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Salcaprozate sodium (SNAC) enhances permeability of octreotide across isolated rat and human intestinal epithelial mucosae in Ussing chambers



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ABSTRACT

Octreotide is approved as a one-month injectable for treatment of acromegaly and neuroendocrine tumours. Oral delivery of the octapeptide is a challenge due mainly to low intestinal epithelial permeability. The intestinal permeation enhancer (PE) salcaprozate sodium (SNAC) has Generally Regarded As Safe (GRAS) status and is a component of an approved oral peptide formulation. The purpose of the study was to examine the capacity of salcaprozate sodium (SNAC), to increase its permeability across isolated rat intestinal mucosae from five regions and across human colonic mucosae mounted in Ussing chambers. Apical-side buffers were Krebs-Henseleit (KH), fasted simulated intestinal fluid (FaSSIF-V2), rat simulated intestinal fluid (rSIF), and colonic simulated intestinal fluid (FaSSCoF). The basal apparent permeability coefficient (P_{app}) of [³H]-octreotide was equally low across rat intestinal regional mucosae in KH, rSIF, and FaSSIF-V2. Apical addition of 20 mM SNAC increased the P_{app} across rat tissue in KH: colon (by 3.2-fold) > ileum (3.4-fold) > upper jejunum (2.3-fold) > duodenum (1.4-fold) > stomach (1.4-fold). 20 mM and 40 mM SNAC also increased the P_{app} by 1.5-fold and 2.1-fold respectively across human colonic mucosae in KH. Transepithelial electrical resistance (TEER) values were reduced in the presence of SNAC especially in colonic regions. LC-MS/MS analysis of permeated unlabelled octreotide across human colonic mucosae in the presence of SNAC indicated that [³H]-octreotide remained intact. No gross damage was caused to rat or human mucosae by SNAC. Attenuation of the effects of SNAC was seen in rat jejunal mucosae incubated with FaSSIF-V2 and rSIF, and also to some extent in human colonic mucosae using FaSSCoF, suggesting interaction between SNAC with buffer components. In conclusion, SNAC showed potential as an intestinal permeation enhancer for octreotide, but *in vivo* efficacy may be attenuated by interactions with GI luminal fluid contents.

1. Introduction

Acromegaly is a hormonal disorder that occurs due to the presence of a pituitary adenoma, resulting in chronic overproduction of growth hormone. It is an orphan disease with an annual prevalence of 30–60 cases/million people (Capatina and Wass, 2015). Treatment includes surgery and therapeutic strategies to improve co-morbidities and quality of life (Lim and Fleseriu, 2017). Peptides are highly potent and selective, and generate lower toxicity compared to small molecules, making them attractive candidates for disease treatment (Lau and

Dunn, 2018). FDA-approved somatostatin peptide analogues for acromegaly include octreotide and lanreotide. The octapeptide, octreotide (molecular weight (MW) 1019 Da) is a first-generation synthetic analogue with a longer circulating $t_{1/2}$ than native somatostatin (Pless, 2005; Yang and Keating, 2010). Injectable formulations of octreotide have been developed for acromegaly treatment over the last 30 years: Sandostatin[®] and Sandostatin[®] LAR depot (Novartis, Geneva, Switzerland) (Fattah and Brayden, 2017). According to a recent extensive survey however, a majority of patients with acromegaly stated that “an oral therapy and/or a treatment to avoid injection” would be

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preferable to current injectable analogues (Strasburger et al., 2016), whose administration is especially pain-inducing due to use of low gauge needles. A once-daily oral formulation of octreotide would therefore be attractive to patients and would improve compliance. The main challenges to address for oral delivery of peptides, however, relates to their metabolic instability in the gastrointestinal (GI) tract and very low permeability across intestinal epithelia (Brayden et al., 2020; Brown et al., 2020; Drucker, 2020).

Octreotide is cyclic and contains D-amino acids and a disulphide bridge, which confer some stability against intestinal peptidases compared to typical linear peptide candidates (Wang et al., 2015). Nonetheless, the hydrophilicity and high MW of octreotide result in low intestinal epithelial permeability and this is the main reason for its low oral bioavailability. Some delivery technologies have improved oral octreotide delivery in preclinical and clinical studies (Fattah and Brayden, 2017). An oral octreotide formulation (Mycapssa[®], Chiasma Pharma, Jerusalem, Israel) uses Transient Permeability Enhancer™ (TPE™), developed by Chiasma Pharma (Jerusalem, Israel), recently received the approval of the US FDA for an octreotide capsule using Transient Permeation Enhancement™ technology for oral use in acromegaly patients (Melmed et al., 2015; “MYCAPSSA,” 2020).

This technology involves formulating octreotide in an oily suspension with intestinal permeation enhancers (PE). Briefly, the capsule consists of the medium chain fatty acid PE, sodium caprylate (C₈) and other excipients, combined with octreotide in an oily suspension, which is then encapsulated in a pH-dependant polymeric enteric coating (Ben-Shlomo et al., 2017). In rat intestinal instillation studies, C₈ and the other excipients in TPE™ induced a transient opening of small intestinal tight junctions, a likely basis for enhancing the paracellular permeability of octreotide (Tuvia et al., 2014). Examination of the pharmacokinetics of octreotide in the TPE™ formulation from a Phase I study indicated that the oral bioavailability of Mycapssa™ was only ~0.25% (Tuvia et al., 2014). Co-formulation of octreotide with other PEs has been investigated to some extent, but none of these studies progressed to clinical trials (Fricker et al., 1996; Fricker and Drewe, 1995; van der Merwe et al., 2004). Overall, This would suggest that there is an opportunity to further increase the oral bioavailability of octreotide using alternative formulations with more efficacious PEs than C8.

The 2019 FDA-approval of oral semaglutide (Rybelsus[®], Novo Nordisk, Copenhagen, Denmark) is likely to increase interest in oral formulations of other suitable peptide candidates (Bucheit et al., 2019). Rybelsus[®] was the first glucagon-like peptide-1 (GLP-1) receptor agonist to be approved by FDA for Type 2 diabetics by the oral route and it is administered once-a-day. Even though its oral bioavailability in humans is 0.4 – 1.0% (RYBELSUS[®], 2020), this is still higher than Mycapssa[®]. The non-enteric coated tablet consists of either 7 mg or 14 mg semaglutide with the PE, 300 mg of salcaprozate sodium (SNAC). SNAC prevents semaglutide degradation by pepsin through modifying the pH around the tablet and by promoting permeability of monomeric semaglutide across the stomach mucosa (Buckley et al., 2018). SNAC was discovered as a lead PE in the Eligen[®] technology by Emisphere (Roseland, NJ, USA), whose researchers hypothesized that it could

chaperone poorly-permeable macromolecules across plasma membranes via a transcellular mode of action (Park et al., 2011). Over the past 20 years, several other mechanisms of action of SNAC were proposed on the basis that it is surfactant-like and is structurally related to salicylic acid and medium chain fatty acids (Maher et al., 2019; Twarog et al., 2019). From a regulatory standpoint, SNAC has generally regarded as safe status (GRAS) and has also been marketed as an oral vitamin B₁₂ supplement (Eligen[®]-vitamin B₁₂, Emisphere, USA) under Medical Food rules for several years (Castelli et al., 2011).

The main aim of this study was therefore to examine the potential of SNAC to promote intestinal permeability of octreotide across rat and human tissue *ex vivo*. We investigated the effects of SNAC on the apparent permeability coefficient (P_{app}) of [³H]-octreotide across isolated rat intestinal tissue mucosae from five regions and from colonic human mucosae mounted in Ussing chambers. A second aim was to develop a liquid chromatography-mass spectrometry (LC-MS/MS) method for octreotide detection to provide evidence that there was no radiolabel degradation during *ex vivo* fluxes of [³H]-octreotide. The third aim was to assess the P_{app} of [³H]-octreotide in biorelevant simulated buffers bathing the apical sides of isolated rat and human tissue mucosae in order to determine if potential components of luminal fluids could interfere with SNAC performance. The results show that SNAC acts as a PE for the peptide with increasing efficacy in rat intestinal mucosae in the proximal-distal region direction as well as in human colonic mucosae in physiological Krebs-Henseleit (KH) buffer. However, efficacy was attenuated in biorelevant buffers, suggesting that SNAC performance *in vivo* may be over-predicted from standard Ussing chamber studies in KH buffer.

2. Materials and methods

2.1. Materials

Octreotide (> 95% purity) was purchased from ChinaPeptides Co., Ltd (Shanghai, China). [³H]-octreotide (specific activity, 0.7 Ci/mmol) was obtained from Moravek, Inc. (Brea, CA, USA). Ecoscint A, liquid scintillation cocktail, was from National Diagnostics (Atlanta, GA, USA). Salcaprozate sodium (SNAC, MW 301 Da, > 99% purity) was obtained from Shanghai Boc Chemicals Co., Ltd (Shanghai, China). Fasted State Simulated Intestinal Fluid-V2 (FaSSIF-V2) and Fasted State Simulated Colonic Fluid (FaSSCoF) were purchased from Biorelevant.com Ltd (London, UK). All other reagents and chemicals were purchased from Sigma-Aldrich (Arklow, Ireland).

2.2. Methods

2.2.1. Media

Composition of buffers are shown in Table 1. KH buffer was prepared in deionized H₂O at pH 7.4 with carbogen bubbling. Rat simulated intestinal fluid (rSIF) was prepared as prescribed (Berghausen et al., 2016). It was prepared by dissolving sodium taurocholate, sodium cholate hydrate, sodium chenodeoxycholic acid, and

Table 1

Buffer compositions on the apical side of rat and human intestinal mucosae in Ussing chambers.

KH	rSIF	FaSSIF-V2	FaSSCoF *
118 mM NaCl	18.7 mM NaCl	68.6 mM NaCl	0.15 mM sodium cholate
4.7 mM KCl	5 mM sodium taurocholate	3 mM sodium taurocholate	0.3 mM Lecithin
2.5 mM CaCl ₂	12.5 mM sodium cholate hydrate	0.2 mM Lecithin	0.1 mM sodium oleate
1.2 mM MgSO ₄	7.5 mM sodium chenodeoxycholic acid	19.1 mM malenic	45.4 mM Tris
1.2 mM KH ₂ PO ₄	5.2 mM Lecithin	34.8 mM sodium hydroxide	120 mM sodium hydroxide
25 mM NaHCO ₃	0.3 mM sodium oleate		
11.1 mM glucose	1.7 mM glyceryl monooleate		
	29.9 mM malenic		

* human colonic mucosae studies only. In all studies KH was used on the basolateral side.

NaCl in 50 ml deionized H₂O and stirred for approximately 30 min at room temperature. Lecithin, sodium oleate, and glyceryl monooleate were added and the mixture was sonicated for 10 min and left stirred overnight to yield a clear solution. Malenic acid and 40 ml deionized H₂O were added and the pH was adjusted to 6.0 with 1 N NaOH. It was stirred until a clear solution was obtained and finally the buffer volume was made up to 100 ml with deionized H₂O. FaSSIF-V2 (pH 6.5) and FaSSCoF (pH 7.8) were made up according to the manufacturer's protocol (Biorelevant Ltd). The buffers were used within 48 h of preparation and were stored at room temperature.

2.2.2. Preparation of dissected rat intestinal tissue for Ussing chamber studies

Male Wistar rats (250–300 g, from Charles River Staffron Walden, UK and the UCD Biomedical Facility, Ireland) were used to study permeability. Rats were housed and used according to the National Institute of Health directive: "Principle of Laboratory Animal Care" (NIH publication number 85–23, 1985). Approval of this project was granted by the UCD Research Ethics Committee for animal experimentation (AREC approval number 14–28-Brayden). Rats were euthanized by stunning and cervical dislocation. After laparotomy, resected duodenum (up to 10 cm from pylorus), upper jejunum (< 11 cm from pylorus), ileum (up to 10 cm above the caecum) and colon were placed in carbogenated KH buffer and dissected according to previous descriptions (Cuthbert and Margolius, 1982). Muscle-stripped stomach mucosae was prepared by a blistering technique (Hopkins et al., 2002). Dissected tissues were mounted between two halves of chambers and maintained in 5 ml temperature-controlled carbogenated KH buffer on each side and with an exposed window area of 0.63 cm². Baseline potential difference (PD, mV) and short circuit current (I_{sc}, μA.cm⁻²) were measured using a DVC 4000 Voltage Clamp (WPI, Hichin, UK) by using a PRO-4 timer (WPI) to switch between open circuit- and voltage clamping to zero, respectively. Transepithelial electrical resistance, TEER (Ω.cm²) was calculated by Ohm's Law (Equation 1).

Equation 1 $TEER = PD/I_{sc}$

Tissues from stomach, duodenum, jejunum, and ileum with minimum TEER values > 30 Ω.cm² and colonic mucosae with minimum TEER values > 70 Ω.cm² were used for permeability studies (Petersen et al., 2012; Ungell et al., 1998). Analogue data was digitised with a Powerlab[®] data acquisition unit and analysed with Chart[®] software package (AD Instruments, UK).

2.2.3. Preparation of dissected human colonic mucosae for Ussing chamber studies

Human colonic specimens were sourced from surgical colon resections. Ethical approval for this study (Protocol 190/2012) was obtained from the St. Vincent's University Hospital Institutional Review Board, and it included informed patient consent. Resections were obtained from patients with suspected colorectal carcinoma. After resection, fresh normal human colonic tissue specimens were obtained from above the diseased margin as designated by a pathologist. Specimens were incubated in ice-cold phosphate buffered saline (PBS). When the tissue arrived at laboratory within 60 min of excision, it was transferred into fresh carbogenated ice-cold KH buffer and kept on ice. Subsequently, the tissue was transferred to a dissection bowl containing carbogenated ice-cold KH buffer (Fig. 1). The tissue was pinned with the luminal side down on a dissection board. The circular and longitudinal smooth muscle of human colonic tissue was dissected from the basolateral side of mucosae (Kisser et al., 2017) using size 5 watchmaker forceps. Stripped mucosae were mounted in pre-equilibrated Ussing chambers (WPI) as described above within 2–3 h of resection. Mucosae with minimum TEER values > 70 Ω.cm² were used for permeability studies (Maher et al., 2009).

2.2.4. Fluxes of octreotide across isolated rat and human intestinal mucosae

The apparent permeability (P_{app}) values of either [³H]-octreotide (0.5 μCi/ml) or unlabelled octreotide (10 μM) were assessed across intestinal tissues from rat and human in the presence of SNAC. [³H]-octreotide and octreotide with or without SNAC (20 mM and 40 mM) were added to the apical compartment bathing mucosae and 0.2 ml samples were taken from basolateral compartment every 20 min over 120 min. Each basolateral sample was replaced an equal volume of KH buffer. Some studies were also carried out in the presence of biorelevant media, rSIF, FaSSIF-V2, and FaSSCoF, which were added to apical compartment only, with KH retained on the basolateral side otherwise. These investigations were carried out in order to better model *in vivo* GI conditions that may affect drug and PE solubility and permeability. Human FaSSIF-V2 is now being widely used by researchers to incubate isolated rat intestinal mucosae in order to determine permeability and solubility in biorelevant buffers (Berghausen et al., 2016; Forner et al., 2017; Wuyts et al., 2015). Given that the bile salt concentrations are 3 mM in FaSSIF-V2 and 25 mM in rSIF, differences *in vitro* permeability of octreotide might be expected as bile salts are also recognised as efficacious PEs (Berghausen et al., 2016; Brown et al., 2020). Therefore, we used both rSIF (to mimic rat luminal contents *in vivo*), as well as FaSSIF-V2 (to model those of the human small intestine). Previous studies demonstrated compatibility between rat isolated small intestinal tissue mucosae using FaSSIF-V2 in the apical compartment of Ussing chambers (Forner et al., 2017; Wuyts et al., 2015). Fed-state simulated intestinal fluid (FeSSIF) was not used as it is too damaging to isolated rat jejunal mucosa (Kristin et al., 2017) and could give rise to false-positive flux data.

The fluxed samples of [³H]-octreotide were mixed with 3 ml liquid scintillation cocktail (Ecoscint A), and radioactivity was quantified using a liquid scintillation counter (Packard Tricab 2900 TR, PerkinElmer, Massachusetts, USA).

Unlabelled octreotide samples were quantified using LC-MS/MS. These samples were mixed with chilled MS grade acetonitrile and stored at –20 °C until analysis. Thawed samples were centrifuged at 15,000 rpm at 4 °C and analysed. The LC-MS/MS system consisted of a triple quadrupole mass spectrometer coupled to a UHPLC system (6470 Triple Quad LC/MS (Model G6470A) Agilent Technologies, California, USA). An AdvanceBio Peptide Map column (2.7 μm, 150 × 2.1 mm) (Agilent Technologies) was used. The injection volume was 2 μl. The chromatographic conditions were: 400 μl/min flow rate for 10 min, with the mobile phase consisting of (A) 0.1% formic acid and (B) acetonitrile. The gradient A:B (v/v) was programmed as follows: from 0 to 4 min the ratio of A:B 90:10 to 20:80 which was maintained for 1 min. The column was re-equilibrated for the next 5 min with the initial ratio of mobile phase. The transition from *m/z* 510.3 to *m/z* 120.1 was used to quantify octreotide, with analysis was done using a multiple reaction monitoring (MRM) model. MRM parameters are from Ismaiel et al., (Ismaiel et al., 2011) with slight modification: Dwell time 200 ms, collision energy 48, Cell Accelerator Voltage 5 and Fragmentor 5. The P_{app} values of [³H]-octreotide and octreotide were calculated according to the following Equation 2:

$$\text{Equation 2 } P_{app} = \frac{dQ}{dt} \times \frac{1}{A} \cdot C_0$$

where dQ/dt was the transport rate across the epithelium (mol/s), A was the surface area of the cell monolayer (0.63 cm²), and C₀ was the initial concentration of [³H]-octreotide or octreotide in the apical donor compartment (mol.ml⁻¹) (Kisser et al., 2017).

2.2.5. Histology of tissue mucosae

Following the 2 h permeability experiments, rat and human tissues were removed from the chambers and fixed in 10% w/v buffered formalin for histology to evaluate potential epithelial damage. After 24 h, the tissues were embedded in paraffin wax. Tissue sections of 5 μm thickness were cut with a microtome (Leitz 1512; GMI, Minnesota, USA) and stained with haematoxylin/eosin (H & E) and examined with

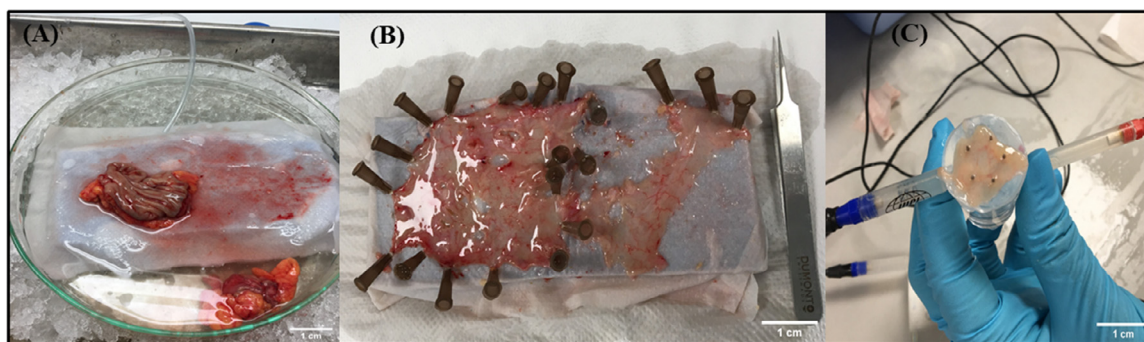


Fig. 1. Representative images of dissected human colonic mucosae for Ussing system. (A) Human colonic specimen, (B) Colonic tissue after removing smooth muscle; the luminal side is faced down on the dissection board, (C) Muscle-stripped mucosae mounted tissue on an Ussing chamber. Horizontal bars = 35 mm.

a light microscope (BX43, Olympus, Tokyo, Japan).

2.3. Statistical analysis

Statistical analysis was carried out using Prism-8[®] software (GraphPad, San Diego, USA). Results are given as mean \pm standard error of mean (SEM). Student's t-tests (unpaired) and ANOVA were used for group comparisons with *post hoc* tests used for the latter. Statistical significance was considered present if $p < 0.05$.

3. Results

3.1. SNAC increases the P_{app} of [³H]-octreotide across rat intestinal mucosae in KH

The effects of 20 mM SNAC in KH buffer were examined on the P_{app} of [³H]-octreotide across rat intestinal mucosae isolated from five regions: stomach, duodenum, upper jejunum, ileum, and colon. The basal P_{app} of [³H]-octreotide ranged from 1.3×10^{-6} cm.s⁻¹ to 2.5×10^{-6} cm.s⁻¹ across mucosae from each region, but they were not different from each other (Fig. 2A). The SNAC concentration was below its critical micellar concentration of 36 mM in KH buffer (Suppl. Fig. 1). In stomach mucosae, apical addition of SNAC increased the P_{app} to a small extent from 1.3×10^{-6} cm.s⁻¹ to 1.9×10^{-6} cm.s⁻¹. In the presence of SNAC, the P_{app} across duodenal mucosae was also marginally increased in the presence of SNAC from 2.1×10^{-6} cm.s⁻¹ to 3.0×10^{-6} cm.s⁻¹, but neither trend was significant. Statistical increases in the P_{app} were however, observed in upper jejunal, ileal, and colonic mucosae: from basal values of 1.9×10^{-6} cm.s⁻¹ to 4.2×10^{-6} cm.s⁻¹, from 2.2×10^{-6} cm.s⁻¹ (basal) to 7.4×10^{-6} cm.s⁻¹, and from 2.5×10^{-6} cm.s⁻¹ (basal) to 7.9×10^{-6} cm.s⁻¹, respectively (Fig. 2A). There was therefore a pattern indicating increasing efficacy of 20 mM SNAC in KH buffer in the order of stomach < duodenum < jejunum < ileum < colon (Fig. 2A). These results indicate regional effects of SNAC as a PE for this peptide in rat intestinal tissue with increasing efficacy observed in the proximal-to-distal direction.

TEER values in the presence of [³H]-octreotide gradually declined to ~40% of the initial TEER over 120 min in tissues from the different GI regions from rats (Fig. 2B-F). These changes in TEER were similar to patterns observed in untreated rat intestinal mucosae (Stuettgen and Brayden, 2020; Twarog, 2020), so there was no indication that [³H]-octreotide itself might be causing TEER reductions across mucosae from the five regions. Following apical addition of 20 mM SNAC however, TEER values were further reduced across four of the regions compared to [³H]-octreotide-exposed controls, except for duodenal mucosae. The times required for significant reductions in TEER when exposed to SNAC compared to the respective tissue controls ([³H]-octreotide alone) were: stomach from 20 - 120 min (20% - 40% reduction), jejunum at 120 min (35% reduction), ileum from 80 - 120 min (30% - 38% reduction), and colon from 15 - 120 min (44% - 83% reduction)

(Fig. 2B-F).

3.2. No effects of SNAC on the P_{app} of [³H]-octreotide across rat jejunal mucosae in rSIF or FaSSIF-V2

We further investigated the permeability of [³H]-octreotide across rat intestinal tissue mucosae by replacing KH buffer on the apical side with simulated intestinal fluids as indicated in Section 2.2.4.

Since 20 mM SNAC induced a significant increase in the P_{app} of [³H]-octreotide across rat upper jejunal mucosae in KH buffer, we re-examined whether this effect pertained in tissues just from this region in the presence of rSIF and FaSSIF-V2 on the apical side. The basal P_{app} for [³H]-octreotide across jejunal mucosae in rSIF and FaSSIF-V2 was 1.6×10^{-6} cm.s⁻¹ and 1.7×10^{-6} cm.s⁻¹, respectively, unchanged from that obtained in KH buffer (1.9×10^{-6} cm.s⁻¹). However, attenuation of the permeation-enhancement effects of SNAC in KH buffer was observed in both rSIF and FaSSIF-V2. The P_{app} of [³H]-octreotide in the presence of 20 mM SNAC in rSIF and FaSSIF-V2 was 1.2×10^{-6} cm.s⁻¹ and 1.9×10^{-6} cm.s⁻¹, respectively, not different from the control P_{app} in these buffers. Yet these values were both lower than the P_{app} value detected in presence of SNAC in KH buffer, (4.2×10^{-6} cm.s⁻¹) ($p < 0.05$) (Fig. 3A). Since the critical micellar concentration (CMC) of SNAC in rSIF and FaSSIF-V2 was lowered to 5.6 mM and 6.3 mM, respectively, an explanation is that the free concentration was of SNAC was reduced in these buffers due to mixed micelles forming in the presence of NaTC and lecithin contributions (Suppl. Fig. 1).

There was a trend of a slight gradual reduction in TEER values over 2 h in control tissues exposed to [³H]-octreotide in rSIF and FaSSIF-V2 buffers. In turn, these reductions were similar to the gradual pattern for TEER reduction seen in KH buffer, indicating compatibility of jejunal tissue with these biorelevant media over this period (Fig. 3B). This result indicated compatibility of these two simulated small intestinal buffers with isolated intestinal tissue mounted in Ussing chambers (Forner et al., 2017; Jantravid et al., 2008; Wuyts et al., 2015). 20 mM SNAC did not cause additional TEER reductions in jejunal tissue in either simulated buffers, in contrast to additional reductions observed in the presence of SNAC in KH.

3.3. SNAC increases the P_{app} of octreotide across human colonic mucosae in KH buffer

Due to difficulty in accessing sufficient human specimens from different GI regions, we were limited to investigating effects of SNAC on the P_{app} of [³H]-octreotide in human colonic mucosae, where surgeries to obtain tissue are common. Following apical addition of SNAC (20 mM and 40 mM) in KH buffer, the P_{app} increased by 1.5-fold to $1. \times 10^{-6}$ cm.s⁻¹ (NS) and by 2.1-fold to 2.7×10^{-6} cm.s⁻¹ ($p = 0.03$) respectively, compared to the control P_{app} of 1.3×10^{-6} cm.s⁻¹ (Fig. 4A) for [³H]-octreotide alone. Therefore, 40 mM SNAC was

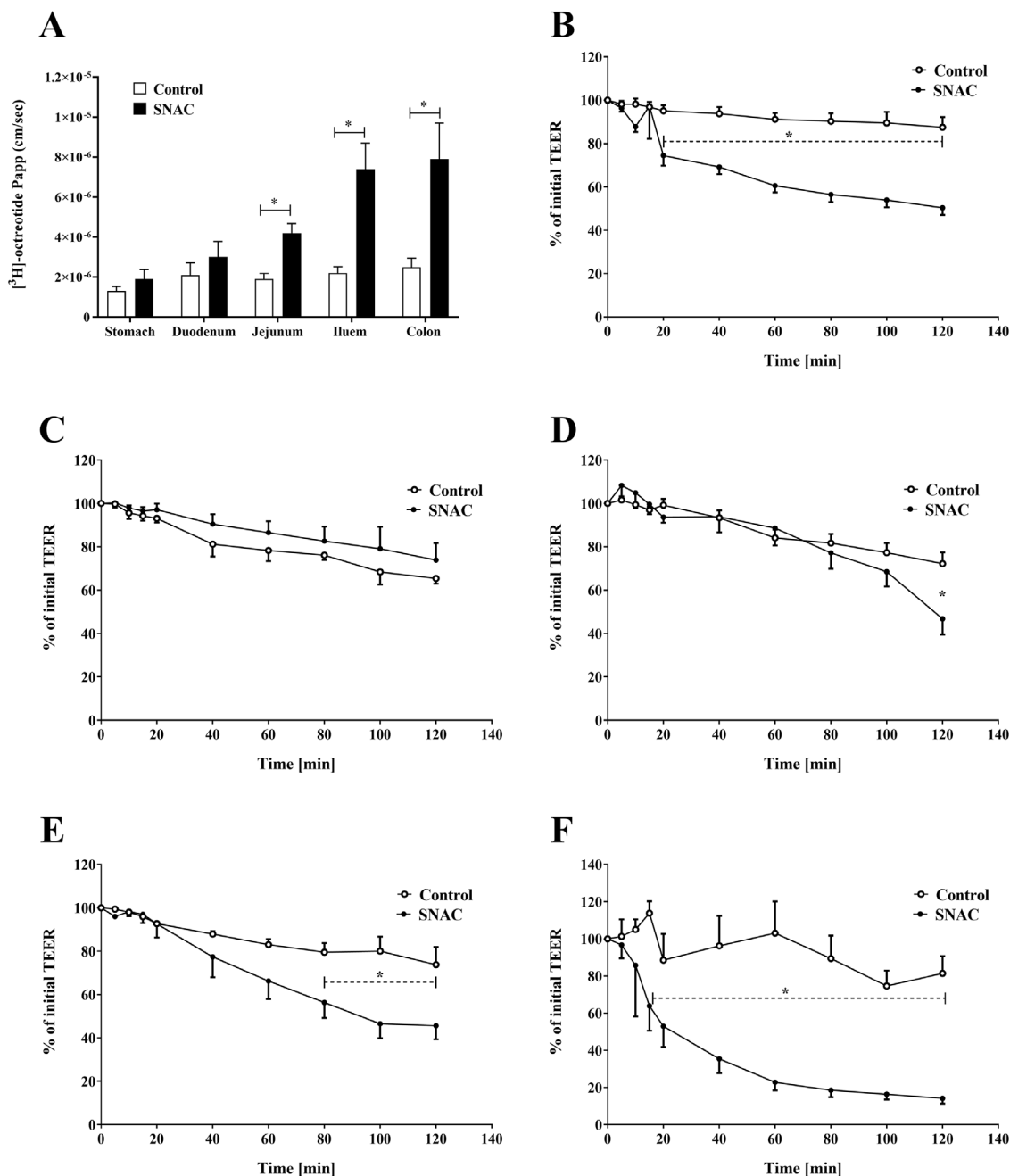


Fig. 2. P_{app} of [^3H]-octreotide and TEER across rat intestinal regional mucosae. A. P_{app} of [^3H]-octreotide in absence and presence of 20 mM SNAC in KH buffer, * $P < 0.05$, (Student's unpaired t -test) comparing P_{app} in the presence of SNAC against regional controls exposed to [^3H]-octreotide alone. B-F. Percentile of initial TEER in tissues exposed either to [^3H]-octreotide alone or to SNAC and [^3H]-octreotide. B. Stomach, C. Duodenum D. Jejunum, E. Ileum, F. Colon. * $P < 0.05$, two-way ANOVA followed by Bonferroni *post hoc* analysis comparing TEER values within regions. Data expressed as Mean \pm SEM ($n = 6$ –7 per group). The dotted line indicates when a significant TEER reduction was seen at particular time point compared to the corresponding control of [^3H]-octreotide alone.

required to statistically achieve same effect in human colonic mucosae as seen for the rat counterpart (20 mM).

We developed an LC-MS/MS method to quantify unlabelled octreotide on the basolateral side in order to confirm the data seen with [^3H]-octreotide in KH buffer and to show that measurement of the radiolabel did not reflect metabolism of the link between tritium and octreotide during transit. Following addition of 40 mM SNAC, the P_{app} of unlabelled octreotide increased to $2.3 \times 10^{-6} \text{ cm.s}^{-1}$ compared the control basal P_{app} of $6.2 \times 10^{-7} \text{ cm.s}^{-1}$, although this trend was not quite significant ($p = 0.23$) (Fig. 4C). The P_{app} values for labelled and unlabelled octreotide were therefore of the same order, indicating that both sets of values were a true measure of intact peptide flux. Had

tritium split from octreotide, the P_{app} for tritium would have been orders of magnitude higher, $\sim 0.68 \times 10^{-4} \text{ cm.s}^{-1}$ (Twarog, 2020).

The effect of SNAC on the TEER of human colonic mucosae was investigated. The mean basal TEER was $99.6 \pm 9.5 \Omega.\text{cm}^2$ ($n = 24$), similar to values reported by others (Maher et al., 2009; Petersen et al., 2012). Following 120 min incubation, the mean TEER of control tissues exposed to [^3H]-octreotide gradually decreased by a small amount, $\sim 25\%$ (Fig. 4B), similar to control tissues in the absence of [^3H]-octreotide, so there was no indication that [^3H]-octreotide reduced TEER in human colonic mucosae *per se*. Upon apical addition of either 20 mM or 40 mM SNAC, there was additional reduction in TEER compared to controls (Fig. 4B and C).

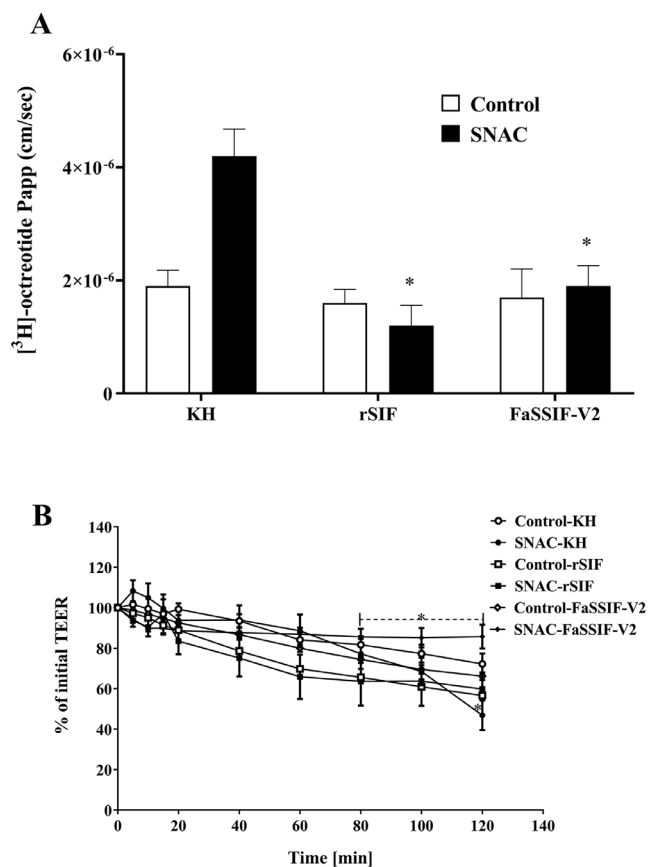


Fig. 3. A. P_{app} of [^3H]-octreotide in the absence and presence of 20 mM SNAC in KH, rSIF, and FaSSIF-V2 in rat jejunal mucosae. $*P < 0.05$, one-way ANOVA followed by Dunnett's *post hoc* analysis comparing the P_{app} in rSIF and FaSSIF-V2 in the presence of 20 mM SNAC to that seen in KH buffer. B. Percentile of initial TEER in the controls and in the presence of 20 mM SNAC in different buffers, $*P < 0.05$, two-way ANOVA followed by Bonferroni *post hoc* analysis to compare reduction in TEER in tissue exposed to SNAC versus the control from individual regions. Data represent Mean \pm SEM ($n = 3-7$). The dotted line indicates when a significant TEER reduction was seen at particular time point compared to the corresponding control of [^3H]-octreotide alone.

3.4. Attenuated effects of SNAC on the P_{app} of octreotide across human colonic mucosae in FaSSCoF

In an attempt to better mimic the *in vivo* situation where the luminal side of intestinal fluid is exposed to bile salts and lecithin, we used a simulated human colonic buffer on the apical side of human colonic tissue. FaSSIF-V2 is considered suitable for the upper small intestinal region (Forner et al., 2017; Jantratid et al., 2008), so we tested the effects of SNAC on the P_{app} of [^3H]-octreotide using a human colonic version, FaSSCoF. The P_{app} of [^3H]-octreotide in FaSSCoF increased by 1.7-fold from $1.5 \times 10^{-6} \text{ cm.s}^{-1}$ (mean basal) to $2.6 \times 10^{-6} \text{ cm.s}^{-1}$, and by 1.9-fold to $2.8 \times 10^{-6} \text{ cm.s}^{-1}$ in the presence of 20 mM and 40 mM SNAC, respectively, but statistical significance was not reached (Fig. 5A). TEER values of control tissues exposed to [^3H]-octreotide in FaSSCoF were unchanged over 2 h, whereas in the presence of SNAC (20 mM, 40 mM), TEER values decreased further (Fig. 5B).

3.5. Minor changes in intestinal mucosae morphology in the presence of SNAC in KH

Histology of rat and human tissue mucosae following exposure to SNAC over 120 min was assessed (Fig. 6A and B). Rat stomach mucosae exposed to KH buffer and 20 mM SNAC were structurally intact with just minor perturbation observed (Fig. 6A). In rat duodenal and jejunal

mucosae, minor cell sloughing at the tip of villi was seen in control and SNAC-exposed tissues. More sloughing was noticeable in rat ileal and colonic mucosae where the tips of villi were absent following exposure to SNAC (Fig. 6A). As seen in Fig. 6B, human colonic mucosae in KH buffer appeared structurally intact with healthy villi present, similar to that of control tissue. Human tissues exposed to 20 mM SNAC showed minor damage to villi tips, whereas 40 mM SNAC caused tip erosion.

Discussion

PEs have potential to increase the bioavailability of poorly permeable drugs and macromolecules (Maher et al., 2019, 2016). The approval of Rybelsus[®] (oral semaglutide) for the treatment of Type 2 diabetes is rightly considered a landmark for oral peptide delivery and offers encouragement to use PE-containing formulations to be used for other peptides even though the oral bioavailability is $\sim 1\%$ (RYBELSUS[®], 2020). Upon closer scrutiny however, rather than opening up the oral peptide field for use with PE formulations, it restricts it to niche peptides as only highly potent peptides of reasonable MW, with acceptable stability, a long $t_{1/2}$, and a large therapeutic index can be considered as candidates at that low and variable level of bioavailability. Perhaps such candidates will emerge through the efforts of medicinal chemistry, which is focussed on producing membrane-permeable stable macrocycles of lower MW than typical linear peptides (Naylor et al., 2017). For this study, we selected octreotide because it displays several of those promising features, although its stability in the GI tract is still relatively low despite its cyclic structure (Wang et al., 2015). Moreover, an oily suspension largely based on the PE, C₈, was recently approved by the FDA despite an oral bioavailability of $\sim 0.25\%$, so there is potential to further improve its systemic delivery. We paired octreotide with SNAC as a well-established PE, present in Rybelsus[®] at a daily level of 300 mg per dose. It is not necessarily the most efficacious PE (that is typically payload dependant), but it has an excellent safety record, GRAS status, and can be produced to Good Manufacturing Practice (GMP) quality in large quantities. SNAC is already on the market as a component of an oral semaglutide formulation (Rybelsus[®]) since 2019.

Here, we demonstrated that SNAC is efficacious at increasing octreotide flux across isolated rat and human intestinal mucosae in the standard KH physiological buffer used for ion transport and drug flux studies across epithelial mucosae mounted in Ussing chambers. This was not a particular surprise as SNAC is well-known to have capacity to increase oral absorption of several co-administered poorly permeable molecules apart from semaglutide including unfractionated heparin, cromolyn, and vitamin B₁₂ (Leone-Bay et al., 1998, 1996; Smith et al., 2016). Yet, exact mechanism of action remains controversial, recently it has been provided a specific mechanism for SNAC in respect of close association with semaglutide in a tablet in the stomach, based on buffering against acid, protection against pepsin, and presentation of semaglutide to the gastric epithelium as a monomer (Buckley et al., 2018). In that study, it was suggested that neither replacing semaglutide with another GLP-1 analogue, liraglutide, nor replacing SNAC with another Eligen[™] PE analogue sufficed to increase flux across gastric epithelial monolayers. The gastric mechanism of action of SNAC in respect of semaglutide that was demonstrated in ligated dogs does not, however, rule out SNAC effects in other GI regions with other payloads where it can exert membrane perturbation due to its surfactant structure and capacity to alter a range of epithelial intracellular parameters (Twarog, 2020).

The basal P_{app} was low and equivalent for [^3H]-octreotide across all five regions, as expected for a hydrophilic peptide of MW ~ 1000 Da. Similarly, Forner et al., also showed that the P_{app} of FITC-labelled dextran 4000 (FD4) was constant across different rat intestinal regional mucosae (Forner et al., 2017). Peterson et al., on the other hand demonstrated regional differences in the basal P_{app} of [^{14}C]-mannitol and FD4, with higher permeability observed in isolated rat jejunal- than

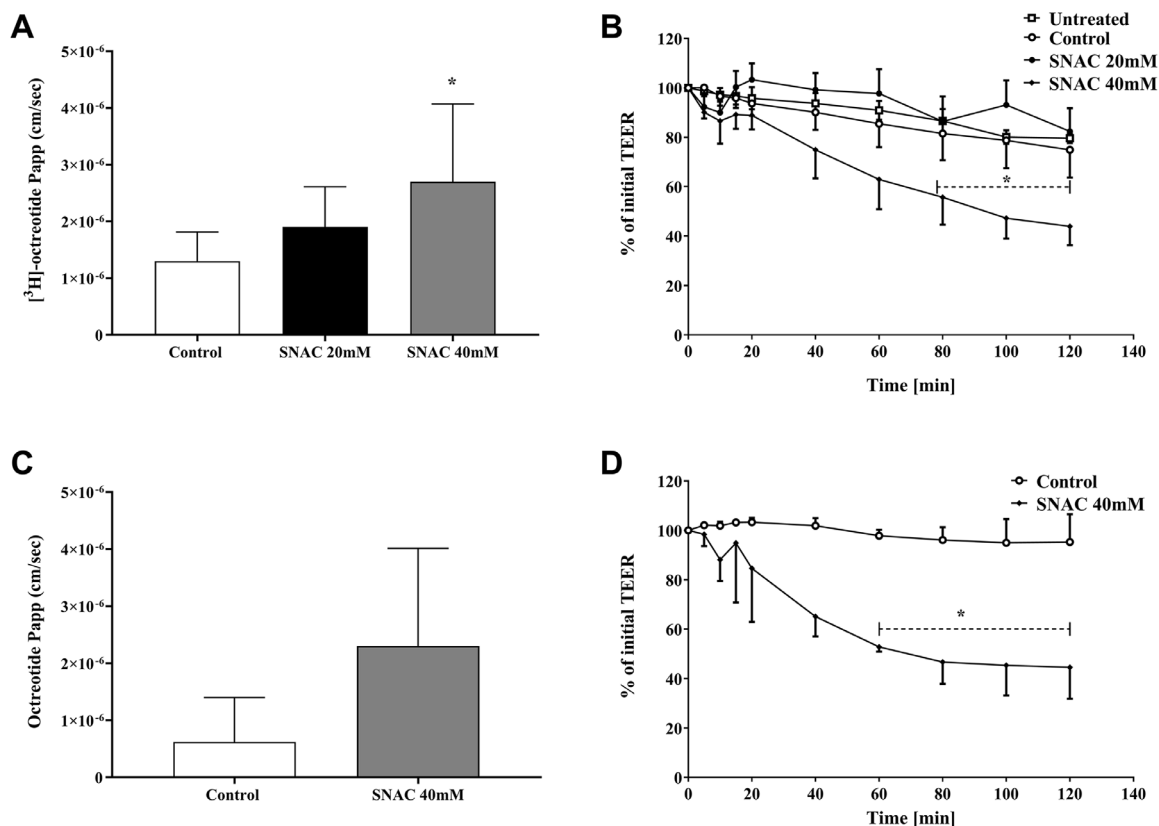


Fig. 4. P_{app} of [^3H]-octreotide and unlabelled octreotide across human colonic mucosae in the presence of SNAC in KH buffer. A. P_{app} of [^3H]-octreotide, B. percentile of initial TEER (with [^3H]-octreotide alone used in controls), C. P_{app} of unlabelled octreotide, D. percentile of initial TEER (with unlabelled octreotide alone used in controls). Mean \pm SEM ($n = 3-6$ per group). * $P < 0.05$, A: one-way ANOVA followed by Dunnett's *post hoc* analysis, C: Student's unpaired *t*-test, and B and D: two-way ANOVA followed by Bonferroni *post hoc* analysis to compare reduction in TEER in presence of SNAC to the control. The dotted line indicates when a significant TEER reduction was seen at particular time point compared to the corresponding control of [^3H]-octreotide alone.

colonic mucosae (Petersen et al., 2012). In the current study and that of Forner et al., the upper jejunal region was used while in Petersen et al., the distal jejunum was used instead. These two jejunal regions may have different basal permeabilities. In addition, while mannitol and FD4 fluxes are mediated by the paracellular pathway, octreotide flux can be mediated by both transcellular and paracellular pathways (Fricker et al., 1992, 1991), another possible source of variation.

SNAC's effects were regional-dependant in rat tissue in KH buffer, with greater efficacy as a PE at increasing the P_{app} of [^3H]-octreotide in the proximal-to-distal direction. To our knowledge, this is the first study to systematically evaluate regional differences of SNAC as a PE for octreotide across rat intestinal mucosae. A recent presentation from Merck (NJ, USA) also investigated the capacity of SNAC to increase flux of octreotide across isolated rat intestinal mucosae mounted in diffusion chambers ("Novel Approaches for Enhancing the Oral Bioavailability and Absorption of Peptides," 2020). Flux was increased by 4.8% w/v SNAC, equivalent to 66 mM, more than a 3-fold higher concentration than used here. No histology was presented in the Merck study and the intestinal region was not stated. Nonetheless, it corroborates some of the findings of this project. In the current study, SNAC's efficacy as a PE was weakest in stomach mucosae for this payload. In Buckley et al., rat gastric mucosae were mounted in chambers and exposed to 10–30 mM SNAC. Their results showed an increase in permeability of semaglutide during an acute 10 min exposure to SNAC followed by removal of the PE-containing buffer, with efficacy gradually declining at 30 and 60 min (Buckley et al., 2018). In our study, the gastric mucosa was exposed chronically to 20 mM SNAC over 2 h, so constant exposure to SNAC in this design would favour a sustained effect on the epithelium, even if acute exposure is a more likely scenario *in vivo* (Buckley et al., 2018). Irrespective, there is no doubt that the efficacy of SNAC as a PE

on rat stomach mucosae is markedly different between these studies: this could be down to differences in stomach dissection technique, or that SNAC is simply more efficacious with semaglutide than with octreotide in stomach mucosa. Taken together, since the stomach appears less amenable to PEs than regions lower down the GI tract, the gastric mode of action for SNAC in the oral formulation of semaglutide is likely to be built primarily upon pH-buffering and monomer induction rather than relying on stomach permeation-enhancement. Small intestinal regional absorption has in fact been demonstrated before for octreotide. Fricker et al., showed higher uptake of octreotide from jejunum than ileum from humans and rats (Fricker et al., 1992, 1991). Despite efficacy observed for SNAC in respect of the P_{app} of [^3H]-octreotide in rat distal intestinal mucosae in the current study, it still took a concentration of 20 mM to achieve it, reflecting its low potency. This is consistent with a requirement for 300 mg in the oral semaglutide tablet, a weight ratio of 21:1 (SNAC: peptide).

Targeting and releasing drug contents in the colonic region has been advocated for formulations that act locally (Bak et al., 2018). Compared to the small intestine, the colon has a lower surface area compensated by a longer transit time (20–30 h), which permits a longer time to release the material (Jones et al., 2016). The observation of increased efficacy of SNAC in lower GI intestinal mucosae is in line with other studies of PEs with isolated rat intestinal mucosae (Maroni et al., 2012; Petersen et al., 2012; Yamamoto et al., 1997). The PE, sodium caprate (C_{10}), also demonstrated superior efficacy in isolated rat colonic mucosae compared to jejunal in respect of promoting flux of macromolecule (Maroni et al., 2012; Petersen et al., 2012; Yamamoto et al., 1997). Recently, our group also showed efficacy of another PE class, piperazine derivatives, on increasing the P_{app} of [^3H]-octreotide across rat colonic mucosae (Stuettgen and Brayden, 2020). It is not known

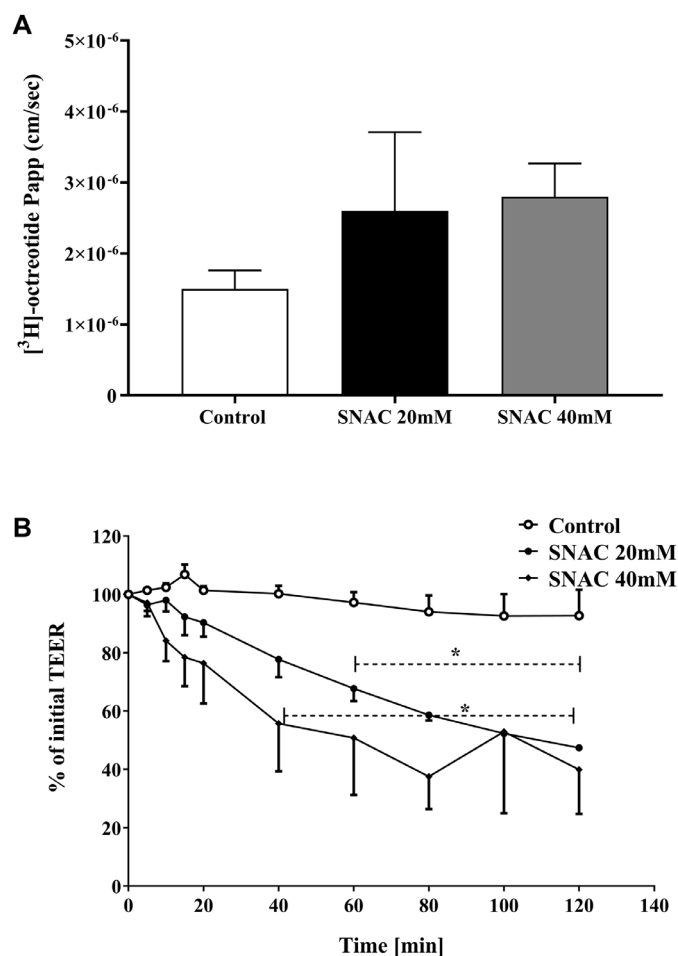


Fig. 5. P_{app} of [^3H]-octreotide in the absence and presence of SNAC using FaSSCoF on the apical side of human colonic mucosae. A. P_{app} of [^3H]-octreotide and B. percentile of initial TEER in FaSSCoF. Data is Mean \pm SEM ($n = 3$). * $P < 0.05$, A: one-way ANOVA followed by Dunnett's *post hoc* analysis, and B: two-way ANOVA followed by Bonferroni *post hoc* analysis to compare reduction in TEER in presence of SNAC to the controls exposed to [^3H]-octreotide alone. The dotted line indicates when a significant TEER reduction was seen at particular time point compared to the corresponding control [^3H]-octreotide alone.

why the colon seems to be especially amenable to many PEs, but it is possible that the plasma membrane lipid composition of colonic enterocytes may be different from the small intestine. A more likely explanation is that the small intestine has adapted to coping with up to 20 mM bile salts in the fed state along with challenges from nutrients, so that it should be more resilient seems intuitive. Even if the colon is arguably the most amenable site for PE to act, it is still not a very attractive option for the systemic delivery of peptides due to the variable GI transit time to reach there with coated oral dosage forms and also due to the complexity of the colon-microbiome interaction (Bak et al., 2018). The histological observations of just mild perturbation observed with SNAC in mucosae from different GI regions from rats are in line with effects of other surfactant-type PEs (Maher et al., 2018; McCartney et al., 2019; Stuetgen and Brayden, 2020).

The basal P_{app} values of [^3H]-octreotide across rat jejunal and human colonic mucosae were not altered in the biorelevant buffers compared to KH buffer. Forner et al., also observed similar P_{app} values for FD4 and atenolol in HBSS versus modified FaSSIF on the apical side of isolated rat jejunal mucosae (Forner et al., 2017). Attenuation of the efficacy of SNAC was however seen here in FaSSIF-V2 and rSIF, and to lesser extent with FaSSCoF. The lack of effect of SNAC on the P_{app} of [^3H]-octreotide seen in these buffers is likely to be due to interaction of

SNAC with components of simulated fluids since basal fluxes were unchanged. Several factors might contribute to the attenuation of SNAC's effects in the simulated fluids. The initial study showing efficacy of SNAC was carried out in rat and human intestinal tissue in KH buffer at pH 7.4, whereas both FaSSIF-V2 and rSIF were used at pH < 6.5. Even with even a small change in pH, different dissolution profiles can be obtained (Berben et al., 2019; Falavigna et al., 2019). A difference in pH might affect the solubility of SNAC (Cumming and Martin, 2000). SNAC is the sodium salt of a weak acid with a pKa of 5.01. At low pH, the solubility of SNAC decreases and the SNAC precipitates as the SNAC free acid form (Cumming and Martin, 2000). Changes in pH could also alter the degree of ionization of octreotide, but this does not seem relevant, otherwise a decrease in the basal P_{app} would have been detected. It is also possible that a change in pH could affect the interaction between SNAC and octreotide. A decrease in pH can also alter rheology of mucus, which might increase the barrier for the peptide or PE to reach the epithelium (Berben et al., 2019; Falavigna et al., 2019).

SNAC might also be entrapped in micelles by key components of biorelevant fluids, bile salts and lecithin, thereby reducing its free concentration. Both sodium taurocholate (NaTC) and lecithin form colloidal structures in simulated intestinal fluids, while FaSSIF consists of mixed-micelles of different sizes (40–100 nm). Gradauer and colleagues also saw similar attenuation in efficacy of the non-ionic surfactant PE, tetradecyl maltoside (Gradauer et al., 2015). In that study, the enhancement capacity of the maltoside to increase permeability of FD4 was reduced in FaSSIF-V2 across Caco-2 monolayers and in rat intestinal instillations. The authors provided evidence for entrapment of the maltoside at concentrations above its CMC in mixed micelles comprising NaTC/lecithin. Whether this occurs for SNAC is unclear as its CMC in KH buffer is 36 mM, above the 20 mM concentration that proved efficacious in rat tissue, but lower than the 40 mM concentration required for human colonic mucosae. However, the CMC of SNAC in rSIF and FaSSIF was 5.6 mM and 6.3 mM, respectively, so it is likely that mixed micelles form in these buffers that lead to reduction in its free concentration, thereby providing a plausible explanation for the lack of effect.

Evaluation of permeability and PE effects using simulated colonic fluid with human mucosa on Ussing chambers has to our knowledge never been conducted before. To date, only dissolution of molecules has been determined in this new buffer, so it needs verification to confirm its suitability for predicting permeability in human colonic mucosae (Augustijns et al., 2014; Vertzoni et al., 2019, 2010). However, incubating human colonic mucosae in KH buffer is well-established and yields correlation with fraction absorbed across human colon *in vivo* (Lennernäs, 2007). The Ussing chamber was originally designed for epithelial electrophysiology, with TEER measurement used to monitor electrical leakiness of tight junctions, as well as the integrity of the mounted tissues. Reduction in TEER is not an indication of toxicity or paracellular mode of action, as transcellular perturbation can also reduce TEER (Maher et al., 2019). Hess et al., demonstrated that SNAC (33–66 mM) could increase the permeability of polar marker molecule, 6-carboxy-fluorescein in isolated rat jejunal mucosae, surprisingly without reducing TEER values at such high concentrations (Hess et al., 2005). Thus, the reduction in TEER induced by SNAC in the mucosae of the current study reveals very little on electrophysiology changes, permeability pathways, or the mechanism of action. It is more likely to simply reflect the mild perturbation seen in the histology images.

In conclusion, the effect of SNAC as a PE for octreotide permeability across in human and rat intestinal membrane was evaluated. Regional differences were found for the SNAC enhancement effect with the best efficacy seen in rat colon, which was confirmed for human colon. Moreover, the influence of rSIF and FaSSIF-V2 on the permeability of octreotide in presence of SNAC was investigated which resulted in attenuation of the PE effect, likely related to entrapment in micellar components of simulated fluid containing NaTC and lecithin, leading to a reduction in the free concentration that can access the plasma

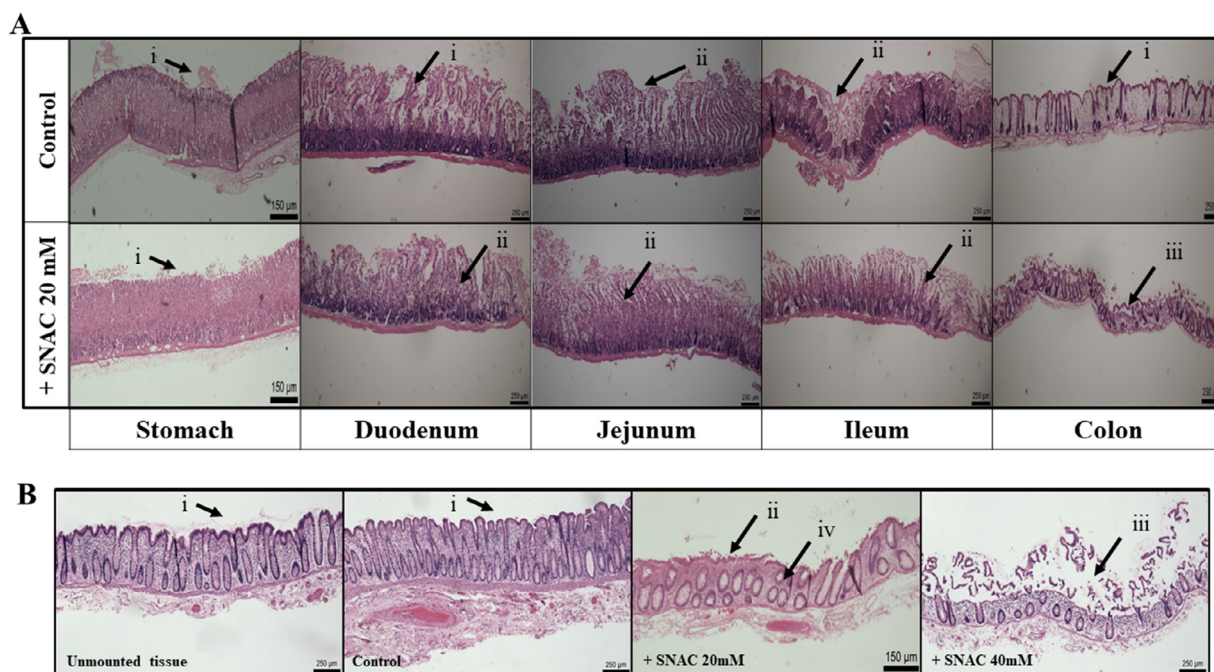


Fig. 6. Representative images of A. rat regional mucosae and B. human colonic stained with H&E following exposure to SNAC in KH buffer for 120 min. Horizontal bars = 150–250 μm . (i) intact villi, (ii) erosion at tip of villi, (iii) erosion of villi and (iv) edema.

membrane. Finally, our results showed compatibility of simulated fluids mapped to intestinal regional mucosae in Ussing chambers. Use of biorelevant compatible buffers for *ex vivo* fluxes which mimic the lumen contents of intestinal regions have the potential to offer more complex mechanistic insights for how PEs act and more accurate predictions of *in vivo* outcomes. Whether SNAC can be formulated in tablets with octreotide to compete with the very low oral bioavailability in humans offered by TPE™ will require initial assessment in large animal studies.

CRediT authorship contribution statement

Sarinj Fattah: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Funding acquisition, Writing - review & editing. **Mohamed Ismaiel:** Methodology, Resources. **Brenda Murphy:** Methodology, Resources. **Aleksandra Rulikowska:** Methodology, Resources. **Jesus M. Frias:** Methodology, Resources. **Desmond C. Winter:** Methodology, Resources. **David J. Brayden:** Conceptualization, Supervision, Investigation, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

David Brayden acts as a consultant to Pharma companies working on oral peptide delivery. The other authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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Supplementary materials

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