ABSTRACT SUMMARY
The milk-derived tri-peptide, Ile-Pro-Pro (IPP) inhibits angiotensin-converting enzyme (ACE) and may contribute to hypertension treatment if presented as an oral pharmaceutical. In contrast to insulin, IPP was stable and was not broken down when exposed to ex vivo intestinal fluids. In vitro permeability of IPP was higher in rat jejunal than colonic mucosae, and it was transported intact. In addition, IPP was not cytotoxic to either human Caco-2 or liver-Hep G2 cells. This study lays the biological groundwork for IPP formulation in an oral nanoparticle format.

INTRODUCTION
Ile-Pro-Pro (IPP) is a bioactive peptide found in bovine milk when casein is hydrolyzed by gastrointestinal enzymes. It has ACE inhibitory potency of 5μM with the potential to reduce blood pressure as an oral pharmaceutical. Its stability has been demonstrated in intestinal fluids and in the presence of Caco-2 brush-border peptidases, however it has poor intestinal permeability. A route of intestinal permeation of IPP may be via the PepT1 carrier, so IPP could be a target for intracellular peptidases. The application of permeation enhancers on the permeability of oral drugs has shown significant results in numerous publications. Few publications investigated cellular toxicity of IPP. The objective of this study was to determine the stability of IPP in rat intestinal fluids and gut homogenates. The permeability of IPP was calculated across rat jejunal and colon mucosae in vitro. Also the cytotoxicity of IPP in Caco-2 and HepG2 cells was investigated. This data prepares the way for formulation into nanoparticles for oral delivery.

EXPERIMENTAL METHODS
Stability of IPP in homogenates and gut washes
A 25cm section of duodenum/jejunum of male Wistar rats (250-300g) was isolated. The section was flushed with 10ml SIF (as per USP) to achieve a ‘gut wash’ (GW). The washed section was placed in HBSS and homogenized to yield ‘washed homogenate’ (WH). Other sections were placed into HBSS and homogenized: ‘unwashed homogenate’ (UH). IPP (4mM) and Insulin (200μM) were incubated at 37°C and agitated at 200 rpm. Samples were taken at 0, 30 and 60 min and analyzed by RP-HPLC with C18 column gradient mobile phase A) water 0.05% TFA and B) acetonitrile 0.05%. Samples were sourced from three rats and each run in triplicate.

Permeability of FITC-IPP across jejunum and colon tissue
A section of jejunum and colon of male Wistar rats (250-400g) was isolated. Isolated muscle-stripped colonic mucosae and unstripped jejunal mucosae were mounted on Ussing chambers. FITC-IPP (500μM) was added to the apical chamber with the addition of 10mM of either sodium caprate (C10) or a second medium chain fatty acid derivative (‘Analogue’). The apparent permeability (Papp) and transepithelial electrical resistance (TEER) were measured over 120 min. Tissue was sourced from five rats.

Cytotoxicity of IPP using MTT and MTS assay
Caco-2 and Hep G2 cells were cultured and seeded on 96 well plates in 200μl DMEM at 37°C. After 24 h, DMEM was removed and replaced with DMEM with IPP (0.1-10mM) or Triton®-X-100 (0.05%) for 1 or 24 h (Caco-2) or 72 h (Hep G2). Cells were treated with MTT or MTS accordingly. All data was analyzed by one-way ANOVA with Dunnett’s post-test.
RESULTS AND DISCUSSION

Incubation of IPP in rat intestinal homogenates and washes showed no evidence of metabolism over 60 min (Fig. 1a). Enzymatic capacity of the three systems was confirmed by breakdown of human insulin (Fig 1b). The greatest capacity for metabolism of insulin was the GW system, which broke down over 60% in 60 min, compared to 40% with the UH.

**Fig. 1.** Stability of A) IPP (4mM) and B) Insulin (200μM) in rat gut wash (GW), washed intestinal homogenate (WH) and unwashed intestinal homogenate (UH). n=3 *P< 0.05, **P< 0.01, ***P< 0.001 compared to control. Each value represents the mean ±SEM.

Permeation of FITC-IPP (Fig. 2.) was slightly higher but not statistically significant, in jejunum compared to colon, perhaps due to the higher presence of PepT1 in jejunum. C10 and the analogue increased the Papp of IPP significantly. In parallel, TEER was significantly reduced by both enhancers up to 70% in 30 min in both mucosae, suggestive of tight junction opening.

**Fig. 2.** Papp of FITC-IPP (500μM) across rat colonic and jejunal mucosae. Increased permeability is shown in the presence of C10 (10mM) and ‘Analogue’ (10mM). n=5 **P< 0.01, ***P< 0.001 compared to control. Each value represents the mean ±SEM.

Comparison of the MTS and MTT cytotoxicity assays indicated that IPP was non-cytotoxic in both the intestinal and liver cell lines, even at very high concentrations of 10mM for 72 h (data not shown).

CONCLUSION

IPP was stable in rat intestinal gut washes and homogenates and was non-cytotoxic to human intestinal and liver cells. Permeation was shown to be increased using permeation enhancers. Therefore, this information supports the development of an oral IPP nanoparticle formulation to potentially reduce blood pressure. Permeability rather than stability is the more important hurdle for IPP.

REFERENCES


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