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Stability, toxicity and intestinal permeation enhancement of two food-derived antihypertensive tripeptides, Ile-Pro-Pro and Leu-Lys-Pro

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ABSTRACT

Two food-derived ACE inhibitory peptides, Ile-Pro-Pro (IPP) and Leu-Lys-Pro (LKP), may have potential as alternative treatments for treatment of mild- or pre-hypertension. Lack of stability to secretory and intracellular peptidases and poor permeability across intestinal epithelia are typical limiting factors of oral delivery of peptides. The stability of IPP and LKP was confirmed *in vitro* in rat intestinal washes, and intestinal and liver homogenates over 60 min. A positive protein control for peptidases, insulin, was significantly digested in each format over the same period. Neither tripeptide showed cytotoxic activity on Caco-2 and Hep G2 cells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, even after chronic exposure. The basal P_{app} of fluorescein isothiocyanate (FITC)-labelled IPP and FITC-LKP across isolated rat jejunal and colonic mucosae were low, but were significantly increased in each tissue type by the medium chain fatty acids (MCFA) permeation enhancers, sodium caprate (C_{10}) and the sodium salt of 10-undecylenic acid (uC_{11}). IPP and LKP were therefore stable against intestinal and liver peptidases and were non-cytotoxic; their P_{app} values across rat intestinal mucosae were low, but could be increased by MCFA. There is potential to make on oral dosage form once *in vivo* pharmacology is confirmed.

Key words: Oral peptides; milk-derived peptides; intestinal peptide transport; permeation enhancers; intestinal peptidases; anti-hypertensive peptides

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1. INTRODUCTION

The body regulates blood pressure in part via the renin-angiotensin-aldosterone system (RAAS). Renin is produced in the kidney and acts on angiotensinogen, producing angiotensin I, which then passes through the pulmonary circulation and is converted by angiotensin converting enzyme (ACE) to the potent vasoconstrictor, angiotensin II (1, 2). ACE inhibitors act competitively, whereas angiotensin receptor antagonists prevent receptor binding of angiotensin II; both cause vasodilation (3). Food-derived ACE inhibitors may provide the opportunity to maintain normal blood pressure and may prevent escalation of hypertension. Meta-analyses of randomised control trials of food-derived antihypertensive peptides concluded that these peptides may lead to reduction of blood pressure. This conclusion could be made, even though these peptides were not presented in optimised oral formulations, although fermented milk matrices may offer some benefits as a delivery system (4, 5). Therefore they could potentially be used as a nutritional treatment for mild hypertension or pre-hypertension, when formulated in functional foods or as nutraceuticals (6-9).

Proteins from food undergo enzymatic hydrolysis by digestive enzymes including stomach pepsin and duodenal serine proteases to release smaller bioactive peptides, which may be bioactive. For example, Ile-Pro-Pro (IPP) was isolated from milk β-casein following fermentation by *Lactobacillus helveticus* (10). Leu-Lys-Pro (LKP) was isolated from chicken and fish muscle after digestion by the bacterial enzyme, thermolysin (11). Both tripeptides are established as ACE inhibitors *in vitro*, with respective IC_{50} values of 5 μM and 0.32 μM (12). Hypotensive effects of the tripeptides on systolic blood pressure were demonstrated in the Spontaneously Hypertensive Rat (SHR) following intravenous administration. It was noted however, that a reduction of 18 mm Hg with oral dose of 5mg/kg IPP and of 18 mmHg with a dose of 60mg/kg LKP could be detected in the rats(13, 14). These studies indicate that

some oral bioavailability leading to a pharmacodynamic effect could be achieved with non-optimised formulations.

Stability of the tripeptides could be an issue, as they may potentially be digested to single amino acids by intracellular carboxypeptidases, however IPP and LKP appeared resistant to metabolism by Caco-2 cell brush border aminopeptidases (15). Permeation of the tripeptides likely requires a contribution from transcellular routing across small intestinal enterocytes. If so, they are likely candidates for the hPEPT1 carrier, similar to captopril (16), and further investigation of their stability to intracellular peptidases is necessary in the event that they exploit this pathway. It is also possible that IPP and LPK can also take advantage of the paracellular pathway due to their hydrophilicity and relatively low molecular weights. Most nutrient absorption occurs in the small intestine due to its larger surface area and leakier epithelial tight-junctions compared to the colon (17). Medium chain fatty acids (MCFA) are powerful permeation enhancers acting in part by re-organising tricellulin and claudin 5 proteins at the tight junction (18), as well as through a mild detergent fluidizing effect on the plasma membrane (19). Several peptides are in oral clinical trials using MCFA-based technologies (20, 21). One of the lead MCFA in clinical trials, capric acid, is found in mM concentrations in dairy products, and its sodium salt (C_{10}) has shown extensive enhancement effects *in vitro* and *in vivo* (22). Recently, an over-the-counter nutritional supplement and antifungal agent, the sodium salt of 10-undecylenic acid (uC_{11}), demonstrated similar permeation enhancement effects to C_{10} *in vitro* using isolated rat intestinal mucosae and *in vivo* in rat intestinal loop instillations (23). However, applying such enhancers to food-derived peptides in oral formulations has not been attempted before.

This study was undertaken to (a) determine the stability of IPP and LKP in rat intestinal fluid, intestinal homogenates and liver homogenates; (b) to determine cytotoxicity of the peptides in Caco-2 intestinal cells and Hep G2 liver cells; and (c) to test the capacity of C_{10} and uC_{11} to increase the permeability of fluorescein isothiocyanate FITC-IPP and LKP across isolated rat small intestinal and colonic tissue mucosae mounted in Ussing chambers. The data show that that IPP and LKP are stable, non-cytotoxic, and are amenable to having their permeability increased across the rat intestinal epithelium. This is the first demonstration that uC11 can improve intestinal permeability of a peptide.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

Synthetic IPP (MW= 325), LKP (MW= 356) and corresponding FITC-labelled versions were obtained from China Peptides (Shanghai, China). CellTitre 96® AQ_{ueous} One Solution Cell Proliferation Assay was supplied by Promega (Madison, USA). C_{10} was obtained from Fluka (Germany); uC_{11} was from Chemos (Germany). Human recombinant insulin USP grade was expressed in *Saccharomyces cerevisiae* from Sigma-Aldrich (UK). All other reagents, chemicals and solvents were analytical grade from Sigma-Aldrich (UK). Caco-2 cells (passage 38-48) were obtained from European Collection of Cell Cultures (Salisbury, UK). HepG2 cells (passage 6-16) were obtained from American Type Culture Collection. Experiments on *post-mortem* rat intestinal tissue were carried out following approval by the local UCD Animal Research Ethics Committee (Protocol: AREC 14-28-Brayden).

2.2 MTS assay

Caco-2 and HepG2 at a density of 2×10^4 cells/well were cultured on 96 well plated in DMEM and EMEM respectively, supplemented with 10% foetal bovine serum, 1% $_L$-

glutamine, 1% penicillin-streptomycin and 1% non-essential amino acids, and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% O_2. The assay was carried out using 1 and 24 h exposure times for the peptides on Caco-2 cells, and for 72 h on HepG2 cells, using Triton X-100™ (0.05%) as a positive control. The time points were selected to mimic acute (1 h) exposure in Caco-2 cells and chronic exposure (24 and 72 h) in each cell type. Cells were treated with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium accordingly. Optical density (OD) was measured at 490 nm . Each value presented was normalised against untreated control and calculated from three separate experiments, each of which included six replicates.

2.3 Preparation of ex vivo rat gastrointestinal enzyme fluids and liver homogenates

Male Wistar rats (250-300 g; Charles River, UK) were euthanized by stunning and cervical dislocation. A small intestine section of 15 cm was removed, flushed with simulated intestinal fluid *san pancreatin* (SIFsp). SIFsp was prepared as per United States Pharmacopoeia: a 25mM KH_2PO_4 buffer solution at pH 6.8 (24). The fluid was collected and extracted three times with 5 ml ice-cold dichloromethane to remove lipids. The extract was filtered using 0.45 µm syringe filters (25), resulting in a fluid designated as '*gut wash'* (GW). The initial tissue was then homogenized in HBSS at 30Hz for 2 min using a Qiagen TissueLyser®. The homogenate was centrifuged at 3695 rpm for 5 min and the supernatant was filtered using 0.45 µm syringe filters resulting in a fluid designated '*intestinal homogenate'* (IH). A fresh liver (~7g) was harvested from a euthanized Wistar rat, placed in ice-cold saline and homogenized in 10 mM PBS pH 7.4 at 30Hz for 2 min. The homogenate was centrifuged 2000 rpm at 4 °C for 5 min, and the supernatant collected was designated as

'liver homogenate' (LH). Each fluid and homogenate was prepared using three independent replicates.

2.4 Stability studies in isolated rat intestinal and liver extracts

IPP and LKP (4mM) were incubated in GW, IH and LH for 60 min at 37 °C and agitated on a shaker at 150 rpm. Recombinant human insulin (200µM) was incubated in GW, IH and LH as a positive control to confirm metabolic activity (25). Samples were taken at 0, 30 and 60 min, centrifuged at 10000 rpm at 4 °C; the reaction was stopped by placing the samples on dry ice. All samples were analysed for direct stability by reverse phase (RP)-HPLC. Each value was calculated from three separate experiments.

2.5 Reverse phase HPLC analysis

Stability of the tripeptides was measured by RP-HPLC using a modified LC-MS method (26). Samples were analysed with a Varian 920 HPLC using a Luna 5µ C18 (2) column 250 x 4.6 mm (Phenomenex, UK). Gradient elution was carried out at a flow rate of 0.5 mL/min, with a mobile phase A, containing water and 0.05% TFA, and a mobile phase B, containing acetonitrile and 0.05% TFA. The gradient sequence was: 100% A from 0-5 min, 5-30% B from 10-25 min, 30-70% B from 25-30 min, 70-5% B from 30-31 min, 5-0% B from 31 to 35 min, and 100% A from 35-37 min. Tray temperature was maintained at 8 °C, the injection volume was 50µL and the UV absorbance was 214 nm.

2.6 Ussing chamber studies: FITC-tripeptide fluxes across isolated rat intestinal mucosae

Male Wistar rats (250-300 g; Charles River, UK) were euthanized by stunning and cervical dislocation. Colon and jejunum were removed, opened along the mesenteric border and rinsed with warm oxygenated Krebs-Henseleit buffer (KH) (27). Fifteen cm of jejunum was excised at a point 10-12 cm proximal to the stomach and pinned with the mucosal side down and mounted in Ussing chambers with a circular window of 0.63 cm^2, bathed bilaterally with

5 ml KH and continuously gassed with 95% CO_2/5% O_2 at pH 7.4 maintained at 37 °C. Colon tissue was excised, cleaned of faecal matter, pinned with the mucosal side down and the muscle layers were removed and mounted in Ussing chambers as described. The transepithelial potential difference (PD; mV) and short circuit current (I_{sc}, µA) were measured across jejunal and colonic mucosae using a DVC-4000 voltage clamp apparatus (World Precision Instruments, Hertfordshire, UK). Analogue data were digitized with a Powerlab® data acquisition unit and analysed with LabChart® software (AD Instruments, Oxford, UK). After an initial 30 min equilibration (jejunum) and 45 min equilibration (colon), PD and I_{sc} were used to calculate the transepithelial electrical resistance (TEER, Ω cm^2) over 120 min (28). FITC-IPP (500µM) or FITC-LKP (500µM) were added to the apical chamber with or without the addition of C_{10} (10mM) or uC_{11} (10mM). Apical additions for MCFA were in calcium-free KH to prevent precipitation. Basolateral samples (100µl) were collected every 20 min over 120 min and transferred to light-protected white 96 well plates. Volumes were replaced with fresh KH, apical-side samples were collected before MCFA were added and also after 120 min. Fluorescence was measured in a spectrofluorimeter (MD Spectramax Gemini) with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The P_{app} (cm/s) was determined using the following equation:

$\boldsymbol{Papp = \frac{dQ}{dt}\frac{1}{A \cdot C0}}$, where dQ/dt is the transport rate across the epithelium (slope of cumulative amount FITC-peptide *versus* time) (mol/s); A is the surface area (0.63 cm^2); C_0 is the initial concentration of the FITC-peptide in the apical compartment (mol/ml).

2. 7 Histology

For experiments with jejunal tissue and colonic mucosae exposed to FITC-peptides and MCFA, tissues were removed after 120 min exposure in Ussing chambers and immersed in

10% (v/v) buffered formalin for at 48 h. Tissues were stained with haematoxylin and eosin (H&E), Alcian blue, and neutral red. Slides were visualized 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 6.3 (Media Cybernetics Inc., USA) acquisition software.

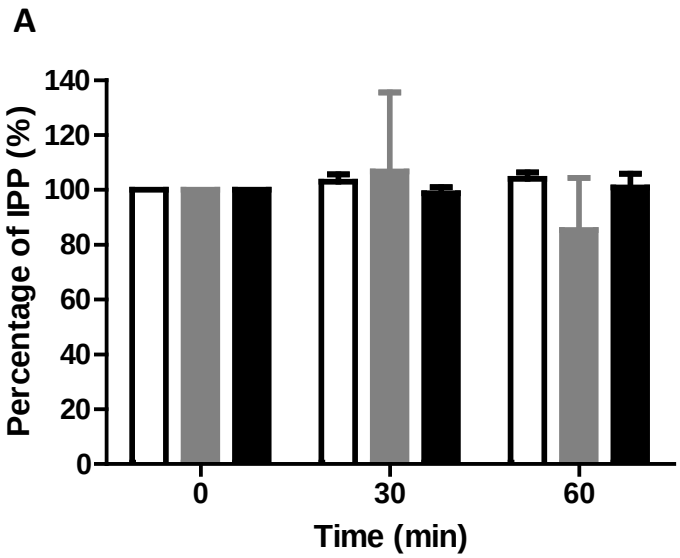
2. 8 Statistics

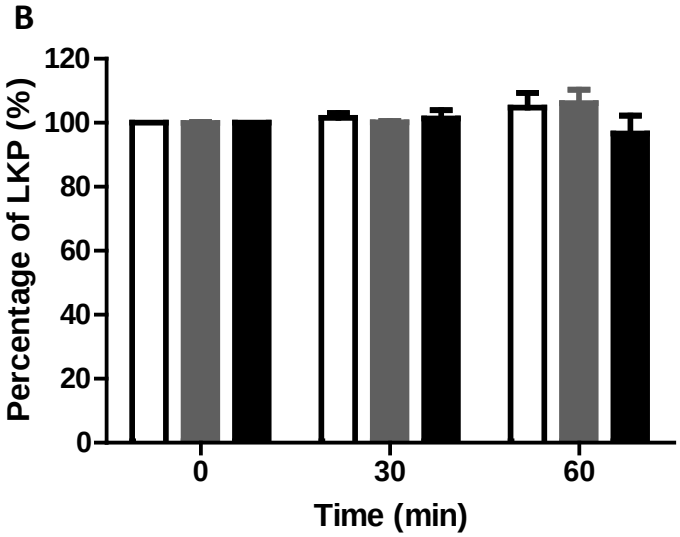
Statistical significance was measured by one-way ANOVA or two-way ANOVA with either Dunnett and Bonferroni *post-hoc* tests. $P<0.05$ was the required level to denote statistical significance.

3 RESULTS

3.1 Tripeptides are stable in rat gastrointestinal and liver extracts

The stability of IPP (4mM) and LKP (4mM) against GW, IH and LH were analysed by RP-HPLC. Incubation of the two peptides in the three extracts showed no evidence of metabolism over 60 min, confirming stability (Fig. 1A, B). Peptidase capacity of the three systems was confirmed by significant breakdown of 200µM human recombinant insulin in each system over the same period (Fig. 1C) showing breakdown similar to a previous study (25). The extract with the greatest capacity for metabolism was the rat gut wash (GW), as indicated by 65% breakdown of insulin compared to that seen in the two homogenates. GW was prepared using SIFsp, which is buffered to the optimum pH for enzymatic activity, whereas, homogenates were prepared in salt solutions (HBSS and PBS). Overall, IPP and LKP appear highly resistant to metabolism by rat intestinal and liver enzymes.


A
Percentage of IPP (%)
140
120
100
80
60
40
20
0
0
30
60
Time (min)


B
Percentage of LKP (%)
120
100
80
60
40
20
0
0
30
60
Time (min)

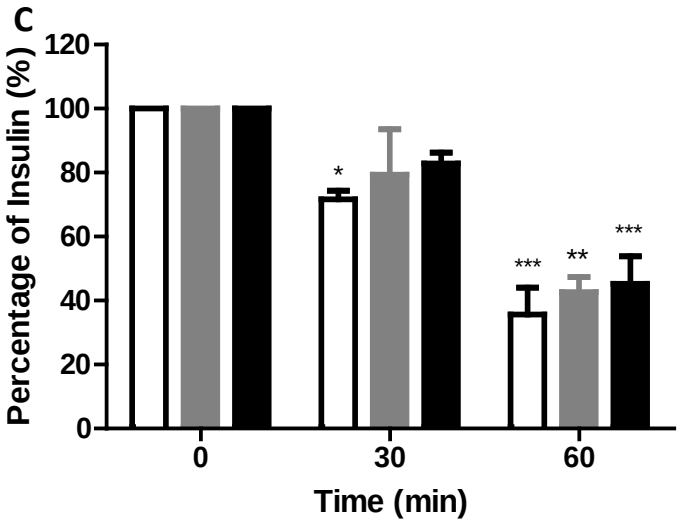
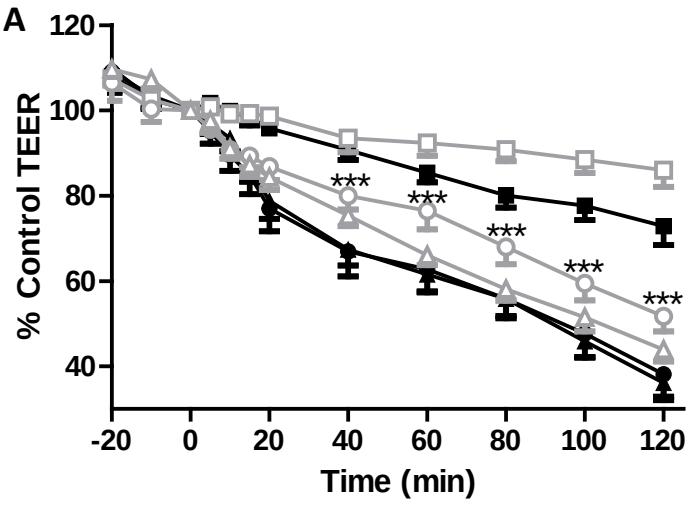

C
Percentage of Insulin (%)
120
100
80
60
40
20
0
*
***
**
***
0
30
60
Time (min)

Fig. 1. Stability of (A) IPP, 4mM; (B) 4mM LKP 4mM; (C) 200µM human insulin in rat gut washes (GW - White), intestinal homogenates (IH - Grey) and liver homogenates (LH - Black). One-way ANOVA with Dunnett's multiple comparison. Mean ± SEM n = 3; * $P <$ 0.05, ** $P < 0.01$, and ***$P< 0.001$ respectively, compared with control (0 min).

3. 2 MCFA increase the Papp of FITC-tripeptides in rat jejunal and colonic mucosae

Jejunal basal TEER values were 56 ± 17 Ω cm^2 (n = 47),within the published range (29). Jejunal TEER gradually decreased over 120 min to 80% of the basal value in the presence of apically-added FITC-tripeptides (500µM), but this was no different from untreated control (data not shown). Apical addition of 10mM C_{10} and uC_{11} significantly decreased TEER to 44% and 40% of basal value respectively, showing significant decreases between 15-120 min compared to control tissue (Fig. 2). Colonic mucosae basal TEER values were 112 ± 32 Ω cm^2 (n = 29), again within the accepted range (29) In the presence of FITC-tripeptides, colonic TEER showed a small decrease of 9% over 120 min, but this was not different from controls. Addition of C_{10} and uC_{11} significantly decreased TEER, to just 3% and 8% of basal values respectively from between 5-120 min compared to tissues exposed to FITC-tripeptides alone (Fig. 2). Both MCFA caused similar TEER reductions in colonic TEER values.



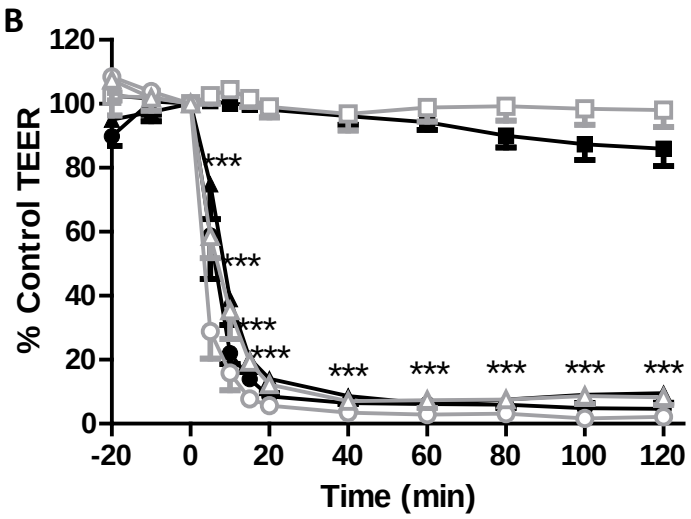


Fig. 2. MCFA decrease TEER across (A) isolated rat jejunum; (B) rat colon. Concentrations were 500µM FITC-IPP (closed symbols) and FITC-LKP (open symbols) and 10mM C_{10} and uC_{11}. FITC-IPP, FITC-IPP + $C_{10,}$ FITC-IPP + uC_{11}, FITC-LKP, FITC-LKP + C_{10}, FITC-LKP + uC_{11}. Two-way ANOVA with Bonferonni's post-hoc test, n = 5; * $P < 0.05$, ** $P < 0.01$, and ***$P< 0.001$ respectively versus FITC-tripeptide counterpart in the absence of the MCFA. Each value represents the mean ± SEM.

The basal P_{app} of FITC-IPP showed no differences across isolated jejunal tissue (1.39 ± 0.73 x 10^{-6} cm s^{-1}) or colonic mucosae (1.35 ± 0.68 x 10^{-6} cm s^{-1}). However, FITC-LKP had a significantly increased basal P_{app} across jejunal mucosae (2.14 ± 0.64 x 10^{-6} cm s^{-1}, $P<0.01$) compared to colonic mucosae (0.71 ± 0.36 x 10^{-6} cm s^{-1}) (Fig. 3). Apical addition of the two MCFAs (at the same concentrations as those that reduced TEER) led to an increased P_{app} of FITC-IPP and FITC-LKP across both jejunal and colonic mucosae. More significant FITC-tripeptide P_{app} increases induced by the MCFA were seen in colonic mucosae compared to jejunal (Table 1).

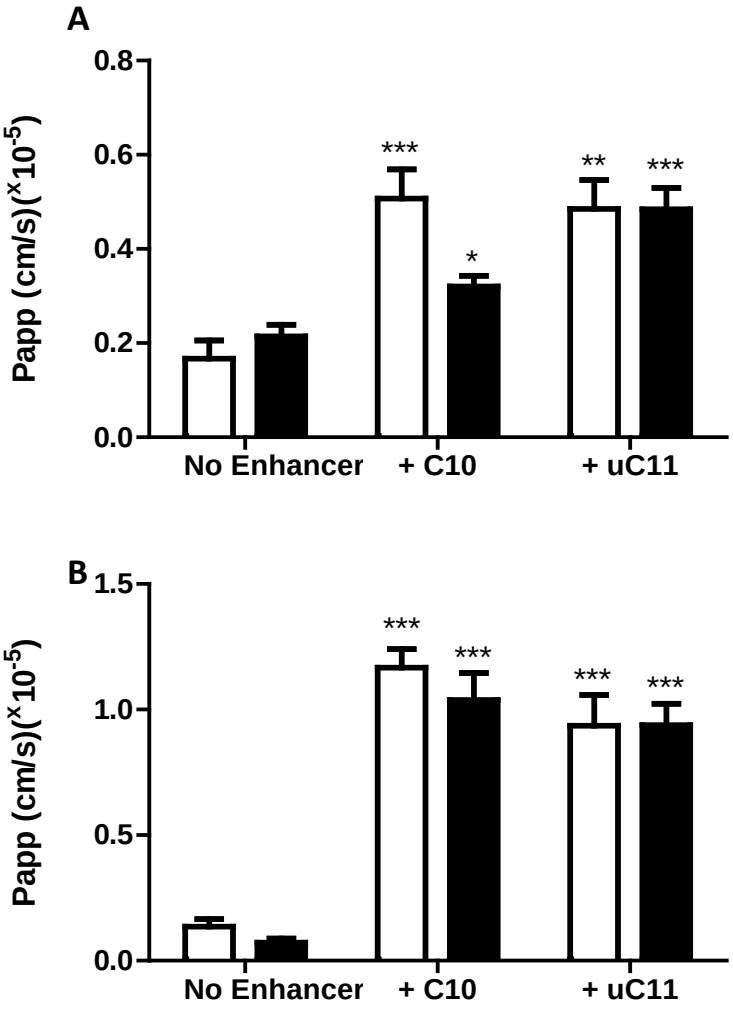


Fig. 3. Effect of apical addition of 10mM C_{10} and uC_{11} on P_{app} of apically-added FITC-IPP (500µM; white column) and FITC-LKP (500µM; black column) in (A) jejunal tissue and (B) colonic mucosae. One-way ANOVA with Dunnett's multiple comparison n = 5; *, **, and *** indicate $P < 0.05$, 0.01 and 0.001 respectively, compared with control. Each value represents the mean ± SEM.

Table 1. Papp values across intestinal mucosae of FITC-tripeptides in the presence of MCFA

	Jejunum		**Colon**	
	P_{app} (10^{-6} cm s^{-1})	**Fold increase**	**P_{app}** (10^{-6} cm s^{-1})	**Fold increase**
FITC-IPP	1.3 ± 0.0	-	1.3 ± 0.3	-
+ C_{10}	5.0 ± 0.6 ***	3.6	11.6 ± 0.7 ***	8.6
+ uC_{11}	4.8 ± 0.6 **	3.4	9.3 ± 1.2 ***	6.9
FITC-LKP	2.1 ± 0.2	-	0.7 ± 0.1	-
+ C_{10}	3.2 ± 0.2 *	1.4	10.3 ± 1.0 ***	14.4
+ uC_{11}	4.8 ± 0.4 ***	2.2	9.3 ± 0.8 ***	13.0

Data are expressed as means ± SEM. One-way ANOVA with Dunnett's multiple comparison test; concentrations as in Fig. 3; n = 5; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control peptides in the absence of MCFA.

3.3 IPP and LKP are not cytotoxic in vitro: MTS and histology

There was no evidence of cytotoxicity for each peptide in two cell lines at different time points (Table 2). The MTS assay data indicate that unlabelled IPP and LKP was not cytotoxic in Caco-2 or HepG2 cells from 0.1-10mM at selected time points. The MTS assay yielded low errors, similar to the findings of others (30). A positive control, Triton®-X-100 (0.05%), massively reduced viability of both cell types at each incubation time point.

Table 2. MTS analysis of IPP and LKP following incubation for 1 h and 24 h on Caco-2 and for 72 h on HepG2 cells.

	1 h **Caco-2**	**24 h** **Caco-2**	**72 h** **HepG2**
IPP			
10mM	101.2 ± 1.4	84.3 ± 1.8	99.7 ± 0.8
5mM	100.7 ± 1.0	89.2 ± 0.4	99.3 ± 0.3
1mM	100.6 ± 0.9	94.6 ± 2.2	101.3 ± 0.6
100µM	99.5 ± 2.9	94.9 ± 1.0	99.3 ± 0.3
LKP			
10mM	93.7 ± 2.8	93.7 ± 5.6	82.2 ± 8.9
5mM	102.0 ± 4.0	101.7 ± 2.9	99.7 ± 2.7
1mM	105.7 ± 2.3	107.0 ± 3.6	107.3 ± 1.7
100µM	98.3 ± 0.6	108.7 ± 2.6	103.0 ± 2.0
Triton®	36.8 ± 3.6 *******	34.9 ± 4.3 *******	18.4 ± 2.0 *******

Triton®: Triton®-X-100 (0.05% w/v). Data are expressed as means ± SEM. One-way ANOVA with Dunnett's multiple comparison test. 1 h and 24 incubations of unlabelled peptides on Caco-2 and 72 h on HepG2 cells.*, **, and *** indicate $P < 0.05$, 0.01 and 0.001 respectively. n=3 independent experiments for each concentration and time point with replicates of six.

The effects of FITC-IPP and FITC-LKP in the presence or absence of C_{10} and uC_{11} were examined by histological analysis of jejunal tissue and colonic mucosae following 120 min exposure in Ussing chambers. It should be noted that the cytotoxicity assays examined

unlabelled peptides whereas the histology study assessed the FITC-labelled peptides. Previous work with these peptides on Ussing chambers noted no impact on the isolated tissue (31), and there is no evidence suggesting that FITC is damaging (32). Control jejunal tissue after 120 min showed an intact epithelium with active goblet cells; exposure to 0.5mM FITC-IPP or FITC–LKP showed no damage (Fig. 4E, F). The MCFA induced cellular sloughing of jejunal villi, however, the overall physiological structure was retained (Fig. 4G, H). Untreated rat colonic mucosae after 120 min also had an intact epithelium (Fig. 4A); FITC-IPP and FITC–LKP caused no damage to the epithelium (Fig. 4B). Effect of MCFA on colonic mucosae shows perturbation of the epithelium, associated with their known mild surfactant actions (Fig. 4C, D). Overall, the histology was complementary to the data indicating absence of cytotoxic effects in the presence of the peptides, suggesting that any mild damage caused was due to the MCFA.

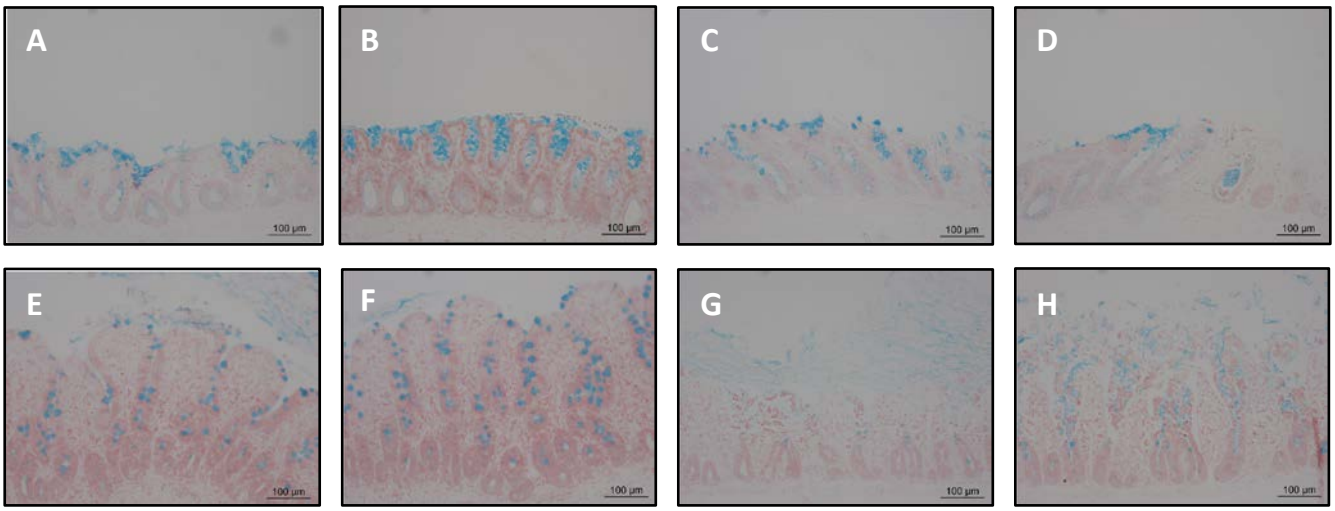


Fig. 4. Alcian blue and neutral red-strained light micrographs of rat intestinal mucosae mounting in Ussing chambers and exposed to FITC-tripeptides (500µM) and MCFA (10mM) for 120 min. (**A**) Untreated colon, (**B**) FITC-IPP in colon, (**C**) 10mM C_{10} in colon, (**D**) 10mM

uC_{11} in colon, (**E**) Untreated jejunum, (**F**) FITC-IPP in jejunum, (**G**) 10mM C_{10} in jejunum, (**H**) 10mM uC_{11} in jejunum. Horizontal bars denote 100µm.

4. DISCUSSION

A previous study showed the resistance of IPP and LKP to brush-border peptidases in Caco-2 monolayers incubated for 60 min in HBSS (15). The data presented here confirm these findings, however, HBSS is not very relevant for the physiology of the GI tract. Due to this, simulated intestinal fluids are used as a more physiological *in vitro* system incorporating modified pH and surfactants, however, they still underestimate potential breakdown (33), as they do not include brush-border and intracellular peptidases. The intestinal gut wash and homogenate used incorporates these enzymes and provides a better prediction of the stability of these peptides *in vivo*. The *ex vivo* gastrointestinal fluids include secretory digestive enzymes in the gut wash (GW) and brush-border peptidases and intracellular enzymes in intestinal homogenate (IH) (25). Each fluid was metabolically active, demonstrating digestion of insulin; GW showed the most degradation of insulin comparable to a similar study (34). As the GW was produced by flushing the small intestinal segment with SIFsp, the resulting fluid is buffered to a pH favourable for digestive enzymes. Whereas, the tissues were homogenised in an un-buffered physiological relevant salt solution. Therefore, GW and IH can be used as complementary methods of determining oral peptide stability to secretory and/or intracellular enzymes. It was also found that IPP and LKP are resistant to metabolism by liver enzymes, suggesting that the tripeptides will not be degraded during first pass metabolism and will be transported into systemic circulation intact. The presence of proline moieties in these tripeptides seem to provide inherent resistance to enzymatic degradation (35, 36), so the stability results are in keeping with that.

The tripeptides showed no cytotoxicity using the MTS assays on human colonic-derived epithelial enterocyte- and liver hepatocyte cell lines. A previous study found no potential for IPP and VPP to induce cytotoxicity or clastogenicity in Chinese hamster lung cells (37). The cytotoxicity data and histology confirm that any impact in the tissue mounted on the Ussing chambers are most likely from the MCFA, which were previously shown to have significant cytotoxicity in the MTT assay at 1 and 24 h exposures in Caco-2 cells (23). Mucosa histology also shows a perturbation of the intestinal and colonic epithelia, comparable to previous studies investigating these MCFA (23). Repair is possible following transient mild damage to the tissue in intact *in situ* rat intestinal instillations using similar enhancers, which caused similar initial perturbation (38).

Due to the stability of IPP and LKP, the main limiting factor in their application must therefore be poor intestinal permeability. Some oral bioavailability was indicated when IPP was delivered in a yogurt matrix, showing 2.1-fold increase compared to placebo control in a small randomised human cohort (39), but overall delivery was still very low. Others showed that after oral administration of a [^{14}C]-IPP analogue to rats, peak plasma radioactivity (albeit low level) was seen after 2 h, indicating some absorption across the intestine (40). Accumulation was seen in tissues associated with the renin-angiotensin system, although the highest was in the duodenum and jejunum. Further to this, the P_{app} of IPP and fluorescein across distal jejunum mounted in Ussing chambers was reported by another group as $5.6 \pm 0.7 \times 10^{-8}$ cm s^{-1} and $5.9 \pm 1.1 \times 10^{-6}$ cm s^{-1} respectively (31), consistent with hydrophilic agents with relatively low passive permeability. In sum, rat *in vitro* and *in vivo* along with human *in vivo* studies have shown a low permeability of IPP, whereas values for LKP are unreported. Upon conjugation of FITC to IPP and LKP, the resulting P_{app} values across proximal jejunum in the current study were $1.39 \pm 0.7 \times 10^{-6}$ and $2.14 \pm 0.6 \times 10^{-6}$ cm s^{-1}

more comparable to fluorescein, which is used as a marker of paracellular transport with permeation enhancers (Table 1). The P_{app} of FITC-tripeptides was indirectly quantified by the FITC moiety, which is covalently bonded to the tripeptides and evidence suggests integrity of FITC-labelled peptides (41). Therefore, successful formulation of IPP or LKP in an oral dosage form will likely require significant permeation enhancement as a design feature.

Addition of C_{10} and uC_{11} at 10mM resulted in significant increases in FITC-tripeptides permeability across both jejunum and colon tissues. C_{10} is regarded as a 'gold standard' intestinal permeation enhancer, however, recently uC_{11} was shown to have similar efficacy in isolated rat colon and in in situ rat intestinal instillations, whereby the P_{app} of FD-4 was increased several fold by both C_{10} and uC_{11} (23). These enhancers reduced colonic TEER values in accordance with previously published results (21). Both MCFA were effective in increasing colonic permeability of FITC-IPP and FITC–LKP. Insulin absorption enhancement by C_{10} using a loop model showed a rank order of efficacy: colon>ileum>jejunum>duodenum (42). This correlates with the increase in P_{app} of FITC-IPP and FITC–LKP in colon (8.62 and 14.49 respectively) compared to jejunum (3.62 and 1.49 respectively). However, uC_{11} had not previously been tested in jejunal tissue, with increases in P_{app} 3.4-fold (FITC-IPP) and 2.2-fold (FITC-LKP). This is in agreement with previous work on other anionic surfactants stating reduced enhancement activity due to reduced decrease in TEER in jejunum/ileum compared to colon (Fig. 2). Studies investigating intestinal permeation enhancement of tetradecyl maltoside (TDM) and dodecyl maltoside (DDM), and coco-glucosides (CG) showed no enhancement of FD-4 permeability across jejunum (27, 43). MCFA, TDM, DDM and CG increase intracellular calcium after 1 h treatment in Caco-2 cells, corresponding to decrease in TEER values in colon (19). However, jejunal epithelia is less sensitive to the effects of these enhancers. 0.2% CG reduced TEER

values in rat jejunum, showing a reduction equivalent to decreases from C_{10} and uC_{11} shown here. Although CG showed a significant decrease in jejunal TEER, the Papp of FD-4 was not affected. However, this is possibly due to the size of the marker used (FD-4). The data presented here is in agreement with the initial hypothesis that many permeation enhancers are more efficacious in colon than the small intestine (44), however, some enhancers including MCFA can still increase permeability across jejunum, the target site for nutrient and bioactive peptide absorption.

Future work is required on formulating antihypertensive peptides such as IPP, VPP (Val-Pro-Pro) and LKP and its prodrug form LKPNM (Leu-Lys-Pro-Asp-Met). These types of agents have been rejected for health claim status by the European Food Safety Authority (EFSA), due to an insufficient "cause and effect" relationship (44-47). Current formulations, often in functional food matrices rely on peptides reaching systemic circulation to affect blood pressure. Due to their low oral bioavailability however, such studies have not shown sufficient reduction or maintenance of blood pressure. Further work in SHR using intestinal permeation enhancers may provide sufficient increased oral bioavailability to elicit a proper sustained hypotensive response. If so, this would provide stimulus to allow formulation of these peptides with MCFA in a solid dosage form.

CONCLUSION

IPP and LKP were stable against secretory, brush-border, and intracellular intestinal enzymes and non-cytotoxic to intestinal and liver cells. C_{10} and uC_{11} significantly increased the permeability of FITC-labelled IPP and LKP in isolated rat jejunal and colonic tissue.

Combining IPP and LKP in a particulate formulation with selected MCFA may have potential as an oral formulation for use in hypertension control.

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