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Sodium caprate enables the blood pressure-lowering effect of Ile-Pro-Pro and Leu-Lys-Pro in spontaneously hypertensive rats by indirectly overcoming PepT1 inhibition

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Abstract

The tripeptides, Ile-Pro-Pro (IPP) and Leu-Lys-Pro (LKP), inhibit angiotensin-converting enzyme (ACE) resulting in lowered blood pressure. Our hypothesis was that the medium chain fatty acid permeation enhancer, sodium caprate (C_{10}), may prevent the decrease in permeability of the tripeptides when PepT1 is inhibited by glycyl-sarcosine (Gly-Sar), a situation that may occur in the presence of food hydrolysates. Using Caco-2 monolayers and isolated rat jejunal tissue, the apparent permeability coefficients (P_{app}) of [^{3}H]-IPP and [^{3}H]-LKP were assessed in the presence of Gly-Sar with and without C_{10}. Gly-Sar decreased the P_{app} of both tripeptides across monolayers and isolated jejunal tissue, but C_{10} restored it. C_{10} likely increased the paracellular permeability of the tripeptides, as indicated by immunofluorescence changes in tight junction proteins in Caco-2 monolayers accompanied by a concentration-dependent decrease in transepithelial electrical resistance (TEER). [^{3}H]-IPP and [^{3}H]-LKP were orally-gavaged to normal rats with Gly-Sar, C_{10}, or with a mixture. Plasma levels of both peptides were reduced by Gly-Sar to less than half that of the levels detected in its absence, but were restored when C_{10} was co-administered. In spontaneously hypertensive rats (SHRs), unlabelled IPP and LKP lowered blood pressure when delivered either by i.v. or oral routes. Oral gavage of Gly-Sar reduced the hypotensive action of peptides in SHRs, but the effect was restored in the presence of C_{10}. In conclusion, there was a reduction in the hypotensive effects of IPP and LKP in SHRs when intestinal PepT1 was inhibited by Gly-Sar, but C_{10} may circumvent this by enhancing paracellular permeability.

Key words: Intestinal peptide transport, PepT1, nutraceuticals, antihypertensive agents, intestinal permeation enhancers, sodium caprate.

1. Introduction

Current numbers of hypertension cases are estimated to be ~1 billion worldwide due to increased sedentary behaviour and widespread adoption of Western diets [1]. A meta-analysis of "*Dietary Approaches to Stop Hypertension*" (DASH) diets suggested that effective adherence could lead to a reduction in the risk of cardiovascular disease by 20% [2]. To date, dietary interventions have achieved only modest reductions in systolic blood pressure (SBP). To improve upon the relatively weak hypotensive actions of peptides currently presented in foodstuffs, use of purified bioactive molecules could be more effective if administered in higher doses in optimised oral formulations [3]. Bioactive di- and tri-peptides are cleaved from food-derived proteins and polypeptides by intestinal peptidases and several seem to possess anti-inflammatory [4], anti-oxidative [5] and anti-hypertensive [6, 7] characteristics. Antihypertensive tripeptides discovered in food include Ile-Pro-Pro (IPP) (molecular weight, MW 325 Da) and Leu-Lys-Pro (LKP) (MW 389 Da). At least part of the blood-pressure lowering mechanism of action of IPP is thought to be due to competitive inhibition of angiotensin-converting enzyme (ACE), thereby preventing conversion of angiotensin I to the potent vasoconstrictor, angiotensin II [8]. This is a similar mechanism of action to the oral small molecule antihypertensive ACE inhibitors, captopril and enalaprilat [9].

IPP and LKP are present in hydrolysates of milk β-casein and fish/chicken muscle respectively. In the Spontaneously Hypertensive Rat (SHR) model blood pressure was reduced by oral administration of IPP [10, 11]. In particular, IPP has been tested as a potential hypotensive agent in human studies as a component of milk [12], yogurt [13], cheese [14], dairy spreads [15], and also when formulated in tablets [16], but results have generally been variable. A meta-analysis of 19 human trials indicated however, that a

combination of IPP and VPP induced an overall decrease in SBP of ~4 mm Hg over a minimum of 8 weeks following doses of 2 – 10 mg peptide /day in a range of food products, a result that might be clinically significant [17]. Previously we have demonstrated that IPP and LKP are substrates for small intestinal epithelial cell uptake via the apical membrane-located intestinal Peptide Transporter 1 , PepT1(SLC15A1) *in vitro* [18], a high affinity pathway that can also be accessed by other food-derived peptides [19-23]. Involvement of PepT1 in mediating di- and tri-peptide transport is indicated by decreased small intestinal epithelial permeation in the presence of the PepT1 inhibitor, glycyl-sarcosine (Gly-Sar). PepT1 substrates typically have good oral bioavailability (e.g. valacyclovir, 50-70% [24]), but there is potential for uptake to be inhibited by PepT1-competing pharmaceutical substrates or ones present in foodstuffs. For example, the fish muscle hydrolysate di-peptide derivative, Val-Tyr (VY), does not reduce blood pressure in SHRs to the same degree when co-administered with captopril due to competition for PepT1; both molecules were more efficacious in the absence of the other [25]. Similarly, the increased apparent permeability coefficient (P_{app}) of the PepT1 substrate, cephalexin, across isolated rat jejunal mucosae induced by Labrasol ®, (comprising acylglycerol- and PEG esters of the medium chain fatty acids, sodium caprate (C_{10}) and sodium caprylate (C_8)), was hindered in the presence of the PepT1 substrates, Gly-Leu and Ala-Ala [26], suggesting competition for PepT1 between cephalexin and dipeptides.

The intestinal permeation enhancer, C_{10}, is found in mM concentrations in milk [27] and also as a methyl ester ethoxylate in coconut oil and palm kernel oil [28, 29]. C_{10} is well-known to increase oral absorption of large MW peptides and reached Phase II trials for an oral insulin as a component of Merrion Pharmaceutical's (Dublin, Ireland) GIPET™ solid dosage form [30]. Despite this, less studied is its potential to increase oral absorption of food-derived bioactives. In one example however, the poorly absorbed soy-derived isoflavone, diadzein,

was entrapped in nanoparticles and co-administered with C_{10} to rats, resulting in a 2-fold increase in plasma levels [31]. Previously, we reported that C_{10} increased the permeation of fluorescein isothiocyanate (FITC)-labelled IPP and LKP by 1.4 to 3.6-fold respectively across isolated rat jejunal tissue mucosae [32]. The *in vitro* membrane perturbation mechanism that C_{10} uses to increase paracellular permeability is due to a combination of elevation of intracellular Ca^{2+} [33], epithelial membrane fluidisation [34], relocation of the tight junction-associated proteins, tricellulin and claudin 5 [35], activation of phospholipase C [27], and increased phosphorylation of myosin light chain kinase (MLCK) [36].

The hypothesis of this study was that C_{10} might overcome a reduction in the *in vitro* and *in vivo* intestinal permeability of IPP and LKP in rats seen in the presence of Gly-Sar. Gly-Sar was used in an attempt to mimic the inhibition of PepT1 that may occur in the presence of peptides generated by lipases from food hydrolysates. First, the effects of Gly-Sar and C_{10} co-administration on the permeability of [^{3}H]-IPP and [^{3}H]-LKP were determined across Caco-2 monolayers and isolated rat jejunal mucosae mounted in Ussing chambers. Secondly, we examined the pharmacodynamics (PD) of IPP and LKP in SHRs in the presence and absence of Gly-Sar and C_{10}. Finally, in initial studies we examined the effects of Gly-Sar and C_{10} on [^{3}H]-IPP and [^{3}H]-LKP absorption following oral gavage to normal rats over a short time frame and compared these data to the PD outputs. All *in vitro* and *in vivo* studies suggested that PepT1 plays a key role in the intestinal permeability of the tripeptides and that C_{10} can overcome inhibition of flux by Gly-Sar.

2. Materials and methods

2.1 Reagents and chemicals

IPP (PubChem CID: 9949212) and LKP (PubChem CID: 24978508) were obtained from China Peptides (China). [^{3}H]-IPP (specific activity 30 Ci/mmol) and [^{3}H]-LKP (specific activity 21 Ci/mmol) were obtained from Cambridge Research Biochemicals (UK). The Alexa Fluor® 494 mouse monoclonal antibodies for occludin, and ZO-1, and the Alexa Fluor® 488 mouse monoclonal antibody for claudin-5 were sourced from Thermo Fisher Scientific (USA). Dako fluorescence-mounting media was obtained from Dako Diagnostics (Ireland). Isoflurane (Iso-Vet®) was obtained from Piramal Healthcare (UK). Pentobarbital sodium (EUTHANAL™) was from Merial Animal Health (UK). Caco-2 cells (passage 48-58) were obtained from European Collection of Cell Cultures (UK). C_{10} (PubChem CID: 4457968) and all other reagents were purchased as analytical grade from Sigma-Aldrich (UK).

2.2 Caco-2 monolayer trans-epithelial transport studies

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 (Biosciences Ltd, Ireland) in 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/ Transwell® on 1.12 cm^2 diameter polycarbonate filters with a pore size of 0.4 µm (Corning Costar Corp., USA) and grown for ~21 days in DMEM [37]. Cells were seeded at a density of 1.5 x 10^5 cells/well in 8 well Nunc™ Lab-Tek II chamber slides and grown for 21 days in DMEM for immunofluorescence experiments. 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 for 120 min to confirm monolayer integrity.

Apical-to-basolateral (A to B) transport of [^{3}H]-IPP and [^{3}H]-LKP were examined across filter-grown monolayers. Transport buffers consisted of calcium-free HBSS supplemented with 12.5 mM glucose and either 10 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 6.5; the apical buffer) or 25 mM HEPES with 2.5mM calcium (pH 7.4; the basolateral buffer) [37, 38]. Buffers were equilibrated with monolayers for 30 min in the presence or absence of apical-side Gly-Sar (10 mM) in HBSS. At time zero, either [^{3}H]-IPP or [^{3}H]-LKP (both 1 μCi/ml) was added to the apical side in the presence or absence of C_{10} (1, 2.5, or 5 mM). Basolateral samples were taken every 30 min for 120 min and apical samples were taken at 0 and 120 min in order to calculate P_{app}. Withdrawn apical and basolateral side samples were replaced with equal volumes of fresh calcium-free HBSS (pH 6.5) or regular HBSS (pH 7.4) respectively. Basolateral and apical samples of 100 μl were mixed with 5 ml Ecoscint A scintillation cocktail (Biosciences Ireland) and measured in a liquid scintillation counter (Packard Tricarb 2900 TR). The P_{app} for each [^{3}H]-IPP and [^{3}H]-LKP were calculated with the following equation:

$$\boldsymbol{P_{app}} = \frac{\boldsymbol{dQ}}{\boldsymbol{dt}} \frac{\mathbf{1}}{\boldsymbol{A \cdot C_0}} \qquad \text{(Eq.1)}$$

where dQ/dt is the slope of the line (linearity required to be > 0.95 for inclusion), *A* is the surface area (1.12 cm^2) and C_0 is the starting concentration of flux marker on the apical side [37]. Basal TEER values were required to be > 1400 Ω.cm^2 in order for monolayers to be included for analysis [39]. Transport experiments were run in triplicate with three independent replicates.

2.3 Immunofluorescence of tight junction proteins in monolayers exposed to C_{10}

Confluent Caco-2 cells grown on 8 well Nunc™ Lab-Tek II chamber slides were exposed to C_{10} (1.0 and 5.0 mM) in calcium-free HBSS for 120 min. Untreated control cells were incubated in HBSS. Following exposure, HBSS was removed and cells were washed gently with ice cold PBS. Cells were fixed in ice-cold methanol for 30 min and washed with PBS. Non-specific background was blocked by incubation with 5% bovine serum albumin in PBS overnight at 4°C. Cells were washed with PBS and permeabilized with 0.1% Triton™ X-100 in PBS for 10 min. Cells were probed with Alexa Fluor® 494 mouse monoclonal antibody against occludin (1:400), Alexa Fluor® 494 mouse monoclonal antibody against ZO-1 (1:400), and Alexa Fluor® 488 mouse monoclonal antibody against claudin-5 (1:400). The slides were washed with PBS and mounted in Dako fluorescence mounting media. Images were captured with an Axioplan Epi-fluorescence microscope (Zeiss, Germany).

2.4 Isolated rat jejunal mucosae transport studies in Ussing chambers

Rat studies were carried out in accordance with the UCD Animal Research Ethics Committee protocol (AREC 14-28-Brayden) and in adherence with European Union Directive 2010/63/EU in relation to the 3Rs and use of *post mortem* tissue. Male Wistar rats (250-350 g; Charles River Labs, UK, and the UCD Biomedical Facility) were euthanized by stunning and cervical dislocation. Jejunal mucosae with intact underlying circular and longitudinal muscle were mounted in Ussing chambers with a circular window areas of 0.63 cm^2 [40]. Mucosae were bathed apically with 5 ml calcium-free Krebs Henseleit buffer (KH), basolaterally with KH buffer, and both sides were continuously gassed with 95% CO_2/5% O_2 with the temperature maintained at 37°C with a heated glass water jacket. The transepithelial

potential difference (PD; mV) and short circuit current (I_{sc}, $\mu A.cm^{-2}$) were measured across tissue using a DVC-4000 voltage clamp (WPI, UK). After an initial 30 min equilibration, PD and I_{sc} were used to calculate the TEER over 120 min. Tissues were equilibrated for 30 min in the presence or absence of apical side 10 mM Gly-Sar in KH. At time zero, [^{3}H]-IPP (1 μCi/ml) or [^{3}H]-LKP (1 μCi/ml) was added to the apical side in the presence or absence of C_{10} (10, 20, or 30 mM). 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} for the apical-to-basolateral (A-B) direction. Withdrawn samples were replaced with an equal volume of fresh calcium-free KH (apical) and KH (basolateral) and they were analysed by on a Liquid Scintillation Counter (Beckman/Perkin-Elmer, USA). P_{app} values of [^{3}H]-IPP and [^{3}H]-LKP were calculated according to equation 1. Basal jejunal TEER values were required to be > 30 Ω cm^2 or were otherwise excluded [40]. Jejunal mucosae transport experiments were carried out with a minimum of five independent replicates.

2.5 *In vivo* rat studies

All live animal procedures adhered to EU Directive 2010/63/EU 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 and male SHRs (16-wk old, Charles River, Germany) weighing 260-320 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. Animals were euthanized at the end of the experiment by an intra-cardiac injection of 0.4 ml pentobarbital sodium (EUTHATAL™, Merial Animal Health Ltd.,UK).

2.6 PD studies in SHRs

Male SHRs (16-wk old, 260-320 g) with SBP over 170 mm Hg were randomised into test groups with 6 rats per treatment. Oral gavage and i.v. administration was carried out with captopril (5mg/kg both routes), and IPP and LKP (1 mg/kg and 5 mg/kg by i.v.; 10mg/kg oral). Oral gavage was carried out using a curved 16 gauge needle to deliver 300 µl to the stomach. The effects of treatments on SBP were compared to that of PBS solution, the vehicle for all test agents. PepT1 inhibition studies compared responses following oral gavage to both IPP and LKP (both 5mg/kg) in the presence of Gly-Sar (100 mM; 16 mg/kg), or in the presence of both Gly-Sar and C_{10} (180 mM; 35 mg/kg). SBP was measured by tail-cuff plethysmography using the CODA® Mouse Rat Tail Cuff Blood Pressure System (Kent Scientific, U.S.A.) at 0, 1, 2, 3, 4, 6, and 8 h post administration after warming the rats in a chamber at 34°C for 5 min. Δ SBP changes were calculated by setting untreated basal readings to zero using GraphPad Prism-5® software.

2.7 Oral absorption of [^{3}H]-IPP (8 µCi/kg; 7.6 mg/kg) and [^{3}H]-LKP in normal rats

Normal Male Wistar rats were administered treatments in PBS by oral gavage as described above. PBS. [^{3}H]-IPP (8 µCi/kg; 7.6 mg/kg) or [^{3}H]-LKP (8 µCi/kg; 8.9 mg/kg) were administered by gavage alone, in the presence of Gly-Sar (100 mM; 16 mg/kg), or in the presence of both Gly-Sar and C_{10} (180 mM; 35 mg/kg). After oral gavage, the rats were anaesthetised with isoflurane (Iso-Vet, 1000 mg/g isoflurane liquid for inhalation, Piramal Healthcare, U.K.) 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 a delivery mask (Blease Medical Equipment Ltd., U.K.). Blood samples (~ 400 µl) were taken via the retro-orbital route at selected time points up to 180 min into 1 ml

Eppendorf tubes and stored on ice at 2-8°C prior to centrifugation (6500g, 5 min) and serum collection. 100 µl serum was mixed with 5 ml scintillation fluid (Ecoscint A scintillation cocktail) and concentrations were read in a Packard Tricarb 2900 TR scintillation counter.

2.9 Histology

Following the studies using isolated jejunal tissue in Ussing chambers and oral gavage studies in normotensive rats, jejunal mucosae were 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 Alcian blue and neutral red. Slides were visualised under a light microscope (NanoZoomer 2.0-HT light microscopy, Hamamatsu) and images were taken with a high-resolution camera (Micropublisher 3.3 RTV, QImaging) and Image-Pro® Plus version 7.1 (Media Cybernetics Inc., USA) acquisition software.

2.10 Statistical testing of group comparisons and PK-PD analysis

Statistical analysis was carried out using GraphPad® Prism-5 software using one-way and two-way ANOVA with Dunnett's and Bonferroni's *post hoc* tests as appropriate. Results are presented as the mean ± standard deviation (SD). A significant difference was designated for all P values < 0.05 but without further denomination into multiple levels, as recently recommended by the Editors of the British Journal of Pharmacology [41].

3. Results

3.1 C_{10} increases the P_{app} of [^{3}H]-IPP and [^{3}H]-LKP across Caco-2 monolayers

The mean basal TEER for Caco-2 monolayers grown on Transwells® was 1984 ± 570 Ω.cm^2 (n=48), within an acceptable range for differentiated monolayers for use in transport studies [39]. Apical addition of 10 mM Gly-Sar had no effect on TEER over 120 min, with values similar to those of untreated monolayers (Fig. 1A). Apical addition of C_{10} (1.0, 2.5, and 5.0 mM) decreased TEER to 49%, 31%, and 15% of basal values respectively over 120 min (Fig. 1A). The basal P_{app} obtained across monolayers for [^{3}H]-IPP in the A-B direction was 6.2 ± 2.9 x 10^{-6} cm.s^{-1}, whereas for [^{3}H]-LKP it was almost 2-fold higher at 11.4 ± 1.7 x 10^{-6} cm.s^{-1} (Table 1). Pre-incubation of monolayers with 10 mM Gly-Sar on the apical side for 30 min caused a decrease in the P_{app} values by 47% for [^{3}H]-IPP (to 3.3 x 10^{-6} cm.s^{-1}), and by 44% for [^{3}H]-LKP (to 6.4 x 10^{-6} cm.s^{-1}). Apical addition of a low concentration of C_{10} (1 mM) did not statistically increase the P_{app} of either peptide in the absence of Gly-Sar, although there was a trend in the case of [^{3}H]-IPP, where the P_{app} increased from 6.2 to 10.1 x 10^{-6} cm.s^{-1}. Co-administration of Gly-Sar with 1mM C_{10} restored the P_{app} values of both peptides to similar values seen in the absence of the inhibitor (Table 1). When concentrations of C_{10} were increased to 2.5 mM and 5 mM however, the P_{app} values of both peptides were increased several fold over those seen in either the presence or absence of Gly-Sar.

3.2 Effect of C_{10} on tight junction-associated proteins in Caco-2 monolayers

Apical addition of 1 mM C_{10} for 120 min induced no overt perturbation or interference with the tight junction proteins probed (data not shown). Compared to untreated control monolayers however, 5 mM C_{10} induced some cell sloughing and ZO-1 was internalised leaving poor membrane definition, indicative of membrane perturbation (Fig. 1B). Claudin-5

and occludin were also relocated and internalised in the presence of 5 mM C_{10}, suggestive of some loss of cell membrane integrity, likely accompanied by an increase in tight junction openings. This data indicates that increasing concentrations of C_{10} led to reorganisation of TJ proteins and is consistent with the reciprocal reductions in TEER and increases in the P_{app} of [^{3}H]-IPP and [^{3}H]-LKP. Overall, C_{10} increased transepithelial flux of the peptides across Caco-2 monolayers due to the internalisation and re-organisation of TJ proteins and is likely to be predominantly a paracellular effect *in vitro*, initiated by membrane fluidisation and perturbation.

3.3 C_{10} enhances flux of [^{3}H]-IPP and [^{3}H]-LKP across isolated rat jejunal mucosae

The basal TEER for isolated jejunal tissue mounted in Ussing chambers was 46 ± 9 Ω.cm^2 (n=48), within the range of TEER values typically reported for this tissue *in vitro* [40]. Jejunal TEER values decreased in untreated controls by 12% on average over 120 min (Fig. 2A). In mucosae pre-incubated with Gly-Sar (10 mM) for 20 min, TEER subsequently decreased by 17% over 120 min, but this was not different to untreated controls. Apical addition of C_{10} decreased TEER to 67%, 52%, and 42% of basal values at 10, 20, and 30 mM concentrations respectively. The P_{app} of [^{3}H]-IPP in the A-B direction was 2.6 ± 0.6 x 10^{-6} cm.s^{-1} and for [^{3}H]-LKP, it was similar at 2.1 ± 0.4 x 10^{-6} cm.s^{-1} (Table 2). When tissue was pre-incubated with apical addition of 10 mM Gly-Sar, a reduction in the P_{app} values were recorded for both peptides: the P_{app} of [^{3}H]-IPP decreased by 42% (to 1.5 x 10^{-6} cm.s^{-1}), while that of [^{3}H]-LKP decreased by 41% (to 1.4 x 10^{-6} cm.s^{-1}). Apical addition of 10 mM C_{10} had no effect on the P_{app} values of either peptide alone. However in the presence of Gly-Sar, C_{10} increased the P_{app} of both peptides back up to basal levels (Table 2). When jejunal tissues were exposed to 20 and 30 mM C_{10}, the P_{app} increased for both peptides above the levels seen in either the presence or absence of Gly-Sar. Histological analysis of jejunal tissue was

carried out following 120 min exposure to increasing concentrations of C_{10} (Fig. 2B). Untreated tissue and tissue pre-incubated with Gly-Sar showed no major morphological changes. Apical addition of 10 mM C_{10} however, induced cellular sloughing of the jejunal villi and increased mucus secretion after 120 min (Fig. 2B.iii). Depth of villi perturbation and damage was more evident at the 20 mM and 30 mM C_{10} concentrations. Overall, the jejunal structure was maintained and the crypts remained mostly unaffected in control tissues and exposed to Gly-Sar, but C_{10} caused concentration-dependent damage at values >10 mM, consistent with the TEER reductions.

3.4 Effects of Gly-Sar and C_{10} on the anti-hypertensive action of the peptides in SHRs

The hypotensive effects of unlabelled IPP and LKP were assessed in SHRs by the i.v. (Fig. 3A) and oral gavage routes (Fig. 3B, C). Captopril (5 mg/kg) statistically lowered SBP by both delivery routes compared to PBS, with no effects of PBS seen by both routes. Both IPP and LKP statistically decreased SBP by the i.v. route at 1 h and 2 h with doses of 1 mg/kg (P versus control, and also at each time point up to 6 h at a dose of 5 mg/kg IPP, and at time points up to 3 h only for 5mg/kg LKP. 5 mg/kg captopril tended to be more effective than either peptide at later time points at the same dose level by the i.v. route, but this was not significant. Concerning oral administration of the two peptides and captopril to SHRs, there was a slower onset of hypotensive action compared to the i.v. route (Fig. 3B, C). Oral doses of 5 and 10 mg/kg of IPP and LKP induced a statistical reduction in SBP from 2-4 h with respect to PBS controls, whereas 1mg/kg doses were ineffective. For the three dose levels of the peptides administered orally, there were dose-dependent blood pressure reductions for each. Captopril (5 mg/kg) statistically reduced SBP at each time point up to 6 h and also at 8 h. It was more efficacious then the two peptides following oral gavage at the 5mg/kg dose level at the 6h and 8h time points. At these later time points there was no statistical reduction

in SBP induced by the two peptides versus PBS in contrast to captopril. The effects of Gly-Sar and C_{10} were then determined in relation to the hypotensive actions of IPP and LKP. Gly-Sar co-administration statistically inhibited the SBP decreases induced by IPP (Fig. 4A) and LKP (Fig. 4B) at 120 min, however the inhibitory effects of Gly-Sar were partially reversed for both peptides to a statistical level in the presence of C_{10}. The data suggest that the IPP- and LKP induced decrease in SBP in the presence of oral Gly-Sar in SHRs could be partially reversed by C_{10}.

3.5 Effects of C_{10} and Gly-Sar on the serum levels of orally-gavaged [^{3}H]-IPP and [^{3}H]-LKP in normal rats

The effects of Gly-Sar and C_{10} on the serum levels of [^{3}H]-IPP and [^{3}H]-LKP were determined over 180 min following oral gavage to normal rats (Fig. 5A). A high concentration of 100 mM Gly-Sar was used in oral gavage studies instead of the 10mM used *in vitro* in order to account for possible dilution in the GI tract [42]. In the presence of Gly-Sar, there was a significant decrease in serum levels both [^{3}H]-IPP (57% reduction) and [^{3}H]-LKP (56% reduction) following gavage (Fig. 5A). When 180 mM C_{10} [43] was co-administered with Gly-Sar and the peptides however, serum levels of [^{3}H]-IPP returned to 84% of levels attained in the absence of Gly-Sar, and that of [^{3}H]-LKP returned to 93%. Surprisingly, C_{10} co-administration with the peptides was without effect on serum levels even at a high concentration of 180 mM; values were similar for both peptides in its presence and absence. Co-administration of C_{10} therefore circumvented the reduction in peptide flux caused by Gly-Sar inhibition of the PepT1-mediated pathway, but C_{10} had no effect on serum levels *per se*. This indicates that C_{10} may reverse the inhibition of absorption of the two peptides by Gly-Sar *in vivo*. Histological analysis of jejunal tissue after oral gavage was

carried out after exposure to treatments and showed no pathological damage from the rats dosed with Gly-Sar, C_{10}, or the combination thereof (Fig. 5 B). There were no differences in histological damage, epithelial perturbation, cell sloughing, or increased mucus production between treated and untreated rats. It was notable that the jejunal histology from gavage studies in rats was in general better preserved than jejunal mucosae from Ussing chamber studies.

4. Discussion

The relatively high P_{app} values [^{3}H]-IPP and [^{3}H]-LKP obtained across Caco-2 monolayers suggest that both molecules have higher permeability across the model than was anticipated for peptides. Examples of well-absorbed small molecules with a similar range of P_{app} values across Caco-2 include (x 10^{-6} cm.s^{-1}): atenolol, 2.2; talinolol, 1.8 [44], and valacyclovir, 4.5 [45], the latter being a substrate of PepT1. Furthermore, the Caco-2 P_{app} values reported here are similar to the P_{app} values of other short chain peptides determined across Caco-2: (x 10^{-6} cm.s^{-1}): e.g. Ile-Phe, 2.4 [46], Thr-Vap-Pro-Ser-Leu, 6.9 [20], and Gly-Pro-His, 1.1 [22], as well as Gly-Sar itself, 9.2 [44]. In addition, the P_{app} values of [^{3}H]-IPP and [^{3}H]-LKP across isolated rat jejunal mucosae the are also in line with values obtained for established small molecule PepT1 substrates across human and rat small intestinal tissue mucosae (x 10^{-6} cm.s^{-1}): oseltamivir, 4.6 [47], captopril, 0.5 [25], and cephalexin, 3.8 [26]. Foltz *et al.* [48] used LC-MS to assay permeated unlabelled IPP and reported P_{app} values of 1.0 x 10^{-8} cm.s^{-1} across Caco-2 monolayers and 9 x 10^{-8} cm.s^{-1} across isolated rat jejunal mucosae, 100-fold lower than the values we report here for tritiated IPP in each model. The discrepancy with our data is unlikely to be due to an artefact of tritium breaking off from the tripeptides during permeation for the following reasons: the radiolabel bond is covalently-linked; the P_{app} values of [^{3}H]-IPP and [^{3}H]-LKP are in line with most other *in vitro* epithelial permeability studies

of PepT1 substrates, and finally Gly-Sar inhibition of [^{3}H]-IPP and [^{3}H]-LKP flux was still present across three bioassays. Thus, although it is unlikely that detachment of the label occurred, this was not directly assessed by column chromatography or LC-MS. Similarly, while Caco-2 permeability values differ between laboratories may be are related to the clone source, differences in basal TEER, passage number, and serum lot, this does not explain the lack of similarity between the two studies for the rat jejunal P_{app} data.

Apical addition of C_{10} in the presence of Gly-Sar increased the P_{app} of both peptides to levels similar to those obtained in the absence of Gly-Sar in both Caco-2 monolayers and rat jejunal mucosae. It is known that C_{10} fluidises the plasma membrane consistent with detergent-like perturbation [34]. Therefore, it is possible that the effect of C_{10} on the apical membrane indirectly nullifies the effect of PepT1 inhibition by Gly-Sar. We speculate that C_{10} allows the P_{app} of the peptides to revert to basal levels by increasing their paracellular flux as a consequence of diverting them from the inhibited transcellular PepT1 pathway through initiating enzymatic and intracellular calcium-mediated events that modulate the tight junction [33]. PepT1-mediated uptake relies on H^+-coupled co-transport, which is supported by an acid microclimate in the small intestine [49]. Alkalinisation of the microclimate can inhibit PepT1-mediated uptake [38], and a lack of pH gradient will reduce the capacity for PepT1 to translocate substrates. The bile salt, sodium deoxycholate, is also a permeation enhancer, but it does not alter the pH of the microclimate in rat jejunum [50], however we have not yet tested whether C_{10} behaves similarly. Therefore *in vitro*, the evidence suggests that C_{10} is acting predominantly by opening the paracellular route through indirect membrane-initiated intracellular mechanisms that lead to alterations in tight junction protein location and function. A model of this interpretation is presented (Fig. 6). In support of this interpretation, increasing concentrations of C_{10} resulted in both a re-distribution of TJ

proteins in Caco-2 monolayers as well as causing cell sloughing from the tips of villi, as expected from a surfactant. C_{10} has a long history of use in man and causes rapidly reversible membrane perturbations in more physiological-relevant models. Therefore the damage caused by C_{10} *in vitro* is likely due to direct exposure to the intestinal epithelia for 120 min without the normal epithelial protective mechanisms available *in vivo*. Further studies in a larger animal model such as oral gavage in pigs would allow for dose-dependent assessment of the contribution of C_{10} to the increase in plasma peptide levels. Unlike larger peptides, IPP and LKP have relatively low molecular weights (325 Da and 356 Da respectively) and are hydrophilic, thus making them suitable candidates for permeation via an enabled paracellular route in the event of PepT1 inhibition. Paracellular permeability typically requires a molecule to be < 500 Da and hydrophilic in order to pass tight junctions. As TJ proteins are transiently relocated and perturbed by permeation enhancers, tissue TEER reduces and the paracellular permeability of tracer molecules (e.g. FITC) increases across intestinal epithelia [51, 52]. However, concerns are often raised when the intestinal barrier is perturbed due to the possible translocation of bacteria, toxins, and bacterial lipopolysaccharide [53]. Previous studies with PEs have shown that the intestinal barrier repaired within 1-3 hours [54-56], and that the molecular diameter of molecules which can cross when the barrier is opened in a temporary fashion is quite low [52].

After co-administration of [^{3}H]-IPP with Gly-Sar or [^{3}H]-LKP with Gly-Sar to normal rats by oral gavage, the plasma levels of the peptides were reduced by 56% and 57% at 180 min respectively compared to values in the absence of Gly-Sar, thereby implicating a role for PepT1 in mediating as least part of their flux pathway *in vivo*. Oral gavage of C_{10} with [^{3}H]-IPP or [^{3}H]-LKP in the presence of Gly-Sar restored serum values at the 180 min time point to the levels obtained with either [^{3}H]-IPP or [^{3}H]-LKP alone and this was consistent with the

in vitro data. Restoring the oral absorption of a PepT1 substrate using an amphiphilic permeation enhancer, Labrasol®, was also previously reported in isolated rat jejunal mucosae for cephalexin [57]. Cephalexin permeability was reduced by 18% in the presence of Gly-Leu and Ala-Ala, but addition of Labrasol® restored permeability to basal levels. Similarly, oral absorption of oseltamivir, an antiviral prodrug and substrate for PepT1, was reduced when co-administered with either Gly-Sar or bovine milk in rats [58]. The authors argued that the inhibition of PepT1 by components of milk is due to intestinal hydrolysis of milk proteins leading to di-and tri-peptide competition for uptake via PepT1 of oseltamivir. On the other hand, a clinical trial showed an increase in oseltamivir plasma $AUC_{0\text{-}24\ h}$ after administration with a high fat high calorie meal including milk compared to fasted state [42]. Perhaps the high fat content of the meal perturbed the intestinal epithelium, thereby acting in part as an emulsion-based permeation enhancer(s) to overcome the effect of PepT1 competition by the milk-derived peptides [59]. Furthermore, Matsui *et al.* reported that co-administration of VY with captopril nullified the individual hypotensive effect of both agents when administered to SHRs, and cautioned against simultaneous intake of ACE inhibitors and foods high in di- and tripeptides [25]. We note that there is some controversy over whether captopril is a true substrate for PepT1, as it has relatively weak affinity for PepT1 in transfected cells and there is speculation by the group of Brandsch that its high oral bioavailability may possibly be accounted for by passive diffusion or the influence of other transporters [60]. Nonetheless, the inhibition of flux of IPP and LKP by Gly-Sar across two *in vitro* bioassays, the inhibition of the blood-pressure lowering effects of the peptides in SHRs by Gly-Sar, along with a reduction in serum levels of the peptides by Gly-Sar in normal rats suggests that the two peptides are indeed substrates for PepT1.

The present study demonstrated that Gly-Sar co-administration partly reversed the

hypotensive effect of both IPP and LKP in SHRs, but that addition of C_{10} restored it. This is the first demonstration that a food-grade agent can enhance *in vivo* effects of bioactive peptides derived from food. Application of pharmaceutical technologies such as food-grade permeation enhancers and delivery systems to food bioactive peptides is an emerging area [61, 62]. Recently, Val-Leu-Pro-Val-Pro was encapsulated in a pol-(lactic-*co*-glycolic) acid-based nanoparticle, and exhibited sustained attenuation of hypertension compared to free peptide after oral administration to SHRs [63]. The European Food Safety Authority (EFSA) regulates health claims for foods, however both IPP and LKP were rejected due to lack of sufficient evidence of reducing blood pressure levels in the general population with foods enriched with IPP and LKP] [64, 65]. However, it should be noted these peptides appear to be more effective in pre-hypertensive and hypertensive subjects compared to normotensive subjects [66]. Use of appropriate delivery systems may therefore yield a food-grade formulation to deliver an efficacious dose of these antihypertensive peptides to attenuate hypertension in pre-hypertensive subjects prior to the requirement for pharmaceutical intervention.

5. Conclusions

C_{10} enhanced the permeability of [^{3}H]-IPP and [^{3}H]-LKP in the presence of Gly-Sar in Caco-2 monolayers and in isolated rat jejunal tissue. Antihypertensive effects of IPP and LKP were confirmed when reduction in blood pressure was seen after both i.v. and oral administration in SHRs. Co-administration of the peptides with Gly-Sar resulted in a decrease in their hypotensive effects, however co-administration with C_{10} in the presence of Gly-Sar reversed the loss of the hypotensive effects of IPP and LKP in the SHRs. The serum levels of [^{3}H]-IPP and [^{3}H]-LKP in normal rats were reduced by ~50% in the presence of Gly-Sar in normal rats following oral gavage, suggesting involvement of PepT1 in the permeation pathway. C_{10}

was able to prevent the reduced serum levels of the peptides when co-administered with Gly-Sar, thereby returning values near to basal levels. C_{10} may overcome PepT1 inhibition indirectly by enhancing paracellular permeability of IPP and LKP.

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Fig. 1

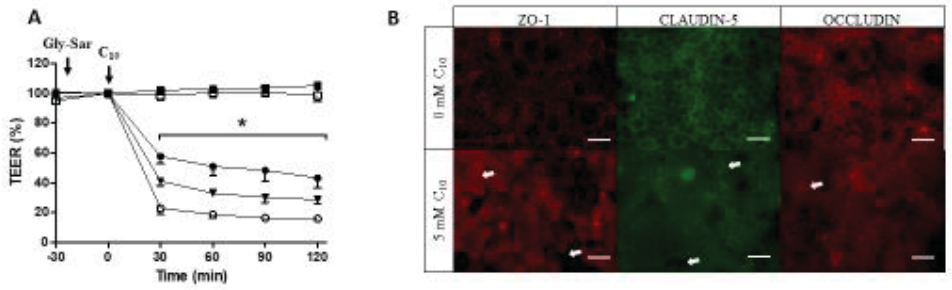

Fig. 1. A. Change in Caco-2 monolayers TEER values at 120 min expressed as % of TEER at $t = 0$ min. Untreated (■), 10 mM Gly-Sar (□), 1 mM C_{10} (●), 2.5 mM C_{10} (▼), and 5 mM C_{10} (○). Two-way ANOVA with Bonferonni's *post hoc* test. * $P < 0.05$ for all three C_{10} concentrations compared with untreated controls. Each value represents the mean ± SD, n = 3 independent replicates. B. Representative immuno-fluorescence analysis of selected tight junction proteins exposed to 5 mM C_{10} for 120 min in Caco-2 monolayers grown on chamber slides. White arrows indicate areas of cell sloughing. Horizontal bars = 10 µm.

Fig. 2

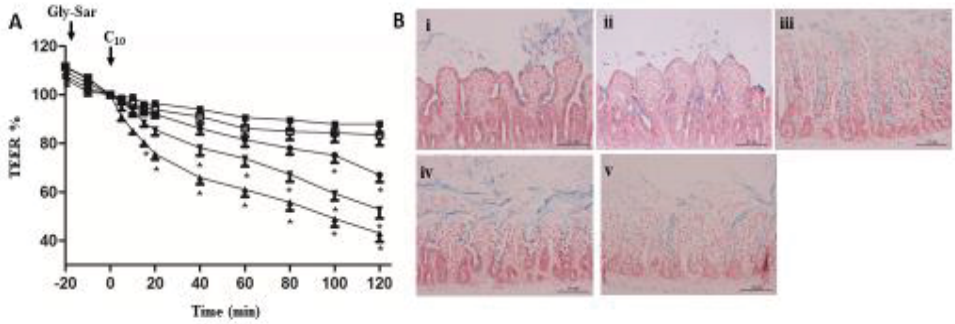

Fig. 2. A. Change in isolated jejunal mucosae TEER expressed as % of TEER at $t = 0$ min. Untreated (■), 10 mM Gly-Sar (□), 10 mM C_{10} (●), 20 mM C_{10} (▼), and 30 mM C_{10} (▲). Two-way ANOVA with Bonferonni's *post hoc* test. * $P < 0.05$ compared with untreated control. Each value represents the mean ± SD, n = 5 independent replicates. B. Alcian blue and neutral red stained representative light micrographs of rat jejunal tissue 120 min after mounting in Ussing chambers. (i) Untreated, (ii) 10 mM Gly-Sar, (iii) 10 mM C_{10}, (iv) 20 mM C_{10} and (v) 30 mM C_{10}. Horizontal bar denotes 10 µm.

Fig 3

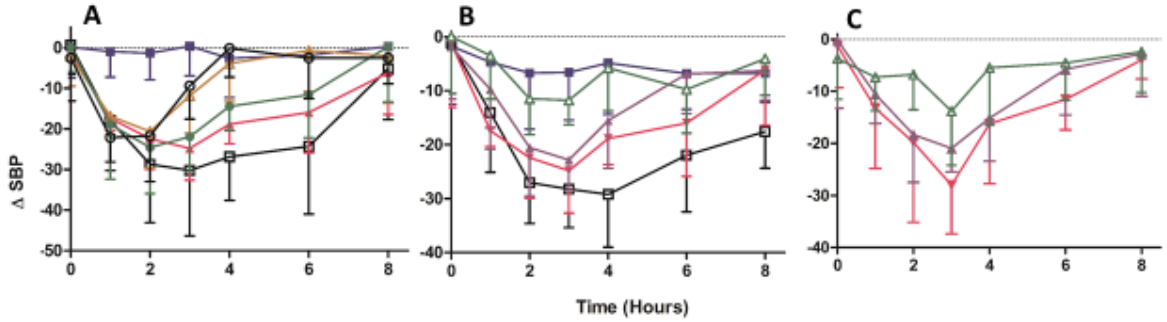

Fig. 3. A. Δ SBP in SHRs. A. I.V. administration. PBS (■), 5 mg/kg captopril (□), 1 mg/kg IPP (Δ), 1 mg/kg LKP (○), 5 mg/kg IPP (▲), and 5 mg/kg LKP (●). B. oral gavage of IPP; PBS (■), 5 mg/kg captopril (□), 1 mg/kg IPP (Δ), 5 mg/kg IPP (▲), and 10 mg/kg IPP (▼). C. Oral gavage of LKP. 1 mg/kg LKP (Δ), 5 mg/kg LKP (▲), and 10 mg/kg LKP (▼). Mean ± SD, n = 6 independent replicates per group.

Fig. 4

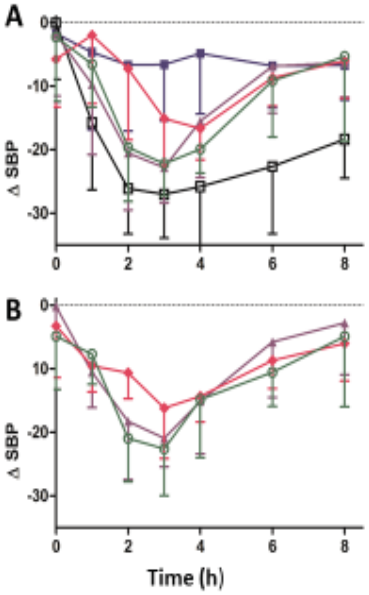

Fig. 4. Δ SBP in SHRs after oral gavage of IPP and LKP. (A) IPP: groups PBS (■), 5 mg/kg captopril (□), 5 mg/kg IPP (▲), 5 mg/kg IPP + Gly-Sar (100 mM) (♦), and 5 mg/kg IPP + Gly-Sar (100 mM) + C10 (180 mM) (○). (B) LKP: groups. 5 mg/kg LKP (▲), 5 mg/kg LKP + Gly-Sar (100 mM) (♦), and 5 mg/kg LKP + Gly-Sar (100 mM) + C10 (180 mM) (○). Mean ± SD, n = 6 independent replicates per group.

Fig. 5

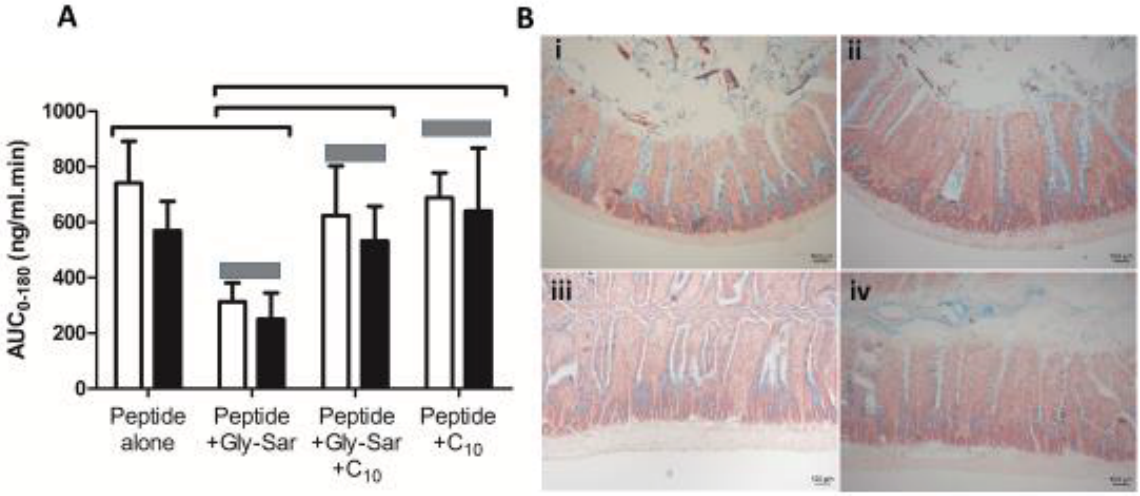

Fig. 5. (A) Area of the curve (AUC) over 180 min of serum [^{3}H]-IPP (white bars) and [^{3}H]-LKP (black bars) following oral gavage co-administration of Gly-Sar (16 mg/kg; 100 mM) and C_{10} (35 mg/kg; 180 mM) to normal rats. Each value represents the mean ± SD, n = 6 independent replicates. Statistical differences at the minimum of the $P < 0.05$ level are seen between the four group types (horizontal grey bars): Peptides + Gly-SAR groups are reduced compared to Peptides alone; the Peptides + Gly-Sar + C_{10} groups are increased compared to the Peptides + Gly-Sar groups; Peptides + C_{10} are increased over both the Peptides alone group and the Peptides + Gly-Sar groups. Peptides comprise IPP and LKP examined separately and statistics are for both peptides. (B) Alcian blue- and neutral red-stained representative light micrographs of jejunal tissue 180 min after oral gavage: (*i*) Untreated (*ii*) 100 mM Gly-Sar, (*iii*) 180 mM C_{10}, (*iv*) 100 mM Gly-Sar and 180 mM C_{10}. Horizontal bars denote 100 μm.

Fig. 6

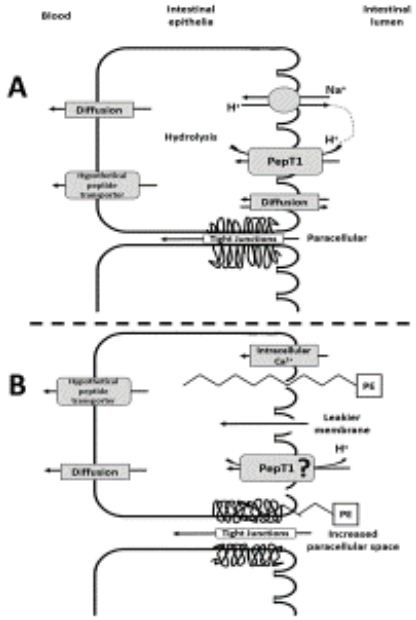

Fig. 6 A. Schematic of IPP and LKP transepithelial transport across small intestinal epithelia in control conditions and B. when exposed to C_{10}.

Table 1. P_{app} of [^{3}H]-IPP and [^{3}H]-LKP across Caco-2 monolayers exposed to C_{10} and/or 10 mM Gly-Sar over 120 min

[^{3}H]-IPP P_{app} (x 10^{-6} cm.s^{-1})				
	Control	*C_{10} (1 mM)*	*C_{10} (2.5 mM)*	*C_{10} (5 mM)*
0 mM Gly-Sar	6.2 ± 2.9	10.1 ± 0.7	12.2 ± 2.7 *	15.7 ± 5.2 *
10 mM Gly-Sar	3.3 ± 1.8[a]	9.6 ± 1.1 *	11.5 ± 3.9 *	20.7 ± 5.3 *
[^{3}H]-LKP P_{app} (x 10^{-6} cm.s^{-1})				
0 mM Gly-Sar	11.4 ± 1.7	12.4 ± 2.4	16.8 ± 3.2 *	22.6 ± 2.1 *
10 mM Gly-Sar	6.4 ± 1.6 [a]	12.1 ± 2.8 *	17.9 ± 1 *	21.2 ± 0.9 *

* $P < 0.05$, C_{10}-exposed monolayers compared with untreated control monolayers. [a] $P < 0.05$, for Gly-Sar exposed monolayers compared with untreated control monolayers. One-way ANOVA with Dunnett's multiple comparison. Mean ± SD, n = 3 independent replicates.

Table 2. P_{app} values of [^{3}H]-IPP and [^{3}H]-LKP across rat jejunal tissue exposed to C_{10} and/or 10mM Gly-Sar over 120 min

[^{3}H]-IPP P_{app} (x 10^{-6} cm.s^{-1})				
Gly-Sar	*Control*	*C_{10} (10 mM)*	*C_{10} (20 mM)*	*C_{10} (30 mM)*
(-)	2.6 ± 0.6	3.4 ± 0.6	4.2 ± 0.8 *	5.3 ± 1.2 *
(+)	1.4 ± 0.3 [a]	3.1 ± 0.8 *	4.1 ± 0.4 *	5.2 ± 0.6 *
[^{3}H]-LKP P_{app} (x 10^{-6} cm.s^{-1})				
(-)	2.1 ± 0.4	2.6 ± 0.7	4.0 ± 0.6 *	5.4 ± 0.8 *
(+)	1.5 ± 0.3 [a]	2.9 ± 0.9 *	3.9 ± 0.4 *	5.2 ± 0.6 *

* $P < 0.05$, C_{10}-exposed mucosae compared with untreated controls; [a] $P < 0.05$, for Gly-Sar exposed mucosae compared with untreated control mucosae. One-way ANOVA with Dunnett's multiple comparison. Mean ± SD, n = 5 independent replicates.