Evaluation of PepT1 transport of food-derived antihypertensive peptides, Ile-Pro-Pro and Leu-Lys-Pro using *in vitro*, *ex vivo* and *in vivo* transport models

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Abstract

Ile-Pro-Pro (IPP) and Leu-Lys-Pro (LKP) are food-derived antihypertensive peptides which inhibit angiotensin-converting enzyme (ACE) and may have potential to attenuate hypertension. There is debate over their mechanism of uptake across small intestinal epithelia, but paracellular and PepT1 carrier-mediated uptake are thought to be important routes. The aim of this study was to determine their routes of intestinal permeability using *in vitro*, *ex vivo* and *in vivo* intestinal models. The presence of an apical side pH of 6.5 (mimicking the intestinal acidic microclimate) and of Gly-Sar (a high affinity competitive inhibitor and substrate for PepT1) were tested on the transepithelial apical to basolateral (A to B) transport of [^{3}H]-IPP and [^{3}H]-LKP across filter-grown Caco-2 monolayers *in vitro* and rat jejunal mucosae *ex vivo*. A buffer pH of 6.5 on the apical side enabled Gly-Sar to reduce the apparent permeability (P_{app}) of [^{3}H]-IPP and [^{3}H]-LKP, but this inhibition was not evident at an apical buffer pH of 7.4. Gly-Sar reduced the P_{app} across isolated jejunal mucosae and the area under the curve (AUC) in intra-jejunal instillations when the apical/luminal buffer pH was either 7.4 or 6.5. However, the jejunal surface acidic pH was maintained in rat jejunal tissue even when the apical side buffer pH was 7.4 due to the presence of the microclimate which is not present in monolayers. PepT1 expression was confirmed by immunofluorescence on monolayers and brush border of rat jejunal tissue. This data suggest that IPP and LKP are highly permeable and cross small intestinal epithelia in part by the PepT1 transporter, with an additional contribution from the paracellular route.

Keywords: Antihypertensive peptides; food-derived bioactives; intestinal transport; PepT1; Ile-Pro-Pro; Leu-Lys-Pro; transport models.

1 Introduction

Food-derived bioactive peptides may provide beneficial physiological effects when released from parent proteins [1]. Recent research has focused on food-derived peptides with potential anti-hypertensive, anti-diabetic, anti-oxidant and anti-inflammatory activities. To date, such peptides have shown modest effects in human trials, and together with the equivocal efficacy, their epithelial permeation pathways across the small intestine are still unknown [2-5]. The antihypertensive lactotripeptide, Ile-Pro-Pro (IPP) was isolated from bovine milk β-casein following fermentation by *Lactobacillus helveticus* [6]. Leu-Lys-Pro (LKP) isolated from the bonito fish muscle also has anti-antihypertensive properties [7]. They are both competitive inhibitors of angiotensin converting enzyme (ACE) albeit with low potency, but nevertheless they induce hypotensive responses in the Spontaneously Hypertensive Rat (SHR) model following either intravenous or oral administration [8, 9].

IPP and LKP must overcome the barriers present in the small intestine to achieve systemic delivery in order to illicit hypotensive action. Oral delivery requires a peptide to be stable in stomach acid, soluble at small intestinal fluid pH, to be resistant to peptidase enzymes in the small intestinal lumen and brush border, and to have sufficient muco-diffusion and epithelial permeability [10]. Peptides typically have low and variable intestinal epithelial permeability, which results in poor oral bioavailability due to their large molecular weight (MW), hydrophilicity, and susceptibility to enzymatic degradation [11]. However, both IPP and LKP have relatively low MWs (325 Da and 356 Da respectively), and therefore may not encounter the same permeability issues as larger peptides. In addition, the presence of the cyclic amino acid, proline, in the primary structure of IPP and LKP provides resistance to enzymatic degradation in the small intestine [12-14]. The possible permeability routes for IPP and LKP

include likely contributions from the H^+ coupled di- and tripeptide co-transporter PepT1 (encoded by SLC15A1) [15], and paracellular transport [16, 17].

There is evidence of both paracellular- and PepT1-mediated uptake of other di- and tripeptides, although there is debate on which route is of more significance [16, 18, 19]. Contribution of PepT1-mediated uptake can be determined *in vitro* in Caco-2 monolayers by saturating PepT1 with glycyl-sarcosine (Gly-Sar), a high affinity dipeptidyl competitive inhibitor and substrate [20, 21]. A decrease in permeability in the presence of Gly-Sar suggests that the candidate peptide is a substrate for PepT1 [22, 23]. However, this conclusion for many molecules relies mainly on tests using Caco-2 monolayers, which may not correlate with *in vivo* in terms of PepT1 expression and function. Foltz *et al.* demonstrated that there was low permeability of IPP across Caco-2 monolayers (apparent permeability coefficient, $P_{app} = 1 \times 10^{-8}$ cm/s) and across distal rat jejunum mounted in Ussing chambers ($P_{app} = 5 \times 10^{-8}$ cm/s), but they did not test its permeability in the presence of a PepT1 inhibitor [19]. PepT1 functions in conjunction with the Na^+/H^+ exchanger (NHE3), which contributes to the small intestinal epithelial acidic microclimate in the immediate layer above the epithelium, thereby providing protons for inwardly-directed symport with substrates [24]. Despite the presence of an unstirred layer, the microclimate is not present in filter-grown Caco-2 monolayers, perhaps due to the lack of goblet cells to produce overlying mucus [25, 26].

The aim of this study was to determine the extent and major route(s) of permeability of [^{3}H]-IPP and [^{3}H]-LKP in Caco-2 monolayers, isolated rat jejunal tissue in Ussing chambers, and intra-jejunal instillations in rats. In order to assess the degree of permeation due to PepT1-

mediated uptake, Gly-Sar was tested as an inhibitor of inward peptide flux in each model. An apical-side pH of 6.5 is required to mimic the small intestinal acidic microclimate [27], therefore, the effect of apical-side/luminal buffer pH of either 6.5 or 7.4 was tested in each model. The effect of these buffers on the jejunal acidic microclimate was also directly assessed in rat jejunal sacs as it was not possible to measure surface pH in Ussing chambers. Some transport models have limitations therefore it is necessary to assess the transport of IPP and LKP in more than one experimental model. The data show that PepT1 plays an important role in the intestinal uptake of these molecules, and that, based on the *in vitro* P_{app} values and levels of radiolabelled peptide achieved in plasma following intestinal instillations, both IPP and LKP have good permeability.

2 Materials

2.1 Reagents and Chemicals

[^{3}H]-IPP and [^{3}H]-LKP (specific activity 21 Ci/mmol) were obtained from Cambridge Research Biochemicals (Billingham, UK). [^{14}C]-mannitol (specific activity 250 µCi/mmol) was obtained from Perkin Elmer (UK). All other reagents, chemicals, and solvents were analytical grade from Sigma-Aldrich (UK). Caco-2 cells (passage 48-58) were obtained from European Collection of Cell Cultures (Salisbury, UK).

2.2 Caco-2 cell monolayers

Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (2 mM), 1% non-essential amino acids, penicillin (100 U)/streptomycin (100 µg/ml) and 10% foetal bovine serum (Gibco, Biosciences Ireland) on 75 cm^2 tissue culture flasks at 95% O_2/5% CO_2 at 37°C in a humidified environment. Cells were seeded at a density of 3 x 10^5 cells/well on 1.12 cm^2 Transwell® filters (polycarbonate, pore size 0.4 µm) (Corning Costar Corp., USA) and grown for 21 days in DMEM for transport experiments [28]. Transepithelial

electrical resistance (TEER, Ω.cm^2) was measured across the monolayers using an EVOM® voltohmmeter with a chopstick electrode (EVOM®, WPI, UK). TEER measurements were made prior to transport studies and then every 30 min over a period of 120 min to confirm monolayer integrity.

2.3 Caco-2 monolayer transport studies

Apical-to-basolateral (A to B) transport of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol were examined across monolayers. The transport buffer consisted of HBSS supplemented with 12.5 mM glucose and either 25 mM HEPES (pH 7.4) or 10 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 6.5) [26, 28]. DMEM was replaced with HBSS and equilibrated for 30 min in the presence or absence of apical-side 10 mM Gly-Sar in HBSS. This was carried out in the presence of apical-side pH adjustment (apical side contained HBSS at pH 6.5, basolateral side contained HBSS at pH 7.4) or under standard pH conditions (both apical and basolateral sides contained HBSS at pH 7.4). At time zero, [^{3}H]-IPP (1 µCi/ml; 3 µM), [^{3}H]-LKP (1 µCi/ml; 3 µM) or [^{14}C]-mannitol (0.1 µCi/ml) was added to the apical side. Basolateral samples were taken every 30 min for 120 min and apical samples were taken at 0 and 120 min in order to calculate the P_{app}. Withdrawn samples were replaced with an equal volume of fresh HBSS. Both basolateral and apical samples of 100 µl were mixed with 5 ml scintillation fluid and measured in a liquid scintillation counter (Packard Tricarb 2900 TR). Basal transepithelial electrical resistance (TEER) values were required to be > 1400 Ω.cm^2 in order to be included [29]. The P_{app} for each marker was calculated according to the following equation:

$$\boldsymbol{P_{app}} = \frac{\boldsymbol{dQ}}{\boldsymbol{dt}} \frac{\mathbf{1}}{\boldsymbol{A \cdot C_0}} \quad (1)$$

where dQ/dt is the transport rate across the epithelium, *A* is the surface area (1.12 cm^2) and C_0 is the starting concentration of flux marker on the apical side [28]. Caco-2 transport experiments were run in triplicate (n=3) with 3 independent replicates.

2.4 Rat intestinal tissue mucosae: dissection and electrophysiology

Studies were carried out in accordance with UCD Animal Research Ethics Committee protocol (AREC 14-28-Brayden) and in adherence with the "Principles of Laboratory Animal Care" (NIH Publication #85-23, revised in 1985). Male Wistar rats (250-350 g; Charles River, UK and UCD Biomedical Facility, Ireland) were euthanised by stunning and cervical dislocation in accordance with recommended procedures. Jejunum was removed 10-12 cm proximal to the stomach, opened along the mesenteric border and rinsed with warm oxygenated Krebs-Henseleit buffer (KH) [14]. Mucosae with underlying intact muscle was mounted in Ussing chambers with a circular window area of 0.63 cm^2 [30], bathed bilaterally with 5 ml KH and continuously gassed with 95% CO_2/5% O_2 and maintained at 37°C. The transepithelial potential difference (PD; mV) and short circuit current (I_{sc}, µA) were measured across jejunal tissue using a DVC-4000 voltage clamp apparatus (WPI, UK). After an initial 30 min equilibration, PD and I_{sc} were used to calculate the TEER over 120 min [31].

2.5 Isolated rat jejunal mucosae transport studies

Transepithelial permeability of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol were measured across jejunal mucosae mounted in Ussing chambers. The apical-side transport buffers consisted of KH (pH 7.4) or KH modified with 10 mM MES (pH 6.5), while the the basolateral buffer was KH (pH 7.4) throughout [32]. Tissues were equilibrated for 30 min in the presence or absence of 10 mM Gly-Sar on the apical side. At time zero, [^{3}H]-IPP (1 µCi/ml; 3 µM), [^{3}H]-LKP (1 µCi/ml; 3 µM) or [^{14}C]-mannitol (0.1 µCi /ml) was added apically. Basolateral samples (100

µl) were taken every 30 min for 120 min and apical samples (100 µl) were taken at 0 and 120 min in order to calculate the P_{app}. Samples of 100 µl were mixed with 5 ml scintillation fluid and read in a liquid scintillation counter as described above. The P_{app} for [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol was calculated according to equation (1) used for monolayers, the only difference being that the surface area for jejunal mucosae was 0.63 cm^2. Basal jejunal TEER values were required to be > 30 Ω cm^2 or were otherwise excluded [30]. Jejunal mucosae transport experiments were carried out as five independent replicates.

2.6 Measurement of rat jejunal surface pH

The pH of the jejunal surface was measured using a micro pH combination electrode (3.6 mm diameter, Sigma Aldrich, UK) and a Hanna HI 2210 pH Meter [27]. Tissue was harvested, flushed with pre-warmed PBS and a pH probe was inserted into the jejunal lumen to record untreated native pH value. Non-everted gut sacs (5 cm in length) were prepared according to previous methods [33], and injected with 0.25 ml of KH (pH 7.4) or KH (pH 6.5) with a 30G needle and maintained in oxygenated KH for 120 min. The sacs and ligation was removed, and flushed with pre-warmed PBS. The pH probe was inserted into the lumen and the pH recorded. Measurements were recorded at 0 and 120 min after incubation. Experiments were carried out using independent replicates from five animals.

2.7 Rat intra-intestinal *in situ* jejunal instillations

All animal experimental procedures in the study adhered to the EC Directive 86/609/EEC for animal experiments and were performed in compliance with the Irish Health Products Regulatory Authority animal licence number AE18982/P037. Male Wistar rats (Charles River, UK) weighing 280-350 g were used. Animals were housed under controlled environmental conditions regarding humidity and temperature with a 12:12 h light/dark cycle. Rats received filtered water and standard laboratory chow *ad lib* and were fasted for 16-20 h

prior to procedure with free access to water. Anaesthesia was induced with isoflurane gas (Iso-Vet, 1000 mg/g isoflurane liquid for inhalation, Piramal Healthcare, UK) at a rate of 4000 ml/min mixed with 4000 ml/min O_2 in an induction chamber. Anaesthesia was maintained with isoflurane 2500 ml/min mixed with 1500 ml/min O_2 using vaporising unit with a delivery mask (Blease Medical Equipment Ltd., UK). Animals were euthanised at the end of the experiment by intracardiac injection of 0.4 ml pentobarbital sodium (EUTHATAL™, Merial Animal Health Ltd., UK).

Isoflurane-anaesthetised rats were placed on a temperature controlled heat pad, and intra-jejunal instillations were performed as previously described, but with minor modifications [34]. Following a midline laparotomy, the jejunum was identified and tied off at both ends 5-7cm apart with a size 4 braided silk suture. Test solutions of 300 µl of [^{3}H]-IPP (8 µCi/kg; 24 µM) or [^{3}H]-LKP (8 µCi/kg; 24 µM) in Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.4) or DPBS buffered with 10 mM MES (pH 6.5), were injected into the lumen using a 1 ml syringe fitted with a 30G needle. 100 mM Gly-Sar was solubilised in either DPBS adjusted to pH 6.5 or in DPBS at pH 7.4 [35]. Blood samples (~400 µl) were taken via the retro-orbital route at 0, 30, 60, 90 and 120 min into 1 ml Eppendorf tubes and stored on ice at 2-8°C prior to centrifugation (6500g, 5 min) and serum collection. Serum (100 µl) was mixed with 5 ml scintillation fluid and read in a liquid scintillation counter. Levels of [^{3}H]-IPP and [^{3}H]-LKP in serum samples were used to determine the area under the curve (AUC).

2.8 Histology and immunofluorescence of monolayers and jejunal tissue

Following the studies in jejunal tissue in Ussing chambers and intra-jejunal instillations, tissue was immersed in 10% (v/v) buffered formalin for 48 h. Tissues were prepared, paraffin-embedded, cut with a microtome, and dried overnight at 60°C. Tissues were stained with haematoxylin and eosin (H&E), Alcian blue and neutral red. Slides were visualised

under a light microscope (NanoZoomer 2.0-HT light microscopy, Hamamatsu) and images were taken with high-resolution camera (Micropublisher 3.3 RTV, QImaging) and Image-Pro® Plus version 7.1 (Media Cybernetics Inc., USA) acquisition software.

The localisation of PepT1 and occludin was determined by immunofluorescence microscopy as previously described [36, 37], but with modifications. Briefly, paraffin- embedded jejunal segments on charged slides were deparaffinised with xylene and ethanol before incubation in pre-warmed (90°C) 10 mM sodium citrate and 2 mM citric acid buffer for 10 min, and then cooled to room temperature over 30 min. Caco-2 cell monolayers were fixed in ice cold methanol for 30 min and washed with PBS. The non-specific background was blocked by incubation with 5% bovine serum albumin in PBS for 45 min at room temperature. The sections were incubated with rabbit polyclonal antibody (Santa Cruz Biotech, Germany) against PepT1 at 4°C overnight. PepT1 was probed with Alexa Fluor® 488-conjugated secondary goat anti-rabbit IgG antibody (Thermo Fisher Scientific, USA). Caco-2 cells were probed with Alexa Fluor® 494 mouse monoclonal antibody (Thermo Fisher Scientific, USA) against occludin. The slides were washed and mounted in Dako fluorescence mounting media (Dako Diagnostics Ireland Ltd, Ireland). Slides incubated without primary antibody were used as negative controls. Epi-fluorescent analysis was performed with Zeiss Axioplan 2 microscope (Zeiss, Germany)

2.9 Statistical Analysis

Statistical analysis was carried out using Prism-5® software (GraphPad, San Diego, USA) using one-way ANOVA and Dunnett's *post hoc* test. Results are presented as the mean ± standard error of the mean (SEM). A significant difference was considered present if $P < 0.05$.

3 Results

3.1 Transport of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol across Caco-2 monolayers

The mean basal TEER value for Caco-2 monolayers was 1980 ± 104 Ω cm^2 (n=36), within the range reported by this lab [29] and others [38]. The addition of Gly-Sar had no effect on TEER and values remained similar to that of untreated monolayers (data not shown). Modification of apical buffer pH from 7.4 to 6.5 also had no effect on TEER, with no difference noted between groups incubated with apical side buffers of either pH value after 120 min. The P_{app} for [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol was measured in the A to B direction using apical side buffers at pH values of either 7.4 or 6.5 (Table 1). When monolayers were pre-incubated with Gly-Sar (10 mM) when the apical side buffer pH was 6.5, there was a decrease in the P_{app} of [^{3}H]-IPP by 42% (Fig. 1A) and [^{3}H]-LKP by 52% (Fig 1B). However, when the apical side buffer pH was 7.4, there was no reduction in the P_{app} of either [^{3}H]-IPP or [^{3}H]-LKP in the presence of Gly-Sar. This indicates that enabling Gly-Sar inhibition of PepT1-mediated uptake of [^{3}H]-IPP and [^{3}H]-LKP by monolayers requires acidic buffer (pH 6.5) conditions on the apical side. To determine if the pH of 6.5 and pre-incubation of Gly-Sar effected the integrity of the monolayer or paracellular transport, the P_{app} of [^{14}C]-mannitol was also tested under the same conditions (Fig 1C). Neither an apical side buffer pH of 6.5 nor Gly-Sar pre-incubation altered the P_{app} of [^{14}C]-mannitol, suggesting there was no effect of either condition on paracellular transport.

When the apical side buffer pH was 7.4, [^{3}H]-IPP had a higher P_{app} value (14.8 x 10^{-6} cm/s) than when the apical side buffer pH was 6.5 (6.8 x 10^{-6} cm/s) (Table 1). This is likely due to the physicochemical characteristics of the tripeptide in apical side buffers with pH 6.5 or 7.5 which can affect the permeation if there is a net neutral or ionised charge [39]. Overall, this

suggests [^{3}H]-IPP and [^{3}H]-LKP both use PepT1-mediated uptake, when the apical side pH is 6.5.

3.2 Transport of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol across isolated rat jejunal mucosae in Ussing chambers

The basal TEER for isolated rat jejunal mucosae was 37 ± 9 Ω cm^{2} (n=40), within the acceptable range [30]. Jejunal TEER gradually decreased over 120 min to 70-80% of the initial basal value in all conditions. The addition of Gly-Sar and/or modification of apical buffer pH from 7.4 to 6.5 had no effect on TEER, and there was no difference in TEER between groups after 120 min (data not shown). The P_{app} of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol was obtained in the A to B direction across isolated rat jejunal mucosae using apical side pH buffers of either 7.4 or 6.5 (Table 2). When tissue was pre-incubated with Gly-Sar (10 mM), a decrease was observed in the P_{app} of [^{3}H]-IPP (Fig 2A) and [^{3}H]-LKP (Fig 2B) at apical buffer pH values of either 7.4 or 6.5. There was no change in the P_{app} of [^{14}C]-mannitol in the presence or absence of Gly-Sar or when the apical buffer pH value was either 7.4 or 6.5 (Fig 2C). To assess the effects of an apical side buffer on the jejunal acidic microclimate, rat jejunal non-everted sacs were prepared, and the surface pH was measured before and after apical addition of KH buffers of 6.5 and 7.4 (Fig 3). The initial jejunal surface pH after the tissue was harvested was 6.14 ± 0.02. After 120 min incubation with the apical buffers, surface pH increased to 6.29 ± 0.05 (for buffer pH 6.5) and to 6.49 ± 0.04 (for buffer pH 7.4). Therefore, maintenance of the acidic surface pH above the epithelium was still achieved even if the buffer of pH 7.4 was present for 120 min. This accounts for the decrease in P_{app} of [^{3}H]-IPP and [^{3}H]-LKP in the presence of Gly-Sar in jejunal mucosae when the apical side buffer pH was 7.4 due to maintenance of the acidic microclimate.

3.3 Absorption of [^{3}H]-IPP and [^{3}H]-LKP from rat intra-jejunal instillations

Intra-jejunal instillations were performed to further investigate the effects of altering apical side buffer pH on absorption of [^{3}H]-IPP and [^{3}H]-LKP when co-administered with Gly-Sar (100 mM) in buffers with pH values of either 7.4 or 6.5 (Table 3). Plasma AUC of [^{3}H]-IPP and [^{3}H]-LKP decreased when co-administered with Gly-Sar in both buffers at pH 6.5 and 7.4 (Fig 4A and B). When apical buffer pH solutions were changed from 7.4 to 6.5, there was no difference between the AUC values of groups in the absence of Gly-Sar. This indicates that the jejunal acid microclimate was intact even at a apical buffer pH of 7.4 [40].

3.4 Histology and immunofluorescence analysis of jejunal tissue and monolayers

Histology analysis was used to assess the effects apical side buffer pH of 6.5 or 7.4 on jejunal mucosae following 120 min exposure in both Ussing chambers and jejunal instillations. Morphology of intestinal villi was unaffected across all experimental conditions; representative images are shown (Fig. 5). Mucus secretion was assessed with Alcian blue and neutral red staining. No histological changes were noted in the presence or absence of Gly-Sar and with apical buffer pH of 6.5 or 7.4. There was no effect on mucus secretion, villi depth, or cell sloughing when exposed to the different experimental conditions. To confirm PepT1 expression in all three models, immunofluorescence was performed on Caco-2 monolayers and on jejunal mucosae from Ussing chamber experiments and instillations following 120 min exposure to apical side buffer of 7.4. PepT1 expression was lower in Caco-2 monolayers (Fig. 6A) compared to jejunal tissue in Ussing chambers (Fig. 6B) and instillations (Fig. 6C). PepT1 was abundantly present in epithelia along the villi in jejunal tissue, whereas a lower intensity was present across monolayers. Even though Caco-2 cells

304 are of colonic origin, they have been shown to express small intestinal transporters including
305 PepT1 at variable levels [41].

4 Discussion

It has been reported that the intestinal transport routes exploited by tripeptides include both paracellular and PepT1-mediated transport [16, 18, 42]. Paracellular permeability was assessed *in vitro* in Caco-2 monolayers by measuring mannitol permeability and TEER [28], whereas PepT1-mediated uptake was assessed by Gly-Sar inhibition of substrate transport [28]. Gly-Sar is a high affinity substrate for PepT1 [28], and competitively inhibits uptake of other PepT1 substrates including IPP and LKP. Reductionist models such as Caco-2 monolayers lack complexities of more physiologically relevant models such as isolated intestinal tissue in Ussing chambers and *in vivo* in situ intra-intestinal delivery. Caco-2 monolayers lack villi formation (although microvilli are present), PepT1 expression varies (moderate to high), and epithelial cell types are limited to enterocytes [41]. *In vivo* the intestinal epithelia is comprised of enterocytes, goblet cells, and Paneth cells [25, 43]. Although the Caco-2 monolayer has an unstirred water layer, it lacks the unstirred acidic microclimate which is required for H^+ coupled transporters: PepT1, the amino acid co-transporter (PAT1), and NHE3 [44]. Therefore, to elucidate the route of transport of IPP and LKP, permeability needs to be assessed across a combination of Caco-2 monolayers as well as isolated jejunal mucosae and *in situ* intra-jejunal instillations. Brandsch has further suggested that prior to assigning a molecule as a PepT1 substrate, there is a need for detailed transport studies in mammalian cells (Caco-2 expressing PepT1) or *Xenopus laevis* ooctyes transfected with PepT1 [45].

Other studies have investigated PepT1-mediated uptake of molecules across isolated intestinal tissue in Ussing chambers. Examples include: Gly-Sar flux across isolated pig jejunum [46] and across isolated jejunum from $PepT1^{+/+}$ and $PepT^{-/-}$ mice [47], as well as

ceftibuten flux across isolated rat jejunum [48]. Here we show PepT1 contributes, at least in part to the uptake of IPP and LKP across isolated rat jejunal mucosae, and this inhibition of PepT1 by Gly-Sar was detected in apical side buffers of both pH 7.4 or 6.5. This proved not to be the case in Caco-2 monolayers, most likely due to the absence of mucus and the unstirred layer which assists the maintenance of an acidic microclimate in the small intestine. The mechanism by which the acidic microclimate is generated is thought to depend on transmembrane H^+ secretion by NHE3 into the negatively charged mucus layer [49]. The presence of jejunal mucus, if undisturbed during the mounting process in Ussing chambers, may have retained the unstirred layer, and its acidic microclimate pH at the apical surface was likely maintained even when apical buffer pH was 7.4. Goblet cells are present in jejunal mucosae which continue to secret mucus in the Ussing chamber as observed by histological analysis. Increased pH in the jejunal microclimate has previously been reported and is dependent on the buffer [50]. Increasing small intestinal luminal pH increases surface pH somewhat, but the acidic microclimate is maintained as previously demonstrated [51]. Addition of the mucolytic, *N*-acetyl-L-cysteine (5%), increased the pH of the microclimate in rats, further suggesting the role the mucus layer plays in maintaining the microclimate [52].

The addition of cephalexin, an established substrate of PepT1, also inhibited the transport of [^{3}H]-Gly-Sar when apical buffer pH was 7.4 across isolated mouse proximal jejunum in Ussing chambers [53]. This is likely due to the continued presence of the acidic jejunal microclimate even in buffers of pH 7.4, and is in line with our findings. Previous studies demonstrated a minor increase in the rat jejunal microclimate pH due to increasing apical pH and indeed, the loss of acidity in the microclimate only occurred at an apical pH of 9 [52]. Addition of the bile salt, sodium deoxycholate did not influence the pH of the microclimate in the rat jejunum [54]. However, Lucas *et al.* determined that in gastrointestinal diseases such

as Crohn's and coeliac disease, the microclimate is less acidic (pH 6.5 and 6.2 respectively) compared to that of healthy human volunteers (pH 5.9) [55]. This can indirectly impede uptake of tripeptides due to loss of pH gradient across the intestinal epithelium.

In situ and *in vivo* models are essential for the characterisation of PepT1-mediated uptake of substrates. When 100 mM Gly-Sar was co-administered with IPP or LKP in instillations, a decrease in [^{3}H]-IPP and [^{3}H]-LKP rat blood plasma was observed. The permeability of the prodrug Val-Val-sanquinivir was decreased in the presence of Gly-Sar in a rat intestinal perfusion study by 68% [56]. Furthermore, blood plasma concentration of cefditoren significantly decreased when co-administered with Gly-Sar in rat intestinal loops [57]. Clyco-trans-4-L-hydroxyprolyl-L-serine (JBP485) blood plasma concentration was decreased when co-administered with Gly-Sar in a rat intestinal perfusion study [58]. Although the in situ rat loop-gut instillations can display large variations in permeability [19], numerous studies show consistency between various *in vivo* jejunal models of PepT1-mediated absorption and inhibition by Gly-Sar [56-58]. Rational design of inhibitors of PepT1 such as, Lys[Z(NO2)]-Pro, have been tested but failed to fully inhibit inward dipeptide uptake in line with Gly-Sar inhibition [59]. For example, valacyclovir absorption was reduced (50%) by co-administration of Gly-Sar in normal mice [60]. In the same study, valacyclovir absorption was reduced to just 10% in a PepT1 knockout mice using jejunal perfusion. In this study, [^{3}H]-IPP and [^{3}H]-LKP were reduced by 30-40% in the presence of Gly-Sar, this data may suggest that they can also permeate paracellularly due to their low MW and hydrophilicity.

It is possible that intake of substrates which compete for PepT1 (such as dietary protein sources), may lead to a reduced oral bioavailability of drugs and bioactives which target

379 uptake via PepT1. Matsui *et al*. reported a loss of hypotensive action from captopril when co-
380 administered with the antihypertensive dipeptide, Val-Tyr (VY), in the spontaneously
381 hypertensive rat (SHR), in contrast with effects seen with either agent alone [61]. The authors
382 suggested this was due to competition between captopril and VY for PepT1, and cautioned
383 that subjects treated with ACE inhibitors for hypertension should avoid combined intake of
384 foods rich in small peptides. On the other hand, when IPP was orally administered in a
385 protein or high fibre meal to pigs, a 1.6-fold and 1.2-fold increase in bioavailability was
386 observed [62]. It is possible that the composition of the meal will have resulted in delayed
387 gastric emptying leading to prolonged exposure time for IPP at the intestinal epithelia.

388

389 Overall, it was shown that [^{3}H]-IPP and [^{3}H]-LKP were highly permeable even when
390 inhibited with Gly-Sar in each model. Previously, Foltz *et al*. reported P_{app} values for IPP as 1
391 x 10^{-8} cm.s^{-1} (Caco-2 monolayers) and 5 x 10^{-8} cm.s^{-1} (isolated rat jejunal tissue in Ussing
392 chambers) [19]. The large difference between IPP P_{app} values in Caco-2 monolayers may be
393 due to low PepT1 expression in the Foltz study [41]. However, the studies used different
394 methods to quantify permeability, here the [^{3}H] label was directly measured, while in the
395 Foltz study IPP was column purified and quantified by LC-MS [19]. Conversely, the values
396 reported here are in line with reported values for bioactive peptides across Caco-2
397 monolayers (P_{app} values ranging from 0.9 – 12.4 x 10^{-6} cm.s^{-1}) [63-66], and known PepT1
398 substrates across isolated jejunal tissue in Ussing chambers (P_{app} values ranging from 0.5 –
399 4.6 x 10^{-6} cm.s^{-1}) [61, 67, 68].

400

5 Conclusions

The routes of intestinal permeation of food-derived bioactive peptides, IPP and LKP were investigated. Comparative test models *in vitro, ex vivo* and *in vivo* transport across intestinal epithelia were utilised. Gly-Sar reduced the permeability of IPP and LKP in Caco-2 monolayers when the apical buffer pH was 6.5 but not when the apical buffer pH was 7.4. In isolated rat jejunum in Ussing chambers and *in situ* intra-jejunal instillations, Gly-Sar reduced IPP and LKP permeability in a nominally pH-independent manner (pH 6.5 or 7.4). On closer analysis, this was likely due to the presence of an intact acidic jejunal microclimate even when apical buffer pH was 7.4 in the jejunal tissue models. [^{14}C]-mannitol permeability and TEER was unaffected by the presence or absence of Gly-Sar and/or when apical buffer pH was 6.5 or 7.4 in the *in vitro* studies. Overall, IPP and LKP permeate both by PepT1-mediated uptake and via the paracellular route in the small intestine and are highly permeable. A combination of the three transport models used in this study may be useful for investigating the transport of other bioactive di- and tripeptides.

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Figure Captions

Fig 1. Effect of apical side buffer pH and/or Gly-Sar (10 mM) pre-incubation on the P_{app} values of **A** [^{3}H]-IPP, **B** [^{3}H]-LKP and **C** [^{14}C]-mannitol across Caco-2 monolayers obtained over 120 min. One-way ANOVA with Dunnett's multiple comparison; *** $P < 0.001$, ns = not significant compared with control. Each value represents the mean ± SEM, n = 3, with 3 independent replicates.

Fig 2. Effect of apical side buffer pH and/or Gly-Sar (10 mM) pre-incubation on the P_{app} values of **A** [^{3}H]-IPP, **B** [^{3}H]-LKP and **C** [^{14}C]-mannitol across isolated rat jejunal tissue obtained over 120 min. One-way ANOVA with Dunnett's multiple comparison; ** $P < 0.01$, *** $P < 0.001$, ns = not significant compared with control. Each value represents the mean ± SEM, n = 5 independent replicates.

Fig 3. Effect of apical side buffer pH on the surface microclimate pH of isolated rat jejunal sacs. The pH was measured at 0, and 120 min in pH of 6.5 or 7.4. One-way ANOVA with Dunnett's multiple comparison; * $P < 0.05$, *** $P < 0.001$, compared with control (0 min). Each value represents the mean ± SEM, n = 6 independent experiments.

Fig 4. Effect of apical side buffer pH and/or the presence of Gly-Sar (100 mM) on the AUC of plasma levels of **A** [^{3}H]-IPP and **B** [^{3}H]-LKP obtained over 120 min following jejunal instillations to rats. One-way ANOVA with Dunnett's multiple comparison; ** $P < 0.01$, *** $P < 0.001$, compared with control. Each value represents the mean ± SEM, n = 6 independent experiments.

Fig 5. Alcian blue and neutral red-stained representative light micrographs of rat jejunal tissue after 120 min in Ussing chambers (*ex vivo*) and instillations (*in vivo*) when apical side buffer pH was 7.4 and 6.5, size bar indicates 100 µm.

Fig 6. Representative immunofluorescence analysis of PepT1 expression: (A) Caco-2 monolayers, (B) jejunal mucosae from Ussing chambers, and (C) jejunal instillations after 120 min exposure to pH of 7.4. PepT1 proteins are stained green and tight junctions are stained red in monolayers. For interpretation of the colour, the reader is referred to the web version of this article.

Fig 1

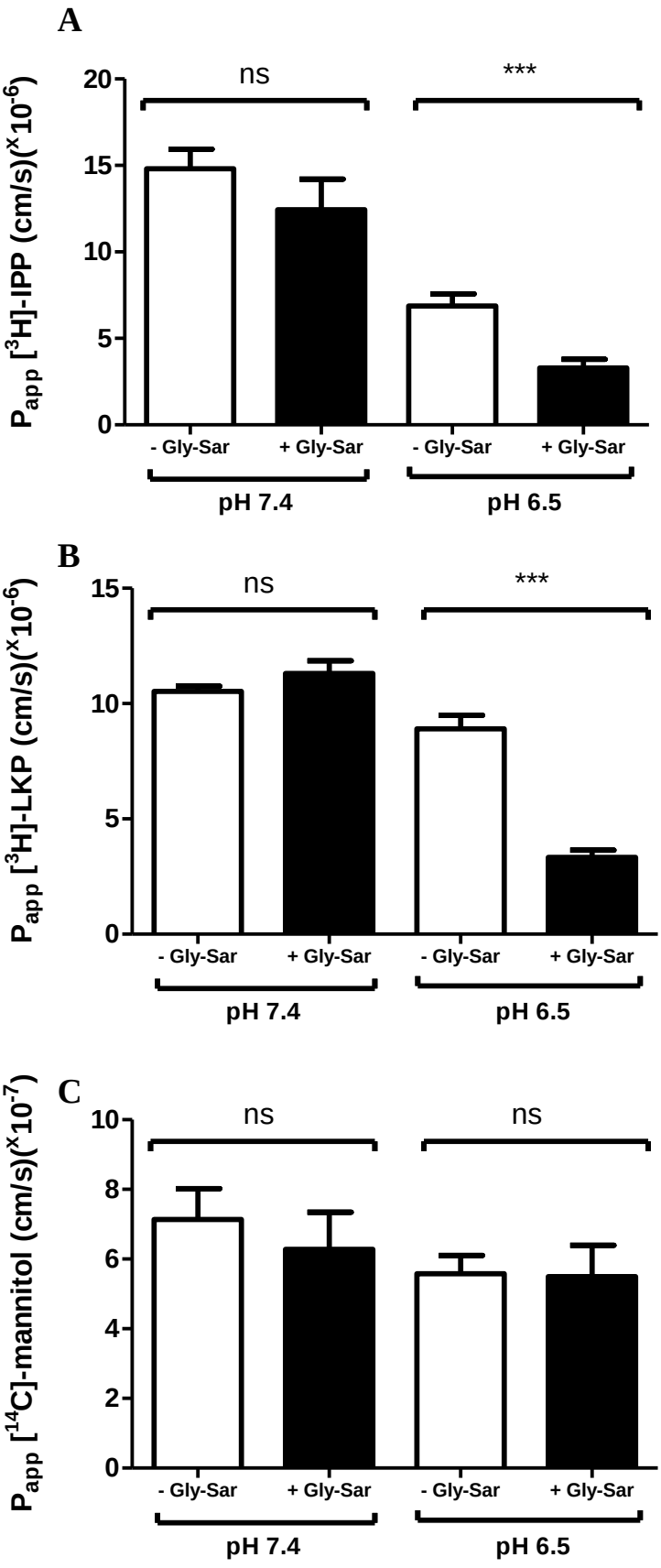
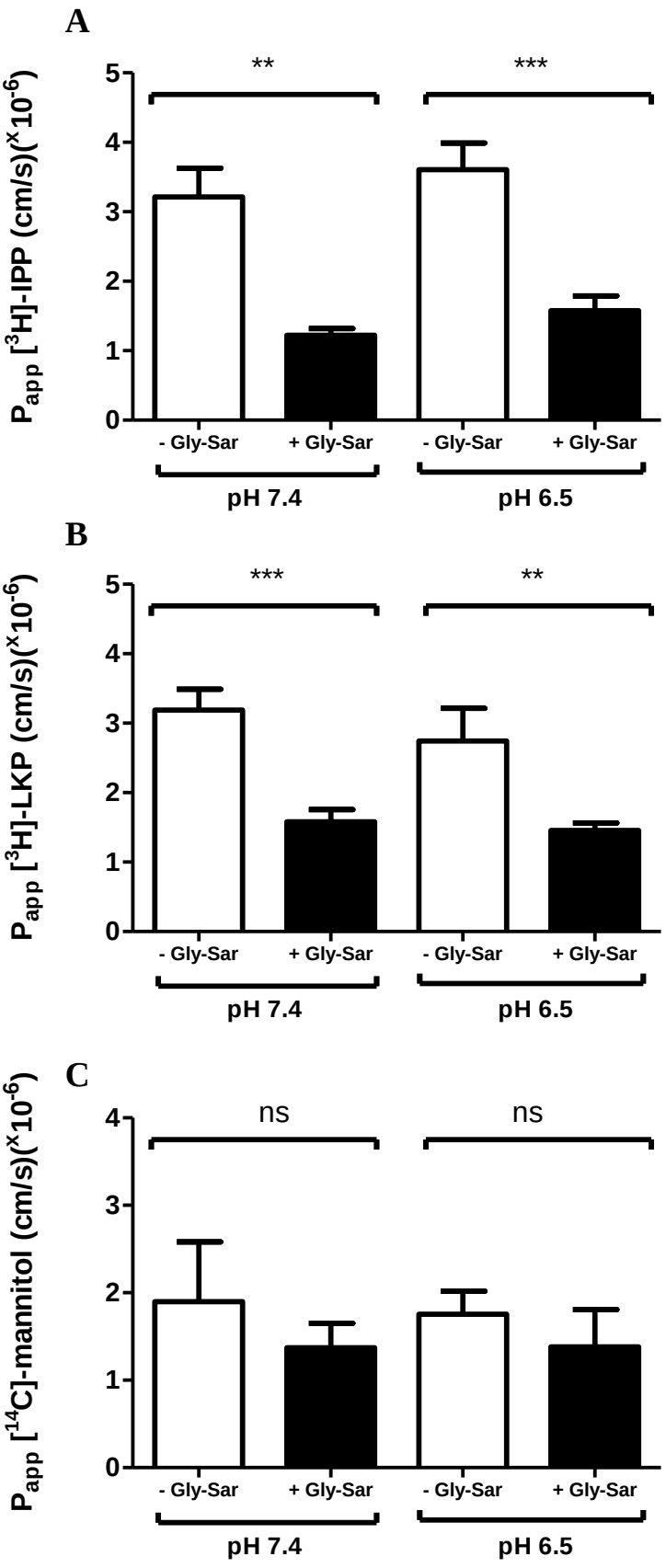

A
ns
***
20
15
10
5
0
P_{app} [^{3}H]-IPP (cm/s)(^{x}10^{-6})
- Gly-Sar
+ Gly-Sar
- Gly-Sar
+ Gly-Sar
pH 7.4
pH 6.5
B
ns
***
15
10
5
0
P_{app} [^{3}H]-LKP (cm/s)(^{x}10^{-6})
- Gly-Sar
+ Gly-Sar
- Gly-Sar
+ Gly-Sar
pH 7.4
pH 6.5
C
ns
ns
10
8
6
4
2
0
P_{app} [^{14}C]-mannitol (cm/s)(^{x}10^{-7})
- Gly-Sar
+ Gly-Sar
- Gly-Sar
+ Gly-Sar
pH 7.4
pH 6.5

Fig 2


A
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P_{app} [^{3}H]-IPP (cm/s)(^{x}10^{-6})
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- Gly-Sar
+ Gly-Sar
pH 7.4
pH 6.5
B
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P_{app} [^{3}H]-LKP (cm/s)(^{x}10^{-6})
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- Gly-Sar
+ Gly-Sar
- Gly-Sar
+ Gly-Sar
pH 7.4
pH 6.5
C
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ns
P_{app} [^{14}C]-mannitol (cm/s)(^{x}10^{-6})
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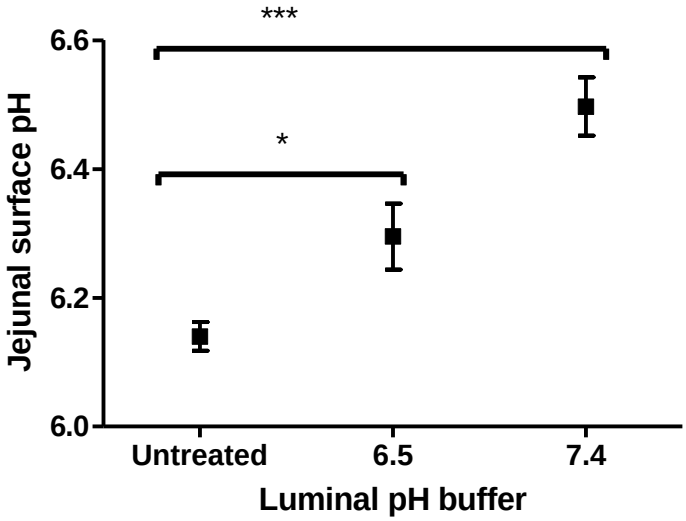
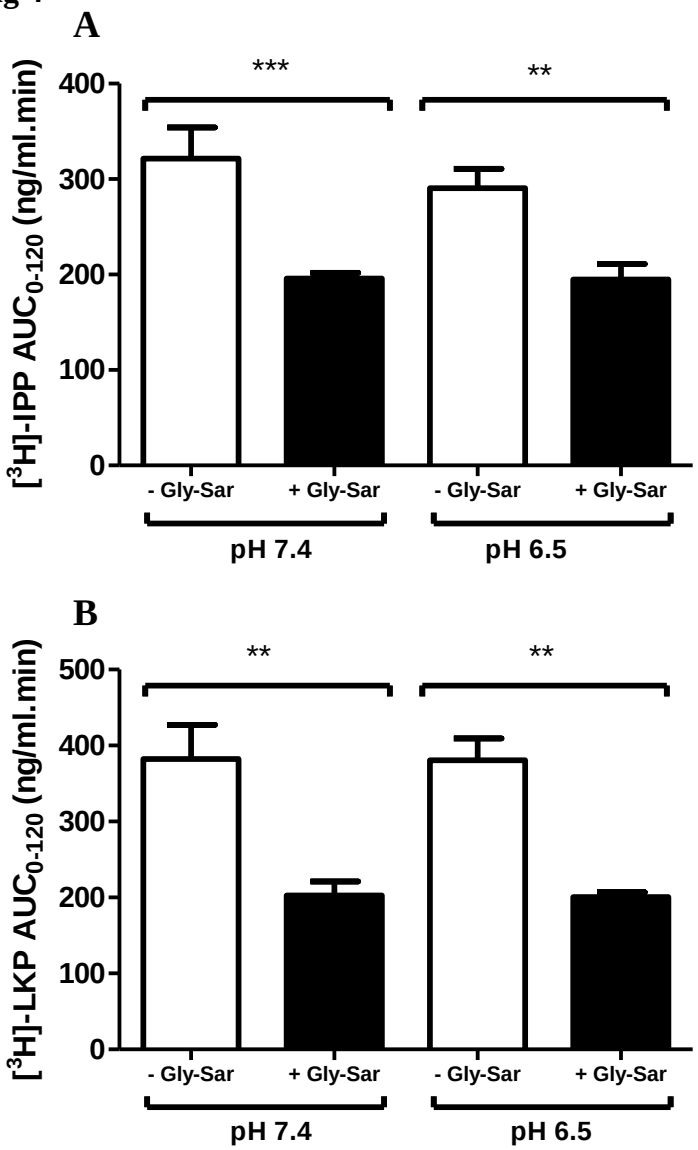
0
- Gly-Sar
+ Gly-Sar
- Gly-Sar
+ Gly-Sar
pH 7.4
pH 6.5

Fig 3


***
*
6.6
6.4
6.2
6.0
Jejunal surface pH
Untreated
6.5
7.4
Luminal pH buffer

458 **Fig 4**



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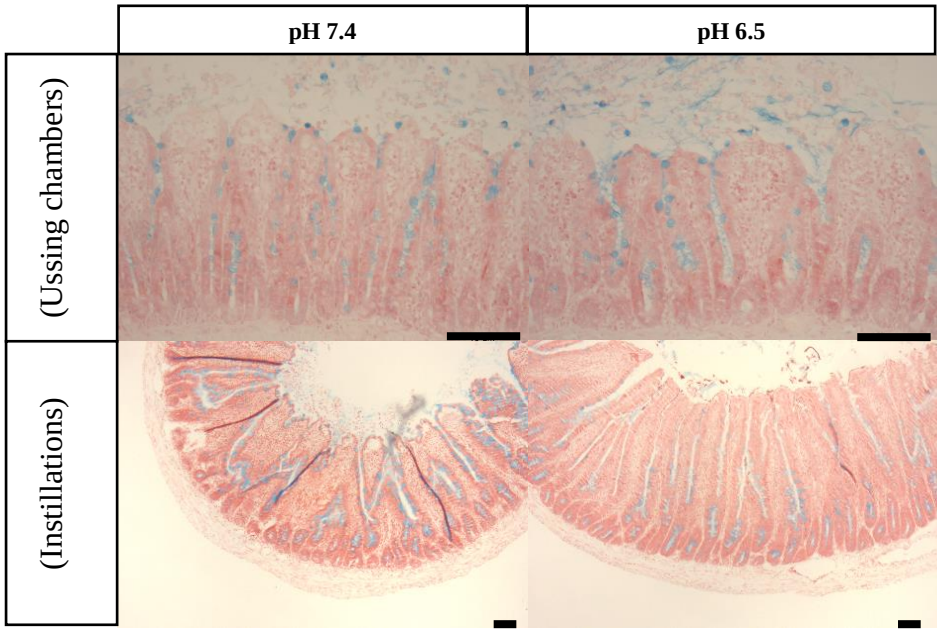
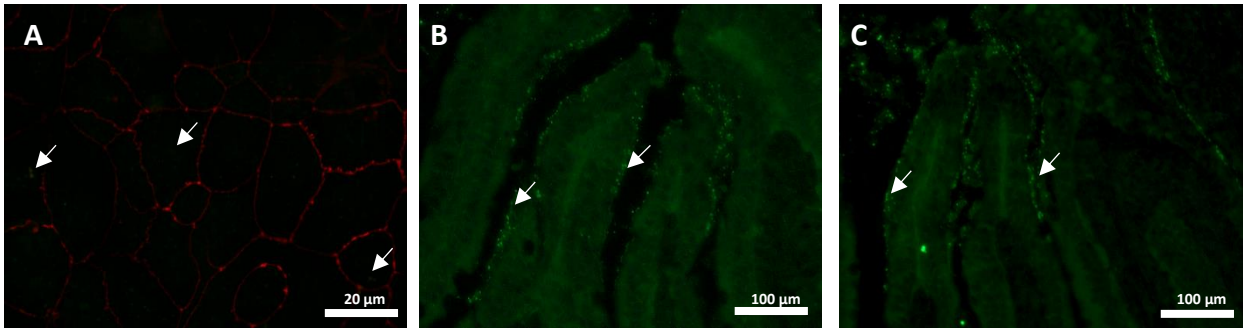
462 **Fig 5**



463

464 **Fig 6**



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466

Table 1 – Effect of apical side buffer pH and/or Gly-Sar (10 mM) on the P_{app} values of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol across Caco-2 monolayers obtained over 120 min periods.

Gly-Sar	pH	[^{3}H]-IPP P_{app} (x 10^{-6} cm.s^{-1})	[^{3}H]-LKP P_{app} (x 10^{-6} cm.s^{-1})	[^{14}C]-mannitol P_{app} (x 10^{-7} cm.s^{-1})
(-)	7.4	14.8 ± 1.1	10.5 ± 0.2	7.1 ± 0.8
(+)	7.4	12.4 ± 1.7	11.3 ± 0.6	6.3 ± 1.1
(-)	6.5	6.8 ± 0.7	8.9 ± 0.6	5.6 ± 0.5
(+)	6.5	3.3 ± 0.5 *******	3.4 ± 0.3 *******	5.5 ± 0.9

One-way ANOVA with Dunnett's multiple comparison; *** P < 0.001, compared with control. Each value represents the mean ± SEM, n = 3, with 3 independent replicates.

Table 2 – Effect of an acidic apical side pH and/or Gly-Sar (10 mM) pre-incubation on the P_{app} values of [^{3}H]-IPP, [^{3}H]-LKP and [^{14}C]-mannitol across isolated rat jejunal tissue in Ussing chambers obtained over 120 min.

Gly-Sar	pH	[^{3}H]-IPP P_{app} (x 10^{-6} cm.s^{-1})	[^{3}H]-LKP P_{app} (x 10^{-6} cm.s^{-1})	[^{14}C]-mannitol P_{app} (x 10^{-6} cm.s^{-1})
(-)	7.4	3.2 ± 0.4	3.2 ± 0.3	1.8 ± 0.7
(+)	7.4	1.2 ± 0.1 **	1.6 ± 0.2 ***	1.4 ± 0.3
(-)	6.5	3.6 ± 0.4	2.8 ± 0.4	1.7 ± 0.3
(+)	6.5	1.6 ± 0.2 ***	1.4 ± 0.1 **	1.4 ± 0.5

One-way ANOVA with Dunnett's multiple comparison; ** P < 0.01, *** P < 0.001, compared with control. Each value represents the mean ± SEM, n =5 independent replicates.

475 **Table 3** – Effect of apical side buffer pH and/or the presence of Gly-Sar (100 mM) on the
476 AUC of plasma levels of [^{3}H]-IPP and [^{3}H]-LKP obtained in rat jejunal instillations over 120
477 min.

Gly-Sar	pH	[^{3}H]-IPP AUC (ng/ml.min)	[^{3}H]-LKP AUC (ng/ml.min)
(-)	7.4	321 ± 32	382 ± 45
(+)	7.4	195 ± 5 ***	202 ± 18 **
(-)	6.5	290 ± 20	380 ± 29
(+)	6.5	195 ± 16 **	200 ± 6 **

One-way ANOVA with Dunnett's multiple comparison; ** $P < 0.01$, *** $P < 0.001$, compared with control. Each value represents the mean ± SEM, n =6 independent replicates.

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