariporide) was developed primarily to inhibit NHE1 [41]. Cariporide is over 10,000 times more potent at inhibiting NHE1 than NHE3, with apparent $IC_{50}$s of 80 nM and 0.9 mM, respectively [41]. The bismethacryloyl derivatives S1611 and S3226 are distinct from the amiloride derivatives and cariporide in that they selectively inhibit NHE3 over NHE1 [36]. Human NHE3 (expressed in LAP-1 cells) is inhibited by S1611 and S3226 with $IC_{50}$s of 50 nM and 20 nM, respectively, while NHE1 is inhibited with $IC_{50}$s of 4.70 μM and 3.55 μM, respectively [36]. To date, there is no evidence for any non-NHE-mediated effects of this latter group of compounds.

There is currently a great deal of interest in the intestinal di/tripeptide transporter PepT1 as a route for targeted drug delivery of pharmaceutical compounds. Much effort is being made to determine whether activity of this transporter may be controlled so that transport capacity can be regulated. The aim of this study was to investigate the effect of a number of inhibitors against their known targets (NHEs) and associated indirect effects on dipeptide transport via hPepT1. In the case of amiloride and derivatives, these compounds are known to inhibit a number of other ion/nutrient transporters [40]. When considering the effects of pharmacological inhibitors it is essential that, especially where there is an interest in a secondary or non-direct effect, the selectivity and possible limitations of these compounds are known so that potential side-effects can be avoided.

## Materials and methods

### Materials

[$^{14}$C]Gly-Sar (56.7 mCi mmol$^{-1}$) was from Cambridge Research Biochemicals (Stockton-on-Tees, UK). $^{22}Na^+$ (722.69 mCi mg$^{-1}$) was from NEN Life Sciences (Zaaventem, Belgium). [$^3$H]leucine (112 Ci mmol$^{-1}$) was from Amersham Biosciences (Little Chalfont, UK).

[$^3$H]lysine (99 Ci mmol$^{-1}$) was from PerkinElmer Life Sciences (Boston, Mass.). EIPA, DMA and MIA were from Sigma-Aldrich (Poole, UK). S1611, S3226 and cariporide (HOE642) were obtained from H.J. Lang (Aventis Pharma Deutschland GmbH, Chemical Research, Frankfurt/Main, Germany). All other chemicals were of the highest standard available and were purchased from Sigma-Aldrich or Bachem (St. Helens, UK).

### Cell culture

Cell culture of Caco-2 cells (passage 105–124) was performed essentially as described previously [12, 20, 21]. Experiments were performed 14–17 days after seeding and 18–24 h after feeding.

### Radiotracer uptake measurements across the apical membrane of Caco-2 cell monolayers

Uptake measurements were performed essentially as described previously [16, 20, 23]. Briefly, Caco-2 cell monolayers on Transwell polycarbonate filters (12 mm diameter) were washed in 4 × 500 ml volumes of modified Krebs' solution (pH 7.4, 22 °C) (mM): NaCl, 137; KCl, 5.4; $MgSO_4$, 0.99; $KH_2PO_4$, 0.34; $NaH_2PO_4$, 0.3; $CaCl_2$, 2.8; glucose, 10 ($Na^+$-free solutions were prepared as above except choline-Cl replaced NaCl and $NaH_2PO_4$ was omitted; glucose-free solutions were prepared by replacing 10 mM glucose with 10 mM mannitol). Solution pH was adjusted at 37 °C by addition of either 10 mM MES (pH 5.0–6.5) or 10 mM HEPES (pH 6.5–8.0), and Tris base. In all experiments, the basolateral solution was pH 7.4. The apical solution was varied between pH 5.0–8.0. For dipeptide and amino acid uptake measurements, [$^{14}$C]Gly-Sar (0.5 µCi ml$^{-1}$, 10–100 µM), [$^3$H]lysine (0.5 µCi ml$^{-1}$, 10 µM) or [$^3$H]leucine (0.5 µCi ml$^{-1}$, 10 µM) were added to the apical solution and uptake allowed to proceed for 1–15 min at 37 °C.

$^{22}Na^+$ (1 µCi ml$^{-1}$, 78–100 nM) uptake experiments were performed as described previously [20, 21, 23]. Cell monolayers were washed in 4 × 500 ml volumes of modified $Na^+$-free and glucose-free Krebs' solution (pH 7.4, 22 °C). The Caco-2 cells were acidified by the $NH_4Cl$ prepulse-and-release manoeuvre by pre-incubation for 15 min at 37 °C in $Na^+$-free and glucose-free Krebs' solution containing 30 mM $NH_4Cl$ (replacing 30 mM choline-Cl). Following pre-incubation, the monolayers were washed in 800 ml pre-warmed (37 °C) $Na^+$-free and glucose-free Krebs. The cell monolayers were then placed into wells containing $Na^+$-free and glucose-free, pH 7.4, Krebs' solution. The composition of the apical and basolateral bathing solutions were identical except that the basolateral solution contained 1 mM ouabain. $^{22}Na^+$ was added to either the apical or basolateral solution and uptake measured over 5min at 37 °C. Various compounds were added to the apical or basolateral uptake solution for the duration of the uptake (see figure legends for details). In all experiments involving use of the amiloride compounds, the vehicle (0.5% DMSO) was also present in the control uptake (and pre-incubation) solutions. DMSO had no effect on NHE, PepT1 or amino acid transport under any experimental condition studied. Following uptake experiments, cell monolayers were washed sequentially in 3 × 500 ml volumes of ice-cold $Na^+$-free Krebs' solution (pH 7.4). Cell monolayer-associated radioactivity was determined by liquid scintillation counting. Sigmoidal concentration-response curves were fitted to the data using Prism version 3 (GraphPad software Inc., San Diego, Calif.). For dipeptide uptake, $IC_{50}$ values were estimated as the concentration that half-maximally inhibited the NHE3-dependent component of Gly-Sar uptake (determined as uptake sensitive to inhibition by 10 µM S1611). For $Na^+/H^+$ exchange activity, $IC_{50}$ values were estimated as the concentration required to inhibit $^{22}Na^+$ uptake by 50%.

### Statistics

Data are expressed as mean ± SE (n). Statistical comparisons of mean values were made using one-way analysis of variance (ANOVA) (using the Tukey-Kramer multiple-comparisons post-test). Significance was assumed if $p<0.05$.

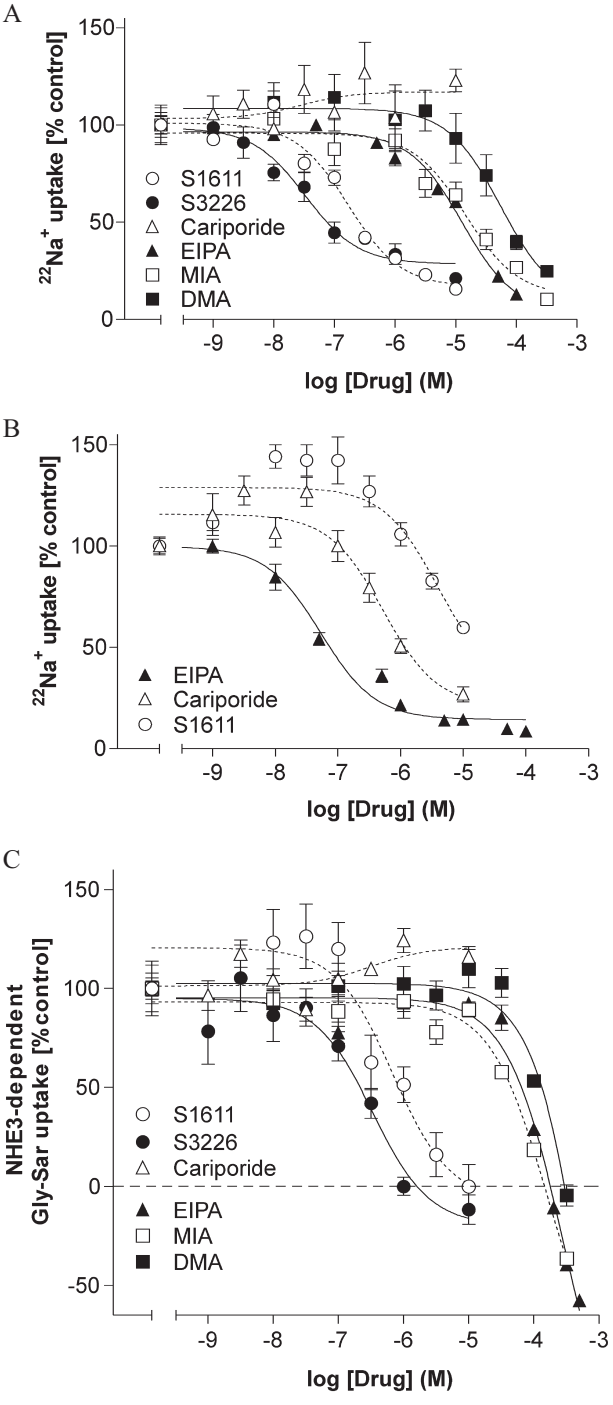
## Results

### The polarised distribution of $Na^+/H^+$ exchange isoforms in Caco-2 cell monolayers

$Na^+/H^+$ exchange was measured across both apical and basolateral membranes of Caco-2 cell monolayers by measurement of $^{22}Na^+$ influx (at extracellular pH 7.4 and an $Na^+$ concentration of 78–100 nM, tracer only) after cells had been acidified by the $NH_4Cl$ prepulse-and-release manoeuvre. The selective NHE3 inhibitors S1611 and S3226 reduced $^{22}Na^+$ uptake across the apical membrane of Caco-2 cell monolayers with $IC_{50}$s of 0.27 µM and 0.08 µM, respectively (fig. 1A). In contrast, the selective NHE1 inhibitor cariporide (1 nM–10 µM) had no effect ($p>0.05$) on apical $^{22}Na^+$ influx (fig. 1A). EIPA, MIA and DMA reduced apical $^{22}Na^+$ uptake in a concentration-dependent manner with $IC_{50}$s of 14 µM, 18 µM and 81 µM, respectively (fig. 1A). The order of potency of inhibition of apical $^{22}Na^+$ uptake was S3226>S1611> EIPA=MIA>DMA>>cariporide, consistent with uptake via NHE3 [34–39, 41, 42].

Basolateral $^{22}Na^+$ uptake clearly has a different sensitivity to S1611, cariporide and EIPA than that observed at the apical membrane (fig. 1A & 1B). When compared to the control, basolateral uptake appears to be stimulated by low concentrations (10–100 nM) of S1611 (fig. 1B). However, at higher concentrations, S1611 reduced $^{22}Na^+$ uptake by up to 40% at 10 µM S1611 (estimated $IC_{50}$

14 µM) (fig. 1B). Basolateral $^{22}Na^+$ uptake was reduced by cariporide with an $IC_{50}$ of 1 µM (fig. 1B). EIPA was the most potent inhibitor of basolateral uptake, reducing $^{22}Na^+$ influx with an $IC_{50}$ of 0.07 µM (fig. 1B). The order of potency for inhibition of basolateral $^{22}Na^+$ uptake was EIPA>cariporide>S1611, consistent with uptake via NHE1 [34–39, 41, 42].




## Pharmacological modulation of the NHE3-sensitive ($Na^+$-dependent) component of dipeptide uptake by NHE inhibitors

hPepT1 function was measured as dipeptide ([$^{14}$C]Gly-Sar) uptake across the apical membrane of Caco-2 cell monolayers at apical pH 6.5 (which represents the physiological pH at the luminal surface in the small intestine [43]) in the presence of extracellular $Na^+$. At a concentration of 10 µM, S1611 should inhibit all NHE3 activity [42] and this seems to be confirmed by figure 1A where 84.3% of apical $^{22}Na^+$ influx was inhibited at 10 µM S1611 which was not significantly ($p>0.05$) different from the maximal inhibition observed with MIA or EIPA. To allow comparison with figure 1A, these data in figure 1C were normalised and the results are expressed as percent control of the NHE3-sensitive (or $Na^+$-dependent) component of Gly-Sar uptake (following subtraction of Gly-Sar uptake in the presence of 10 µM S1611 which represents the NHE3-insensitive or $Na^+$-independent component). The NHE3-selective inhibitors S3226 and S1611 reduced the $Na^+$-dependent component of Gly-Sar uptake with $IC_{50}$s of 0.23 µM and 0.95 µM, respectively (fig. 1C). As observed with apical $^{22}Na^+$ uptake (fig. 1A), there was no significant difference ($p>0.05$) in Gly-Sar uptake in the presence of either 3 or 10 µM S1611 (fig. 1C). Cariporide (1 nM–10 µM) was without effect ($p>0.05$) on apical Gly-Sar uptake (fig. 1C). The amiloride analogues EIPA, MIA and DMA inhibited the $Na^+$-dependent component of Gly-Sar uptake with $IC_{50}$s of 69 µM, 48 µM and 138 µM, respectively (fig. 1C). Therefore, the relative ability of the NHE inhibitors to inhibit the $Na^+$-dependent

Figure 1. Pharmacological inhibition of apical $^{22}Na^+$ influx (NHE3 activity), basolateral $^{22}Na^+$ influx (NHE1 activity) and apical dipeptide uptake (hPepT1 activity). $^{22}Na^+$ (1 µCi ml$^{-1}$, 78–100 nM) uptake (pH 7.4, $Na^+$ free, 5 min) was measured across either the apical (*A*) or basolateral (*B*) membranes of Caco-2 cell monolayers after the cells had been acidified by the $NH_4Cl$ prepulse-and-release manoeuvre (see Materials and methods for details). $^{22}Na^+$ influx was measured in the presence of increasing concentrations of S1611 (open circles, dashed line), S3226 (closed circles, solid line), cariporide (open triangles, dashed line), EIPA (closed triangles, solid line), MIA (open squares, dashed line) or DMA (closed squares, solid line) with all drugs added to the cis compartment as appropriate. Uptake was measured in $Na^+$- and glucose-free Krebs' solution (pH 7.4) with the basolateral solution containing 1 mM ouabain. Results are the mean ± SE (n=6–12) and are expressed as a % of control (absence of inhibitor). Data for S1611 and EIPA (apical only) were adapted from Anderson et al. [21] and Thwaites et al. [23], respectively. (*C*) [$^{14}$C]Gly-Sar (0.5 µCi ml$^{-1}$, 100 µM) uptake (15 min) across the apical membrane of Caco-2 cell monolayers was measured in $Na^+$-containing solutions (apical pH 6.5) in the presence of increasing concentrations of S1611, S3226, cariporide, EIPA, MIA and DMA (key as above). Results are the mean ± SE (n=11–15) and are expressed as % control [NHE3-dependent Gly-Sar uptake which represents total uptake after subtraction of uptake in the presence of 10 µM S1611 (a concentration which inhibits all NHE3 activity)]. All data were fitted to sigmoidal concentration-response curves using Prism version 3.0.

component of Gly-Sar uptake (S3226>S1611>EIPA= MIA>DMA>>cariporide) (fig. 1C) was identical in rank order of potency to that observed for inhibition of apical $^{22}Na^+$ uptake (NHE3 activity) (fig. 1A). However, a striking observation was the ability of the lipophilic amiloride analogues EIPA and MIA to reduce Gly-Sar uptake beyond the maximal component that can be attributed to any involvement of NHE3 (fig. 1C).

**EIPA modulation of dipeptide uptake in the presence and absence of functional NHE3 activity**

Gly-Sar uptake was reduced by 500 µM EIPA in both the presence and absence of functional NHE3 activity (fig. 2A). NHE3 is active at extracellular pH 6.5 but inactive at pH 5.5 [16, 20, 28, 34]. At pH 5.5, apical Gly-Sar uptake was independent of extracellular $Na^+$ (fig. 2A). This confirms our previous observations that the $Na^+$ dependence of Gly-Sar uptake is itself dependent on pH where no effect is observed at extracellular pH 5.5 when NHE3 is inactive [16, 20, 34]. However, even though NHE3 is inactive at pH 5.5 (in either $Na^+$-containing or $Na^+$-free conditions), 500 µM EIPA reduced Gly-Sar uptake in both $Na^+$ and $Na^+$-free conditions (fig. 2A) suggesting that EIPA is able to inhibit dipeptide uptake by a non-NHE3-mediated mechanism. At 'physiological' apical pH 6.5 [43], Gly-Sar uptake was, in part, dependent on extracellular $Na^+$ (due to a requirement for NHE3 activity) (fig. 2A), as described previously [16, 20]. At pH 6.5, 500 µM EIPA inhibited Gly-Sar uptake both in the presence of extracellular $Na^+$ (and NHE3 activity) and in the absence of functional NHE3 (in $Na^+$-free conditions) (fig. 2A). At pH 6.5, the $Na^+$-independent component of Gly-Sar uptake was reduced by EIPA in a concentration-dependent manner (fig. 2B), where 500 µM ($p<0.01$) and 300 µM ($p<0.05$) EIPA significantly reduced uptake, whereas concentrations of 200 µM EIPA and below had no effect ($p>0.05$) (fig. 2B). Gly-Sar uptake in the absence of extracellular $Na^+$ was reduced by 500 µM EIPA throughout the apical pH range pH 5.0–7.4 (fig. 2C). The greatest inhibitory effect of EIPA was observed at pH 5.5 ($p<0.001$ vs control). Amiloride and its 5-amino nitrogen analogues (e.g. EIPA) are weak bases which inhibit $Na^+/H^+$ exchange in their protonated forms [40]. The $pK_a$ of EIPA has not been reported but is likely to be similar to those of amiloride and MIA ($pK_a$ 8.7–8.8 and 8.1, respectively) [40]. Therefore, these observations are consistent with EIPA being a more potent inhibitor of $Na^+$-independent dipeptide uptake via hPepT1, in its protonated form (fig. 2C). In contrast, the ability of EIPA to inhibit $Na^+$/noradrenaline transport in PC12 cells is increased as extracellular pH is alkalinised, and is also increased as extracellular $Na^+$ is lowered [44]. This suggests that the membrane-permeable weak base crosses the membrane and binds to an intracellular unloaded $Na^+$-binding site on the $Na^+$/noradrenaline transporter [44].

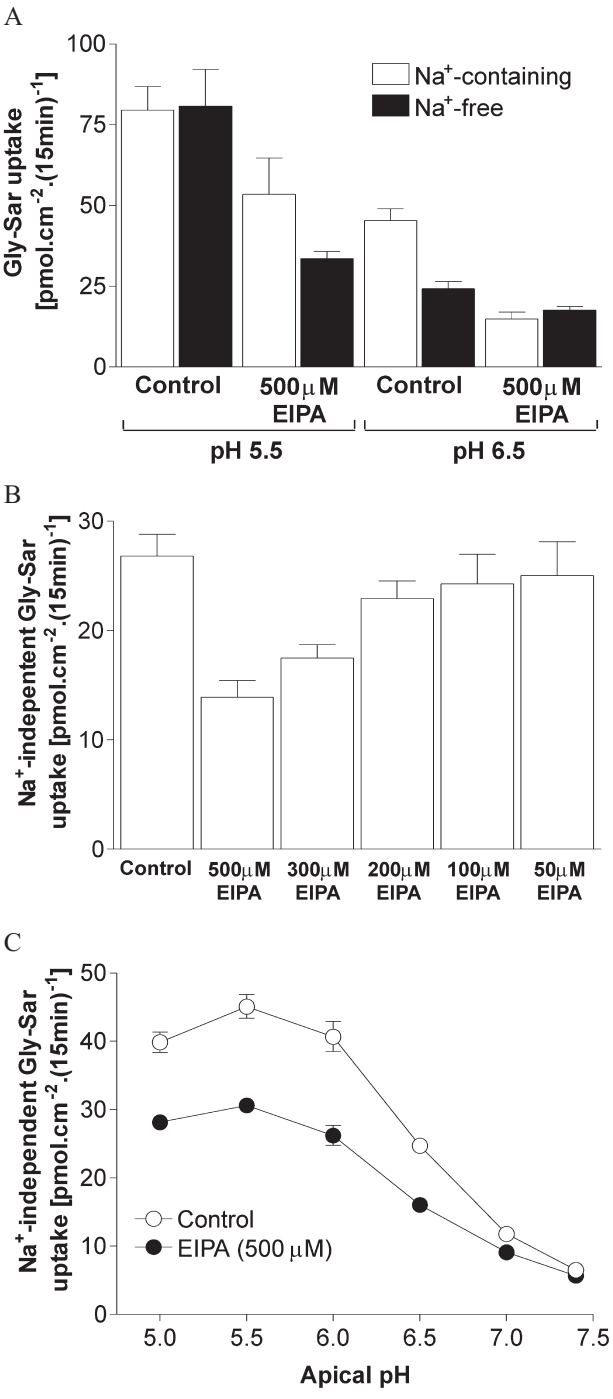



Figure 2. $Na^+$-independent inhibition of dipeptide uptake by EIPA. (*A*) Gly-Sar (0.5 µCi $ml^{-1}$, 10 µM) uptake (15 min) across the apical membrane of Caco-2 cell monolayers was measured in the presence (open columns) and absence (filled columns) of extracellular $Na^+$ and in the presence and absence of 500 µM EIPA at apical pH 5.5 and 6.5. Results are the mean ± SE (n=5). (*B*) Apical Gly-Sar (0.5 µCi $ml^{-1}$, 10 µM) uptake (apical pH 6.5, 15 min) was measured in the absence of extracellular $Na^+$ and in the presence of apical EIPA (50–500 µM). Results are mean ± SE (n=5). (*C*) Apical Gly-Sar (0.5 µCi $ml^{-1}$, 10 µM) uptake (15 min) was measured in the absence of extracellular $Na^+$ and in the presence of 500 µM EIPA over the pH range pH 5.0–7.4. Results are the mean ± SE (n=10).

We have previously shown that 3 µM S1611 reduces Gly-Sar uptake at apical pH 6.5 in the presence of extracellular $Na^+$ down to the levels of uptake observed in the absence of $Na^+$ [20]. Similarly, maximal inhibitory effects of S1611 on apical $^{22}Na^+$ and Gly-Sar uptake were observed at 3 µM and above (fig. 1A, C). Figure 3A demonstrates the ability of 3 µM S1611 to inhibit Gly-Sar uptake at apical pH 6.5 in the presence of $Na^+$ ($p<0.001$ vs control). EIPA (100 µM) had a similar inhibitory effect to that observed with S1611 ($p<0.001$ vs control, $p>0.05$ vs S1611). At these concentrations, both compounds are apparentty inhibiting Gly-Sar uptake via the same mechanism as no additivity is observed when both compounds are used together (fig. 3A, $p>0.05$ vs S1611 alone, $p>0.05$ vs EIPA alone). However, when the concentration of EIPA was raised to 500 µM, an additional component of Gly-Sar uptake was inhibited ($p<0.01$ vs 100 µM EIPA).

We have shown previously that the relative $Na^+$ dependence of dipeptide uptake increases with the incubation period used, presumably due to the fact that over very short incubation periods the $H^+$ electrochemical gradient will not be run down even in the absence of extracellular $Na^+$ [16]. Similarly, when Gly-Sar uptake is measured over 1 min, there is no effect of S1611 ($p>0.05$ vs control), whereas after a 15-min incubation Gly-Sar uptake was reduced ($p<0.001$) by almost 50% in the presence of S1611 (fig. 3B). At each time point, Gly-Sar uptake was lower in the presence of 500 µM EIPA than control conditions or in the presence of S1611. Although there is a clear increase in the S1611-sensitive component with time (and this putative NHE3-sensitive component will also be inhibited by EIPA), there is an additional component of inhibition of Gly-Sar uptake in the presence of 500 µM EIPA. Thus the proportion of uptake sensitive to S1611 increases with time (0% at 1 min, 14% at 2 min, 24% at 5 min and 42% at 15 min) due to the increased requirement for NHE3 activity, whereas the additional S1611-insensitive EIPA-inhibitable component remains fairly constant with time (22% at 1 min, 35% at 2 min, 26% at 5 min and 36% at 15 min). These data support the hypothesis that EIPA is inhibiting dipeptide uptake both indirectly (through inhibition of NHE3) and directly (via direct inhibition of hPepT1).

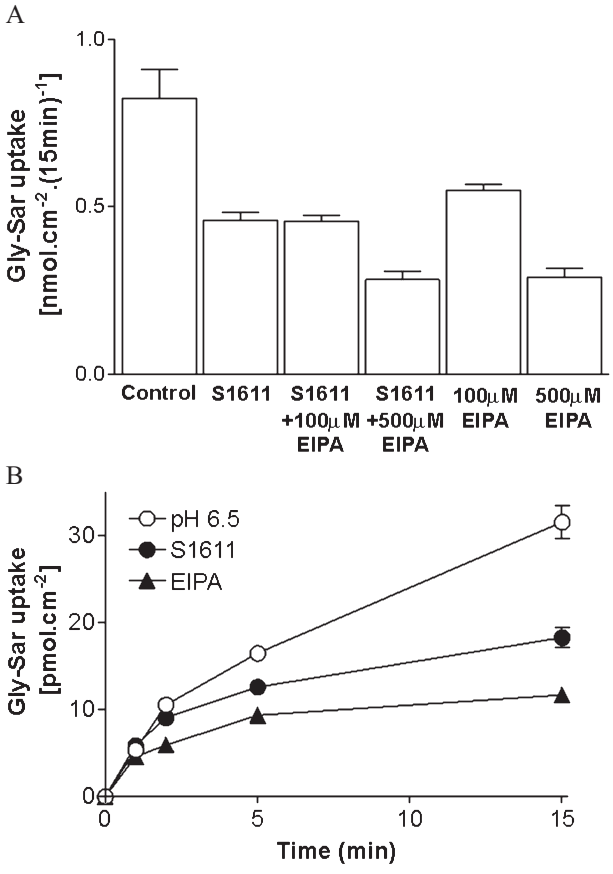



Figure 3. EIPA and S1611 inhibit dipeptide uptake by both similar and distinct mechanisms. (*A*) Gly-Sar (0.5 µCi $ml^{-1}$, 100 µM) uptake (15 min) across the apical membrane of Caco-2 cell monolayers was measured at apical pH 6.5 in the presence of extracellular $Na^+$ and the absence and presence of apical EIPA (100 or 500 µM) and/or 3 µM apical S1611. Results are the mean ± SE (n=5). (*B*) Apical Gly-Sar (0.5 µCi $ml^{-1}$, 10 µM) uptake (1–15 min) was measured at apical pH 6.5 in the presence of extracellular $Na^+$ at apical pH 6.5 in the presence and absence of 500 µM EIPA or 3 µM S1611. Results are the mean ± SE (n=5).

**The effects on $Na^+$-independent dipeptide uptake of EIPA, MIA, DMA and amiloride**

Gly-Sar (10 µM) uptake (at apical pH 6.0, 15 min) across the apical membrane of Caco-2 cell monolayers measured in the absence of extracellular $Na^+$ [34.26±1.34 (n=12) pmol $cm^{-2}$ $(15min)^{-1}$] was reduced ($p<0.001$) by 500 µM EIPA [to 20.70±1.03 (n=12) pmol $cm^{-2}$ $(15\ min)^{-1}$] and 500 µM MIA [to 25.94±1.51 (n=11) pmol $cm^{-2}$ $(15\ min)^{-1}$]. In contrast, 500 µM DMA caused only a small reduction (to 29.64±1.02 (n=12) pmol $cm^{-2}$ $(15\ min)^{-1}$, $p>0.05$) and amiloride was without effect (37.37±1.17 (n=11) pmol $cm^{-2}$ $(15\ min)^{-1}$, $p>0.05$). MIA is relatively lipophilic (94% distribution in octanol versus an aqueous phosphate buffer) compared to DMA (6%) or amiloride (5%) [40]. The relative lipophilicity of EIPA has not been reported although is likely to be MIA-like. Thus, the most potent members of the amiloride group of compounds to inhibit the $Na^+$-independent NHE3-insensitive component of dipeptide uptake are apparently those which are most lipophilic. However, this may not have anything to do with their ability to access the target site, as EIPA is most effective (fig. 2C) when it is in the protonated form which will be less membrane permeable. In addition, the pH dependence of the EIPA effect on dipeptide uptake suggests that the inhibitory mechanism is distinct from the intracellular inhibition of the $Na^+$/noradrenaline transporter [44].

**Is the $Na^+$-independent inhibitory effect of EIPA on dipeptide uptake a direct effect on hPepT1, a general effect on membrane transport or an effect on cell viability, membrane potential or $pH_i$?**

Amiloride and analogues have multiple effects on many cellular processes depending upon the concentration of drug used [40]. This raises the possibility that the reduction in dipeptide uptake in the presence of 500 µM EIPA could be due to some general effect on cell viability. Although Gly-Sar uptake across the apical membrane of Caco-2 cell monolayers in the absence of extracellular $Na^+$ was reduced ($p<0.001$) in the presence of 500 µM EIPA, no inhibitory effect was seen on either leucine (a dipolar amino acid) or lysine (a dibasic amino acid) uptake (fig. 4). Rather leucine and lysine uptake were stimulated (both $p<0.001$) in the presence of 500 µM EIPA (fig. 4). $Na^+$-independent lysine uptake is via a membrane potential-sensitive carrier [45]. Since EIPA stimulates dibasic amino acid uptake via this membrane potential-sensitive system, the effect on dipeptide transport cannot be due to a general depolarisation of the membrane potential as this would cause a decrease in lysine uptake (rather than the increase observed). Nor can there be a general non-specific effect of EIPA on cell viability as this would reduce uptake of all three substrates.

Although EIPA does not appear to affect membrane potential, it could affect the pH gradient across the apical membrane by crossing the cell membrane and changing $pH_i$. Indeed the uncharged unprotonated form of amiloride (which will be most abundant at alkaline pH values around the $pK_a$ of 8.7–8.8) will cross membranes, become protonated (and trapped) in the intracellular compartment [40, 46] and alkalinise $pH_i$ (and thus increase the driving force for PepT1 transport). Amiloride and analogues can collapse pH gradients in this way by accumulating within and alkalinising acidic compartments [40]. Alternatively, if the protonated charged form of amiloride or EIPA (which will be more abundant at relatively acidic pHs) crosses the membrane it could accumulate in the relatively alkaline intracellular environment, release a $H^+$ and thus decrease $pH_i$ (which would decrease the driving force for PepT1 transport). The greater lipophilicity of the amiloride compounds in their unprotonated rather than protonated forms [40] indicates that any effect on $pH_i$ is most likely to be observed at more alkaline $pH_o$ values. This suggests that the pH-dependent inhibitory effects of EIPA in figure 2C are not due to effects on the transapical membrane pH gradient.

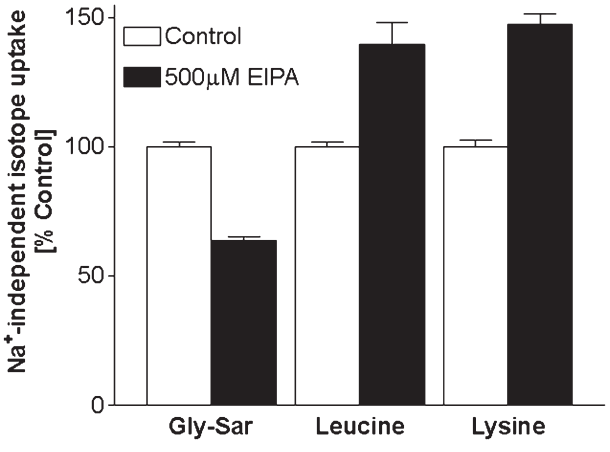



Figure 4. The effects of EIPA on $Na^+$-independent dipeptide and amino acid uptake. Uptake (15 min, pH 6.0, $Na^+$-free) of Gly-Sar, leucine and lysine (all 0.5 µCi $ml^{-1}$, 10 µM) was measured across the apical membrane of Caco-2 cell monolayers in $Na^+$-free conditions and in the presence and absence of 500 µM EIPA. Results for each substrate are expressed as % control (uptake in absence of EIPA) and are the mean ± SE (n=10).

Nevertheless, to discriminate between direct effects on transport and indirect effects on gradient, and to discount the possibility that EIPA was running down the $H^+$ electrochemical gradient, $Na^+$-independent Gly-Sar uptake was measured in the presence and absence of EIPA (500 µM) following a 10-min pre-incubation also in the presence or absence of 500 µM EIPA (fig. 5). At the more alkaline pH of 8.0, apical Gly-Sar uptake was reduced in the presence of EIPA (fig. 5A, $p<0.001$ vs control). However, apical Gly-Sar uptake in the absence of EIPA, but following a 10-min pre-incubation with EIPA, was also significantly ($p<0.05$ vs control) reduced (fig. 5A). At pH 8.0, EIPA will behave like a permanent weak base and will enter the cells and alkalinise $pH_i$. After the pre-incubation, if extracellular EIPA is removed, unprotonated EIPA will exit the cells towards the extracellular alkaline (pH 8.0) environment thus leaving protons in the intracellular compartment and decreasing $pH_i$ (in much the same way that $NH_4Cl$ is used to acidify cells). This non-specific effect could thus account for the inhibition of Gly-Sar uptake at pH 8.0 observed when EIPA is present during the pre-incubation alone (fig. 5A). However, the inhibitory effect of EIPA at pH 8.0 when present only during the uptake (fig. 5A) can only be due to a specific effect on the transporter since under these conditions any non-specific effect of EIPA on the gradient would cause an increase in $pH_i$ which would only increase the driving force for hPepT1 activity.

As expected, apical Gly-Sar uptake at pH 5.0 was significantly reduced ($p<0.001$) by 500 µM EIPA (fig. 5B). If EIPA was being transported across the apical membrane in its protonated charged form (which will be most abundant at pH 5.0), once exposed to the relatively alkaline intracellular environment some EIPA is likely to dissociate and release protons which would decrease $pH_i$ and, therefore, reduce hPepT1 activity indirectly through a reduction in the driving force. However, unlike at pH 8.0, apical Gly-Sar uptake at pH 5.0 (in the absence of EIPA) was unaffected ($p>0.05$) by a pre-incubation with EIPA (fig. 5B), suggesting that at pH 5.0, EIPA is not having any non-specific effect on $pH_i$. This suggests that the $Na^+$-

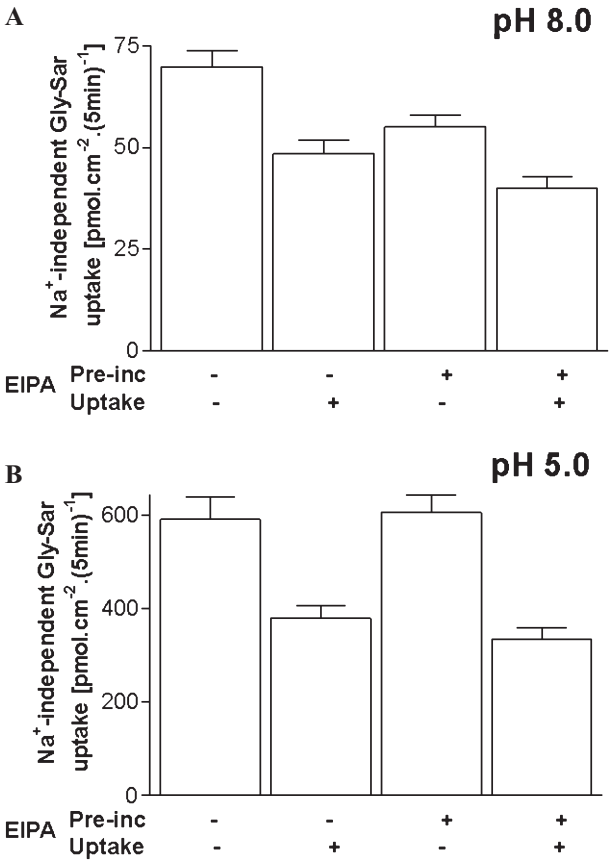



Figure 5. The effects of pre-incubation with EIPA on $Na^+$-independent dipeptide uptake. Gly-Sar uptake (100 µM, 5 min) was measured across the apical membrane of Caco-2 cell monolayers in $Na^+$-free conditions at apical pH 8.0 (*A*) or pH 5.0 (*B*) in the presence (+) and absence (–) of 500 µM EIPA (Uptake). Before the uptake measurements were made, all cell monolayers were pre-incubated for 10 min at 37 °C in an $Na^+$-free solution [pH 8.0 (*A*) or pH 5.0 (*B*)] in the presence (+) or absence (–) of 500 µM EIPA (Pre-inc). Between the pre-incubation and uptake periods, the cell monolayers were washed for 3 min in warm (37 °C) $Na^+$-free Krebs' solution at pH 8.0 (*A*) or pH 5.0 (*B*) (as appropriate) in the presence or absence of 500 µM EIPA (as appropriate). Data are the mean ± SE (n=12).

independent inhibition of Gly-Sar uptake by EIPA at acidic apical pH values such as pH 5.0 (figs 2C, 5B) is due to a direct effect of EIPA on hPepT1 rather than an indirect effect due to an EIPA-induced intracellular acidification.

## Discussion

The absorption of many nutrients and nutrient-like drugs across the small intestinal wall is mediated by cotransport through ion-coupled transport proteins where the energy for the movement is stored in transepithelial and transmembrane ionic gradients. Therefore, the absorptive capacity of the human intestinal epithelium is not simply dependent upon the expression of ion/nutrient cotransport proteins but is also exquisitely sensitive to the ionic composition of the microenvironments bathing both intra- and extracellular surfaces of the brush-border membrane. During the absorptive process, the intestinal epithelial cell must be able to establish and maintain the transmembrane ionic gradients so that optimal absorption is achieved. Thus, the maximal absorptive capacity of the intestinal epithelial brush-border surface for transport of small di/tripeptides and peptide-like drugs via hPepT1 is also dependent upon the coexpression and cooperative functional activity of the $Na^+/H^+$ exchanger NHE3 so that peptide-cotransported protons are recycled back across the brush-border surface to maintain the driving force for further hPepT1-mediated transport [16, 20, 21, 23, 28, 29]. Indeed, NHE3 is normally quiescent at resting $pH_i$ values but is activated by transport through the $H^+$-coupled di/tripeptide transporter hPepT1 [23]. Since optimal hPepT1-mediated transport is only accomplished in the presence of functional NHE3 activity [16, 20, 21], peptide absorption has the potential to be regulated directly (via effects on hPepT1) and indirectly (via effects on NHE3 and the maintenance of the driving force).

In this study we used a panel of selective $Na^+/H^+$ inhibitors with a broad range of affinities to identify the polarised distribution of $Na^+/H^+$ exchangers in monolayers of the human intestinal epithelial cell line Caco-2. This pharmacological identification of NHE3 at the apical membrane and NHE1 at the basolateral membrane is consistent with previous investigations using Caco-2 cells and mammalian small intestinal tissues using a variety of techniques aimed at functional and protein localisation [23, 26, 27, 38, 39]. The identical rank orders of potency of this panel of NHE inhibitors for inhibition of NHE3 activity and hPepT1 transport (fig. 1) in the presence of extracellular $Na^+$ confirm that inhibition of NHE3 leads to an indirect reduction in hPepT1 activity by decreasing the driving force for transport (the $H^+$ electrochemical gradient) [16, 20, 21]. The slight differences in $IC_{50}$ observed with the two measurements may be due to different experimental conditions used (e.g. apical pH, extracellular $Na^+$ concentration and intracellular pH following the $NH_4Cl$ prepulse-and-release manoeuvre) as, for example, the potency of amiloride (and analogues) for inhibition of $Na^+/H^+$ exchange is known to be sensitive to both extracellular $Na^+$ concentration and pH [40]. Amiloride (and analogues) has a higher affinity for inhibition of $Na^+/H^+$ exchange at low $Na^+$ concentrations so becomes a more effective inhibitor as the extracellular $Na^+$ concentration is decreased [40]. Similarly, amiloride and analogues inhibit $Na^+/H^+$ exchange in their protonated forms so are likely to be more effective at pH values well below the $pK_a$ ($pK_a$ 8.7–8.8 for amiloride) [40]. In addition, the effect on NHE3 is direct whereas the effect on hPepT1 is indirect through a reduction in the transmembrane ionic driving force. Despite the slight differences in affinity the rank

orders of potency support the conclusion that NHE3 inhibition leads to an indirect inhibition of hPepT1.

The relatively high affinity of the NHE3-selective inhibitors S1611 and S3226 (fig. 1) suggest that they will prove to be excellent tools for investigating the importance of NHE3 in optimal nutrient and drug absorption across the small intestinal wall. For example, previous studies have demonstrated clearly that S1611 can only reduce the intestinal epithelial absorptive capacity for PepT1 substrates under conditions in which NHE3 is active [20, 21]. In the absence of extracellular $Na^+$, S1611 has no effect on dipeptide transport [20]. In contrast, in this study, we report for the first time that analogues of amiloride can also inhibit dipeptide transport, via hPepT1, in a non-NHE3-dependent manner. In addition to their effect on the NHE3-dependent component of Gly-Sar uptake, EIPA and MIA also reduced dipeptide transport in the absence of extracellular $Na^+$ and at apical pH values at which NHE3 will be inactive. The results presented in this study suggest that this $Na^+$-independent effect of EIPA is direct on hPepT1 as there are no indirect effects on membrane potential, $pH_i$ or the transmembrane pH gradient, or any general non-specific inhibitory effect on other membrane transport proteins. Nor are the characteristics of the pH-dependent and $Na^+$-independent effects of EIPA on hPepT1 activity consistent with simple physicochemical properties of the amiloride analogues. The exact mechanism responsible for the EIPA-induced inhibition of hPepT1 activity (in the absence of $Na^+$) is not clear but it seems highly unlikely that EIPA is transported via hPepT1 as EIPA does not possess the recognition elements within its structure to suggest that it would be a high-affinity substrate for hPepT1 [47]. The effects of amiloride and analogues on other transport proteins are generally thought to be the result of non- or uncompetitive binding. The effect on the $Na^+$/noradrenaline transporter is thought to result from EIPA binding to an internal empty $Na^+$ site [44]. The effects of amiloride and analogues on $Na^+/H^+$ exchangers may involve binding at or near to an $Na^+$-binding site as well as a non-cation-binding site [24]. Two regions of the NHEs, transmembrane domain 4 and the region between transmembrane domains 8 and 10, have been demonstrated to be involved in determining the affinity of the various NHE isoforms for amiloride and analogues [24]. Which part of the hPepT1 transporter is sensitive to binding by amiloride and analogues is not known.

Amiloride and its analogues have proven to be very useful tools for the investigation of ion transport processes in many cell types [40]. However, these compounds have multiple effects and it is sometimes difficult to determine whether the effects are direct on the target function of interest or involve other mechanisms activated or inhibited downstream or upstream of the original target. Clearly, interpreting the results of many studies using these compounds requires detailed knowledge of the relative ability of these drugs to inhibit different transport and cellular processes [40]. At concentrations higher than those required to inhibit $Na^+/H^+$ exchange, amiloride and analogues interact with a number of other $Na^+$-dependent transport systems [40, 44]. In addition, amiloride and analogues may affect many other cellular processes that are either dependent or independent of $Na^+/H^+$ exchange [40].

Using the human intestinal cell line Caco-2 we have identified the small intestinal epithelium and the close functional relationship between NHE3 and hPepT1 as a potential site for drug-drug interactions [16, 20, 21, 23]. There is both pharmaceutical and clinical relevance in these observations. In human volunteers following an overnight fast, the absorption rate and absolute bioavailability of the aminopenicillin amoxicillin (an hPepT1 substrate) was reduced by a single oral dose (10 mg/100 ml) of the commonly prescribed diuretic amiloride, 2 h before oral administration of amoxicillin [48]. At the concentration used (approximately 400 µM), amiloride will reduce, at least partly, the activity of the $Na^+/H^+$ exchanger NHE3 suggesting that the effect of amiloride is to inhibit NHE3 activity and reduce the driving force for hPepT1 activity. At the level of the enterocyte, experiments using the selective NHE3 inhibitor S1611 support this hypothesis where S1611 inhibits apical Gly-Sar uptake (by decreasing $V_{max}$) in Caco-2 cell monolayers but only in the presence of extracellular $Na^+$ [20]. A recent study investigated the in vivo effects of amiloride on amoxicillin permeability following perfusion through the human jejunum [49]. In contrast to the earlier study [48], no effect of amiloride on amoxicillin absorption was observed, although this could have been due to the dose of amiloride (110 µM amiloride, in a pH 6.5 buffer including 147 mM $Na^+$) used, which was too low to effectively inhibit the amiloride-insensitive $Na^+/H^+$ exchanger NHE3 (particularly in the presence of a high concentration of $Na^+$). As stated above, the $IC_{50}$ for amiloride-induced inhibition of NHE3 is 100–438 µM and a concentration of 1 mM is required to inhibit NHE3 completely [34–39].

In the study we report that $Na^+/H^+$ exchange inhibitors can inhibit the capacity of hPepT1 by two distinct modes. S1611, S3226, EIPA, MIA and DMA all reduce hPepT1 activity indirectly by inhibition of NHE3. In addition, at concentrations higher than those required to inhibit NHE3, EIPA and MIA also have direct inhibitory effects on hPepT1. These observations emphasise the importance of detailed knowledge of the pharmacology of any potential drug compound. In addition, they highlight that care needs to be taken before firm conclusions can be made as to whether an effect is direct, on a target protein, or indirect due to activation of a cascade of events following inhibition or activation of an ion-transporting membrane protein [40].

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