Research Article

Efficacious Intestinal Permeation Enhancement Induced by the Sodium Salt of 10-undecylenic Acid, A Medium Chain Fatty Acid Derivative

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ABSTRACT. 10-undecylenic acid (UA) is an OTC antifungal therapy and a nutritional supplement. It is an unsaturated medium chain fatty acid (MCFA) derivative, so our hypothesis was that its 11-mer sodium salt, uC₁₁, would improve intestinal permeation similar to the established enhancer, sodium caprate (C₁₀), but without the toxicity of the parent saturated MCFA, decylenic acid (C₁₁). MTT assay and high-content screening (HCS) confirmed a cytotoxicity ranking in Caco-2 cells: C₁₁ > C₁₀ = uC₁₁. Five to ten millimolars of the three agents reduced TEER and increased the Papp of [¹⁴C]-mannitol across Caco-2 monolayers and rat intestinal mucosae, a concentration that matched increases in plasma membrane permeability seen in HCS. Although C₁₁ was the most efficacious enhancer *in vitro*, it damaged monolayers and tissue mucosae more than the other two agents at similar concentrations and exposure times and was therefore not pursued further. Rat jejunal and colonic *in situ* intestinal instillations of 100 mM C₁₀ or uC₁₁ with FITC-dextran 4000 (FD4) solutions yielded comparable regional enhancement ratios of ~10 and 30%, respectively, for each agent with acceptable tissue histology. Mini-tablets of uC₁₁ and FD4 however delivered more FD4 compared to C₁₀-FD-4 mini-tablets in both regions, as reflected by a statistically higher AUC, and with no evidence of membrane perturbation. The unsaturated bond in uC₁₁ therefore confers a reduction in lipophilicity and cytotoxicity compared to C₁₁, and the resulting permeation enhancement is on a par with or superior to that of C₁₀, a key component of formulations in current phase II oral peptide clinical trials.

KEY WORDS: 10-undecylenic acid; Caco-2 cells; intestinal permeation enhancement; medium chain fatty acids; oral peptides.

INTRODUCTION

Improving the intestinal permeation of peptides, proteins and macromolecules remains one of the great challenges in drug delivery. Enabling such oral formulations will improve patient compliance and enable parenteral-to-oral switching for commercial benefit, while the more physiological oral route may reduce potential side effects seen with bolus injection (1). In the past 5 years, major advances have occurred with at least 10 technologies reaching clinical trials for oral peptides (2). Common features of these advanced solid dose formulations are the following: pH-dependent polymer coatings that protect the tablet or capsule until it reaches the duodenum, incorporation of pancreatic peptidase inhibitors and co-formulation with permeation enhancers (PEs) with a history of safe use in man, some of which are listed as excipients. Examples of PEs that have reached the

clinic in oral peptide formulations include a broad range of surfactants: carnitines, alkyl maltosides, sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC), bile salts and medium chain fatty acids (MCFA) (reviewed in 3), while payloads cover molecular weights ranging from 1,000 to 7,000 Da: calcitonin, insulin, PTH (1–32), GLP-1 and octreotide. The most clinically advanced oral peptides are calcitonin (4) and octreotide (5), but these are atypical in that their high potency suggests that the very low oral bioavailabilities achieved may still be commercially viable and they are relatively inexpensive to synthesize compared to other peptides. Many peptides including insulin, however, will require minimum oral bioavailabilities of >15%, so unless oral peptide delivery systems are not to become a narrow commercial niche for one or two products, new formulation strategies are needed. One of the most clinically advanced intestinal PEs is sodium caprate (C₁₀). It is currently a component of Merrion Pharmaceutical's GIPET™ oral peptide formulation that is in phase II trials (6). C₁₀ has a complex mechanism of action involving increases in epithelial paracellular transport *in vitro* across tricellular tight junctions (7) as well as transcellular epithelial drug transport due to surfactant detergent-like effects on plasma membranes at concentrations above its critical micellar concentration *in vivo* (8). It reversibly perturbs epithelial membranes at

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concentrations of the same order as those required to induce permeation enhancement in rat intestinal instillations (9), suggesting relatively narrow safety margins for repeat oral dosing regimens. This would suggest that new enhancers are needed where, if possible, permeation enhancement and mucosal damage can be more clearly dissociated across a wider dose range.

The 11-mer MCFA sodium salt, undecylenic acid (C_{11}), has physicochemical properties similar to the 10-mer sodium salt, C_{10} , suggesting a capacity for permeation enhancement. A separate mono-unsaturated species of C_{11} is the sodium salt of 10-undecylenic acid (uC_{11}), which is derived from its parent acid ($CH_2=CH(CH_2)_8COOH$ (UA)). ClonMedica (Ireland) markets two nonprescription topical antifungal products containing salts of 10-undecylenic acid (UA) as active ingredients: Caldesene® powder for the prevention and treatment of nappy rash and Desenex® powder and ointment for the treatment of athlete's foot. Salts of UA also inhibit *Candida albicans* morphogenesis and are used to prevent denture stomatitis by reducing biofilm formation on denture liners (10,11). The salts have a history of oral ingestion in man, spanning over 60 years. They are currently used as a systemic antifungal agents, dosed orally either as the free fatty acid, typically in an oil-based soft gel capsule or as salt powders in a hard gelatin capsules for the alternative treatment of intestinal fungal dysbiosis and chronic rhinosinusitis. The adult daily dosage of UA is 450–750 mg administered in three divided doses (12,13). The oral LD_{50} of UA in rats was 2.5 g/kg (UA, Material Safety Data Sheet). Thorne Research (USA) supplies soft gel capsules of salts of UA in olive oil (Formula SF722®), as a nutritional supplement “to correct an imbalance in the microbial flora of the digestive tract”. The manufacturer's recommended regimen is 250 mg UA, administered BID or TID. The extent of Formula SF722® use is not publicly available, but the product is described on the company website (14) as “one of our best selling products for over 20 years”, implying extensive human ingestion of UA. Despite debate over the efficacy of UA as a nutritional supplement, continued supply over many years would suggest that ingestion of UA and its salts is generally safe in man. The physicochemical properties of the sodium salt of UA, uC_{11} , therefore lie between those of C_{10} and C_{11} . The terminal alkene of uC_{11} reduces its hydrophobicity compared to the saturated C_{11} salt, with a resultant increase in solubility and critical micelle concentration (CMC) (15,16). UA and its salts have been used as penetration enhancers in transdermal drug delivery (17,18), and there have been attempts to improve oral delivery of testosterone using a UA ester (19,20); however, the use of either UA and/or uC_{11} specifically as intestinal permeation enhancers has never been investigated. uC_{11} is a powder, more amenable to commercial-scale solid dose manufacturing than the acid form and was therefore more appropriate for the current oral drug delivery study.

The aim of this study was to assess the efficacy of uC_{11} as an intestinal permeation enhancer in a range of *in vitro* and *in vivo* bioassays and to compare it against C_{10} and C_{11} . We also used high-content multi-parametric analysis and tissue histology to compare its capacity to damage epithelia *in vitro* and *in vivo*, respectively. The data reveal that uC_{11} is a highly efficacious enhancer with a benign *in vitro* and *in situ* toxicity profile and suggest that this molecule can be added to the select range of agents with potential for clinical application as a PE.

MATERIALS AND METHODS

Materials

The acid of C_{11} was obtained from Sigma Aldrich (Ireland). UA was obtained from Chemos GmbH, (Germany). C_{11} and uC_{11} sodium salts were not commercially available and were synthesized by mixing the acids with NaOH at a 1:1 molar ratio in deionized water at 50°C. One percent isopropyl alcohol was included to reduce foaming on mixing. The resulting salts, uC_{11} and C_{11} , were oven-dried overnight at 65°C to form powders, which were tested by differential scanning calorimetry and thermo-gravimetric analysis to confirm >95% purity (data not shown). Ninety-eight percent pure C_{10} was obtained from Fluka. Male Wistar rats (Charles River Laboratory, UK) weighing approximately 300 g were used for all animal studies. Caco-2 cells were obtained from the European Collection of Cell Cultures and used between passages 52 and 60.

Critical Micelle Concentration

High-resolution ultrasonic spectroscopy (HR-US) was used to measure the CMC of MCFA salt solutions in Ca^{2+} -free Dulbecco's modified Eagle's medium (DMEM) (21). Ultrasonic wave velocity (v (m/s)) was measured between 5 and 15 kHz using a HR-US 102 instrument (Ultrasonic Scientific Ltd., Ireland). It was equipped with two sample cells allowing for differential sample measurement to increase velocity measurement resolution (to 0.2 mm/s). MCFA stock solutions were prepared in degassed Ca^{2+} -free DMEM. One millilitre degassed medium was added to the cell and the base v value determined at 37°C. Stock MCFA solutions were then centrifuged (>10,000g) to ensure that they were completely degassed following preparation. v was measured, while the sample was titrated by adding MCFA stock solution through a septum in the lid of the measuring cell using a manual Hamilton dispenser and 250 μ l Hamilton syringe (Gastight, 1700 series). Each injection from the manual dispenser loaded 4.97 mg of solution into the measuring cell with stirring.

Fluxes Across Caco-2 Monolayers

Caco-2 cells were grown on Corning Costar polyester Transwell® filters (pore size, 0.4 μ m; diameter, 12 mm) for 21–28 days in DMEM and incubated at 37°C with 5% CO_2 . [^{14}C]-mannitol flux across differentiated Caco-2 monolayers and apparent permeability coefficients (Papp) were measured according to previous methods (22). The fluxes of fluorescein isothiocyanate (FITC)-dextran (FD4, FD10, Sigma Ireland) were measured in the same way as mannitol fluxes with the exception that HBSS (Gibco®, Life Technologies™) supplemented with 12.5 mM D-glucose (Sigma) and buffered with 25 mM HEPES (Gibco®) was used instead of DMEM. To prevent MCFA precipitation, Ca^{2+} -free HBSS was used in the apical compartment. Dextran solutions (5 mg/ml, $\times 10$) were prepared in Ca^{2+} -free HBSS on the day of use (final dextran concentration in apical compartment of 250 μ g/ml). For [^{14}C]-mannitol fluxes, a T_0 apical sample of 50 μ l was taken; all of the basolateral (1,500 μ l) media was sampled throughout and completely replaced with fresh HBSS at each sampling point. Samples containing [^{14}C]-mannitol were mixed with scintillation fluid and read in a scintillation counter (Packard Tricarb 2900 TR). FITC-containing

samples were transferred onto a Nunc 96 well white Maxisorp® plate (Thermo Scientific). The T_0 apical sample was diluted to 1:10 (20 μ l in 180 μ l HBSS) and then to 1:100 on the plate. The FITC signal intensity was measured (excitation/emission wavelengths of 490/525 nm, respectively) using a Spectra Max Gemini fluorescence intensity microplate reader (Molecular Devices, CA, USA). Papp values for FITC-dextran were calculated in the same way as mannitol. Each treatment was added in duplicate to each plate and repeated on three separate occasions.

MTT Cytotoxicity Assay

Caco-2 cells were cultured using published methodology (22). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out on Caco-2 cells grown on 96 well plates according to previous descriptions (23), but with one modification. At 24 h, DMEM was replaced with Ca^{2+} -free DMEM in order to avoid precipitation of MCFA salts (24). MCFA exposures were carried out for 1, 8 or 24 h. The IC_{50} was defined as the concentration of test compound required to reduce the absorbance of the MTT-formazan crystals by 50%.

High Content Analysis

In vitro high-content analysis (HCA) was developed to screen discovery candidates for potential hepatic drug toxicity in HepG2 cells (25) and consequently for intestinal cytotoxicity in Caco-2 cells (26). It was used here to study sublethal multiparametric responses to MCFA exposure in Caco-2 cells. Cells were seeded in 200 μ l DMEM per well in 96 well plates (Nunc® Thermo Scientific) at a density of 6,000 cells/well. After 24 h, medium was replaced with 300 μ l Ca^{2+} -free DMEM containing MCFAs and the plates incubated for 1, 8 and 24 h. One hundred fifty microlitres of medium was removed from each well and a dye mix (150 μ l of $\times 2$ stock in Ca^{2+} -free medium) was then added to each well. It contained 0.8- μ M Hoechst 33342 (measures cell number (CN), nuclear area (NA) and nuclear intensity (NI); 1 μ M Fluo-4 AM (measures intracellular calcium (IC); 20 nM tetramethyl rhodamine methyl ester (TMRM) (measures mitochondrial membrane potential (MMP) and 1 μ M TOTO®-3 iodide 642/660 (measures plasma membrane permeability (PMP)). Cells were incubated with the dyes for 50 min before positive controls were added for each parameter—100 μ M carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (a mitochondrial membrane uncoupler), 20 μ M ionomycin (a calcium ionophore) and 0.05% (v/v) Triton® X-100 (membrane perturbant). Positive controls were prepared at $\times 6$ concentrations in $\times 1$ concentration dye mix in Ca^{2+} -free DMEM. Fifty microlitres medium was removed from each positive control well and replaced with 50 μ l of the positive control agent. The plate was incubated for a further 10 min before image acquisition using an In-Cell® 1000 High Content Analyzer (GE Healthcare, UK) using multi-target analysis. The excitation/emission wavelengths used were Hoechst 33342: 360/460 nm, Fluo 4-AM: 480/535 nm, TMRM: 535/600 nm and Toto-3®: 620/700 nm. The Hoechst

channel was used for cell counts and 10 random fields per well were imaged using a $\times 10$ objective magnification. For each other parameter, eight random fields per well were imaged using a $\times 20$ objective magnification. Exposure times were optimized for Hoechst (150 ms), Fluo-4 AM (500 ms), TMRM (600 ms) and TOTO®-3 iodide (200 ms).

Fluxes Across Isolated Rat Colonic Mucosae

Ussing chamber electrophysiology and flux studies using dissected isolated rat colonic mucosae were carried out according to our most recent description (27) and in accordance with the UCD Animal Research Ethics committee policy on use of animal postmortem tissue. Sheets of intestinal mucosa with underlying lamina propria were mounted in Ussing chambers (WPI, UK) with a circular window surface area of 0.63 cm². Tissues were bathed bilaterally with 5 ml Krebs-Henseleit buffer (KH) and were continuously gassed with 95% O₂/5% CO₂ at 37°C. Ca^{2+} was omitted from KH in the apical chamber in order to avoid MCFA precipitation. The potential difference (PD, (mV)) across the tissue was measured in the open circuit configuration using Ag/AgCl electrodes attached to an automated voltage clamp (EVC 4000, WPI, UK) before being clamped at zero PD to permit recording of short circuit current (I_{sc} , (μ A cm⁻²)). Colonic mucosae with TEER values below 70 Ω cm⁻² were excluded to ensure only fully functional tissues were used (28). In addition to monitoring TEER, Papp values for [¹⁴C]-mannitol (0.2 μ Ci ml⁻¹) and FD-4 (250 μ g ml⁻¹) across tissue segments were calculated as for Caco-2 studies. Apical-to-basolateral flux of each paracellular marker was determined by basolateral side sampling (200 μ l) at time zero (T_0) and every 20 min for 2 h, and the Papp was calculated as above. Volumes were replaced with fresh KH at each sampling point. A T_0 apical side sample (100 μ l for [¹⁴C]-mannitol and 20 μ l for FITC-dextran) was also taken. Capacity to generate an inward electrogenic chloride secretory response to basolateral application of the muscarinic agonist, carbachol (CCh), was used to confirm tissue function following MCFA exposure at 120 min.

Mini-Tablet Production and Characterization

Mini-tablets containing C₁₀, C₁₁ and uC₁₁ salts were formulated with FD4. Microcrystalline cellulose (Avicel® PH102, FMC Biopolymer, UK) was used as a tablet filler and disintegrant. All components were sieved through 150–425 μ m screens prior to use. Powder components were weighed directly into a 30 ml skirted tube and mixed by inversion to give a total blend weight of 3 g for ~60 tablets. Each 50-mg tablet contained 20% w/w FD-4; MCFA-containing tablets in addition had 60% w/w C₁₀ or uC₁₁ (i.e. 30 mg) and 20% Avicel®, while control tablets had 80% Avicel® and no MCFA. Tablets were produced using a 3.8-mm round concave multiple-tip tool (Natoli Engineering, MO, USA) on a manual single-station hydraulic press with compression force gauge (MTCM 1, LobePharma, NJ, USA). Tablet powder blend was hand-filled into the die and a compression force of 500 psi applied for each mini-tablet formulation. Tablet hardness was determined using a tablet hardness tester (Pharmatron, Switzerland). An arbitrary method was used to

determine relative disintegration times of different mini-tablet formulations. Disintegration time was determined by placing a mini-tablet into an Eppendorf tube containing 1 ml Medium 199 (Sigma, Ireland) at 37°C to ensure that MCFA dissolution was not solubility-limited. The tube was closed, inverted and placed on a plate incubator (37°C) with rotation speed set at 200 rpm. Disintegration time was defined by visual observation as the time taken for complete disintegration of the mini-tablet without any remnants of a core. Tablet content uniformity was assessed by measuring the uniformity of FD4 in tablets from each tablet batch produced. Tablets were dissolved in 10 ml deionized H₂O, giving a theoretical content of 1 mg ml⁻¹ FD4. Triplicate samples of the dissolved tablet were then diluted 1:10, yielding a theoretical FD4 concentration of 2 µg ml⁻¹, which was measured as above. Mini-tablet content uniformity, hardness and disintegration time are shown in Suppl. Table 1. The CMC of the C₁₀, C₁₁ and uC₁₁ in calcium-free buffer were obtained as described above (21) and were 26, 6.2 and 17.6 mM, respectively.

In Situ Intestinal Instillation Studies

Procedures were performed under licence B100/4433 from the Irish Department of Health and with the approval of the UCD Animal Research Ethics Committee. Animals were housed under controlled conditions of temperature and humidity with a 12:12 h light/dark cycle. Rats received filtered water and standard laboratory chow ad lib up to 48 h prior to the instillation procedure at which point water was supplemented with 5% (w/v) glucose and animals fasted 16–20 h prior to the procedure with free access to glucose water. Isoflurane-anaesthetized rats were placed on a heat pad, and *in situ* instillations were performed as previously described (9) with minor modifications. Following midline laparotomy either the upper jejunum or proximal colon was identified and tied off proximally with a size 4 braided silk suture. Following removal of faecal matter, another suture tie was made 5 cm distally from the first tie to create a loop. Treatment solutions were injected into lumens using a 1-ml syringe with 30G needle. Each MCFA was tested at a concentration of 100 mM (equivalent to 27 mg kg⁻¹ C₁₀ and 29 mg kg⁻¹ uC₁₁) and co-administered with FD4 (40 mg kg⁻¹). Solutions were prepared in tissue culture grade PBS without calcium and magnesium. The volume of MCFA/FD4 solution was based on body weight and ranged from 368 to 500 µl per loop. To calculate absolute bioavailability (BA), FD4 (40 mg kg⁻¹) was administered via the tail vein of some rats.

For jejunal mini-tablet instillations in either jejunum or colon, a small incision was made between the vasculature approximately 6.5–7 cm distally from the proximal suture tie to allow the tablet placement using a fine tweezers. A second distal tie proximal to the incision was then made once the mini-tablet was in place. For colonic tablet instillation, it was inserted using a fine tweezers through the same incision initially made to remove faecal matter. Three hundred microlitres PBS was subsequently instilled to aid tablet disintegration and dissolution. The abdomen was closed by

suturing the epidermal layer using Mersilk W505 silk sutures with a size 4 cutting needle. Blood samples were taken by retro-orbital bleeds at 0, 5, 15, 30, 45, 60, 90, 120, 150 and 180 min into 0.5-ml Eppendorf tubes and stored at 2–8°C for a minimum of 60 min prior to centrifugation (6,500g, 10 min) and serum collection. Serum was stored at –20°C until analysed. Animals were euthanised at the end of the experiment with intracardiac injection of 0.5 mL pentobarbital sodium (EUTHATALTM, Merial Animal Health Ltd., UK). Based on an average rat weight of 330 g, each mini-tablet represented the administration of 30 mg kg⁻¹ FD4 and 90 mg kg⁻¹ MCFA enhancer.

Rat Serum FD4 Assay

Serum FD4 levels were quantified as previously described (9). Thawed serum was diluted 1:10 in borate buffer on a Nunclon® 96 well white Maxisorp plate. The FD4 standard curve samples included on each plate were prepared in borate buffer and stored at –20°C with the serum samples until required such that the FD4 in the standard curve also underwent a freeze-thaw cycle. The FITC signal intensity was measured as above. Pharmacokinetic (PK) characteristics from serum data were calculated using WinNonLin® software (Pharsight Inc., USA). Non-compartmental analysis was used with the peak concentration (*C*_{max}) and the time taken to reach *C*_{max} (*T*_{max}) calculated from the serum concentration profiles. The area under the serum-concentration curve, AUC_{0–3 h}, was calculated using the linear trapezoidal rule. The BA of FD4 over the 3-h intestinal instillation was calculated using the following:

$$\text{BA}(\%) = \text{AUC}_{0-3 \text{ h}} / \text{AUC}_{i.v.\infty} \times 100$$

where AUC_{0–3 h} is the area under the plasma concentration curve over the 3-h instillation period and AUC_{i.v.∞} is the area under the plasma concentration *versus* time (0–∞) after i.v. administration of 40 mg kg⁻¹ FD-4 (0.01 mM).

Tissue Histology

Intestinal issue samples for morphological examination following treatments were fixed for 24 h in 10% (w/v) formalin and subsequently embedded in paraffin wax. Tissues from Ussing chambers or instillations were incubated in formalin. Five-micromolar tissue sections were cut on a microtome (Leitz 1512; GMI, USA), mounted on adhesive coated slides, stained with haematoxylin/eosin (H&E) and examined under light microscopy (Nikon Labphoto; Nikon, Japan).

Statistical Analysis

Results from Ussing chamber and instillation studies were analysed by one-way ANOVA with Bonferonni's ad hoc posttest. HCA was conducted on three independent plates with two treatment replicate wells per plate. All data was normalized to the untreated control, and each treatment time was analysed by one-way ANOVA with Dunnett's ad hoc posttest. All data is presented as the mean±SEM with minimum statistical significance set at *p*<0.05.

RESULTS

uC₁₁ Permeation Enhancement Across Caco-2 Cell Monolayers

Effects of uC₁₁, C₁₀ and C₁₁ on permeability across monolayers were determined by measuring effects on TEER and the Papp values of flux markers. Minimum concentrations of 3, 8.5 and 10 mM of each of C₁₁, C₁₀ and uC₁₁ induced a statistical reduction in TEER to below 20% of initial value (T₀) within 30 min (Fig. 1a, b). This data suggests that TEER is most sensitive to C₁₁, but effective concentrations were of the same order for the three MCFAs. In parallel, there were significant concentration-dependent increases in the Papp of [¹⁴C]-mannitol, FD-4 and FD-10 in the presence of C₁₁ and uC₁₁ (Fig. 2a, b). These concentrations mapped to those that caused TEER reductions. Similar increases in Papp were seen for each marker at 5 mM C₁₁ and uC₁₁ and also for 10 mM C₁₀. These data suggest that the potency and efficacy order for inducing permeability across Caco-2 monolayers is similar for the three agents.

Cytotoxicity Assessment of uC₁₁: MTT and HCA

MTT assay results for uC₁₁, C₁₁ and C₁₀ on Caco-2 intestinal epithelial cells over time are shown in Fig. 3a–c. Following 24 h exposure, the IC₅₀ was ~5 mM for both uC₁₁ and C₁₀, whereas it was less than 2.5 mM for C₁₁. Exposure for 1, 8 and 24 h revealed that cells were equally resilient to both 5 mM C₁₀ and uC₁₁ but less resilient to C₁₁. Just 20% loss in viability at 5 mM uC₁₁ and C₁₀ occurred at 8 h, whereas 5 mM C₁₁ killed 20% of cells after only 60 min and 50% at 8 h. C₁₁ was therefore considerably more cytotoxic than both uC₁₁ and C₁₀ in the MTT assay, whereas the latter two agents were equivalent and not lethal even at very high concentrations and exposure times. HCA was then used to examine multiple contemporaneous cellular effects of uC₁₁ and to relate them to efficacy as a permeability enhancer. Initial Caco-2 cell responses to uC₁₁ (1–5 mM, 60 min) were an increase in nuclear intensity (NI) and intracellular Ca²⁺ concentration (IC) accompanied by mitochondrial hyperpolarization (Fig. 4a–f). Maintenance of high IC

preceded a reduction in mitochondrial membrane potential (MMP) and an increase in plasma membrane permeability (PMP) and nuclear fragmentation from 5 to 20 mM, which ultimately lead to death. Importantly, PMP changes seen at 60 min with 8.5 mM uC₁₁ correlated with increased Papp values across monolayers seen at similar concentrations and suggest a mild surfactant effect on the plasma membrane. Similarly, HCA also revealed that 60 min exposure to 8.5 mM C₁₀ and 2.5 mM C₁₁ resulted in significant increases in PMP, confirming the similarity between the concentrations of MCFAs that cause PMP increases in Caco-2 cells and those that increase fluxes across monolayers (data not shown). Of note is that the initial IC increase in response to uC₁₁ occurred at concentrations lower than those that induced PMP increases, and this implicates calcium regulation of tight junction proteins and paracellular transport as an earlier step than perturbation. Most changes in parameters occurred at concentrations of uC₁₁ and exposure times below those that affected viability according to MTT assay. After 8 h exposure to concentrations >5 mM uC₁₁, all parameters were significantly altered as cells began the apoptotic process. The cell number changes are a balance between cell division with cell death and give a more accurate assessment than the MTT snapshot.

uC₁₁: Fluxes Across Isolated Rat Colonic Mucosae

uC₁₁ displayed equal capacity as C₁₁ for TEER reduction and Papp increases of [¹⁴C]-mannitol and FD-4 (Fig. 5). C₁₁ produced significant increases in mannitol P_{app} at lower concentrations than uC₁₁. A 5-fold increase in [¹⁴C]-mannitol Papp was obtained following exposure to 5 mM C₁₁, whereas 10 mM C₁₀ and uC₁₁ were required to induce similar increases in the [¹⁴C]-mannitol Papp, however maximal effects were seen at 20 mM uC₁₁ and C₁₁. For FD4 fluxes, 5 mM C₁₁ was sufficient to induce a significant Papp increase compared to the minimum concentrations of 10 mM C₁₀ and uC₁₁ required, but 20 mM C₁₁ was more efficacious than the same concentration of C₁₀. Histological assessment revealed that 10 mM concentrations of MCFA that increased Papp across colonic tissue also caused some mucosal

Fig. 1. a uC₁₁ and b C₁₁ reduced TEER across Caco-2 monolayers. 10–15 mM uC₁₁ and 3 mM C₁₁ caused a reduction in TEER comparable to 10 mM C₁₀ (red). N=3

Fig. 2. **a** uC_{11} and **b** C_{11} increased the P_{app} values obtained over 2 h for $[^{14}\text{C}]$ -mannitol, FD-4 and FD-10 across Caco-2 monolayers. C_{10} data was included for comparison (10 mM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus untreated controls. $N=3$

perturbation compared to controls, (Fig. 6a–d), which is consistent with the relationship between Caco-2 cell PMP and P_{app} increases following exposures. Notably, C_{11} caused more damage compared to uC_{11} and C_{10} . The results obtained on rat colonic mucosae therefore further supported of potential of uC_{11} as an intestinal permeation PE with similar characteristics to C_{10} and C_{11} , but the latter is more toxic by several *in vitro* criteria. Despite the damage caused by all agents, mucosae retained secretory ion transport function since Isc responses to CCh were still substantial for each MCFA (Fig. 6e).

Rat Intestinal Instillations With MCFA Solutions

C_{11} was omitted from *in vivo* study due to its high cytotoxicity. *In vivo* instillation of FD-4 (40 mg kg^{-1}) in solution containing 100 mM uC_{11} or C_{10} for 3 h increased FD-4 serum concentration in jejunum and colon (Fig. 7a–d). The selection of such a high dose was warranted owing to previous data showing a requirement for 5–10-fold higher doses of C_{10} *in vivo* compared to *in vitro* concentrations (9). The possible reasons for such a high dose are many: a more robust epithelium *in vivo*, dilution, intestinal absorption of the enhancer, and difficulties in estimating free concentrations above the CMC when MCFA vesicles are present. PK data

for both agents is summarized (Table I). The similarity in the BA increase achieved by uC_{11} in both jejunum and colon is further *in vivo* evidence of its potential application as an intestinal PE. The BA of FD-4 following control solution instillation was higher from jejunum (2.1%) compared to the colon (1.7%). BA from colon was more than double that from the jejunum when co-instilled with either MCFA. Enhancement ratios (ER's) of 10.5 and 10.2 were achieved using C_{10} and uC_{11} , respectively, in the jejunum, increasing to 28.9 and 29.2 in colon, representing BA values of almost 22 and 50%, respectively, for both agents (Table I). H&E is shown for colonic sections and did not reveal evidence of damage following 3-h instillations with uC_{11} or C_{10} (Fig. 7e–g).

Rat Intestinal Instillations With MCFA Mini-Tablets

FD-4/ uC_{11} and FD-4/ C_{10} mini-tablets were instilled to rat jejunum and colon at a dose level of 120 mg kg^{-1} (100 mM) and the data is shown in Fig. 8 and Table II. In jejunum, C_{10} tablets surprisingly did not alter FD-4 BA, whereas a significant increase was obtained from uC_{11} tablets and there was less variability from the uC_{11} tablet compared to C_{10} . Similar to solution studies, basal FD-4 absorption from control tablets was higher in jejunum compared to colon. uC_{11} and C_{10} mini-tablet formulations were effective in

Fig. 3. MTT assay on Caco-2 cells over time. **a** uC₁₁, **b** C₁₀ and **c** C₁₁. black bar 1 h, gray bar 8 h and white bar 24 h. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus medium controls. $N = 3$ independent plates, each in duplicate

enhancing FD4 absorption across colon, but uC₁₁ tablets increased FD4 absorption across both jejunum and colon to a greater extent than C₁₀ tablets (Fig. 8c, d). H&E-stained colonic sections following exposure to equal concentrations of C₁₀ and uC₁₁ in tablet instillations did not reveal mucosal perturbation following exposure to uC₁₁ or C₁₀ compared to control (Fig. 8d–f). Importantly, the aqueous solubility was somewhat lower for uC₁₁ (155 mg ml⁻¹ or 0.75 M) compared to C₁₀ (385 mg ml⁻¹ or 1.98 M), resulting in disintegration times of 34.3 and 26.4 min, respectively (Suppl. Table 1). The lower aqueous solubility may have resulted in a slower colonic release of uC₁₁, prolonging mucosal perturbation and increasing FD4 absorption. This could explain the longer $T_{\max}$ (84 min) in the colon for the uC₁₁/FD-4 tablet compared to the C₁₀/FD-4 tablet (45 min) (Table II).

DISCUSSION

uC₁₁ was identified as a new intestinal PE with the advantage of having an established history of safe oral ingestion in man due to its use for over 20 years as a

nutritional supplement and alternative medicine at doses in excess of 500 mg per day. The unsaturated carbon double bond in uC₁₁ reduces lipophilicity, increases the CMC and solubility relative to the saturated form, C₁₁. Thus, the physicochemical properties of uC₁₁ lie between those of the C₁₀ and C₁₁ as indicated by a CMC value of 17.6 mM compared to values of 26 and 6.2 mM, respectively, and this is likely to be the key factor contributing to the balance between efficacy as a PE and cytotoxicity.

C₁₀ is advanced in the clinic as a major component of the GIPETTM solid dose formulation for oral peptides being developed by Merrion Pharmaceuticals (Ireland) (6). Still, while C₁₀ is an approved food additive without a recommended maximum limit of ingestion, it is still not yet incorporated into an approved oral therapeutic dosage form nor are humans normally exposed to the high dose levels required in oral peptide clinical trials compared to those in food and drink. Of relevance is that similar to C₁₀, uC₁₁ is a solid at room temperature and is suitable for commercial-scale solid dose pharmaceutical manufacturing. Initial assessment of C₁₀ and uC₁₁ by HCA in Caco-2 cells indicated that both MCFAs have very similar sublethal cytotoxicity profiles, and this tallied with the MTT assay revealing a high IC₅₀ for C₁₀ and uC₁₁ even after 24 h. In contrast, C₁₁ was more cytotoxic than the other two molecules in HCA and MTT assays, where 50% of cells were killed with 5 mM concentrations for 8 h compared to 20% for uC₁₁. Taken together with the more damaging histology seen in isolated intestinal mucosae following exposure to C₁₁ compared to uC₁₁, such toxicity potential ruled out further study of C₁₁ as a PE due to its likely overly robust surfactant effects.

The intestinal PE capacity of uC₁₁ was similar to C₁₀ across several *in vitro* and *in vivo* intestinal models. Initial studies demonstrated uC₁₁'s capacity to increase mannitol and FD fluxes in the MW range of 0.182–40 kDa across Caco-2 monolayers. However, despite demonstration by HCA of a similar capacity of the two agents to increase Caco-2 plasma membrane permeability in HCA assays, flux data suggested that uC₁₁ has a lower capacity to increase mannitol flux than C₁₀, with an EC₅₀ (concentration to increase P_{app} by 50%) ~1.7-fold higher. Surprisingly, FD flux studies across monolayers demonstrated that the capacity of uC₁₁ to increase relative flux was proportional to the MW of the ad-mixed FD, i.e. the EC₅₀ values decreased with increasing MW ($R^2 = 0.99$). This is understood, however, in the context of a reduced basal P_{app} across monolayers with increased flux marker MW (29). Solution studies on rat colonic tissue mounted in Ussing chambers and *in vivo* instillations to rat jejunum and colon produced additional evidence of uC₁₁'s intestinal PE efficacy, but importantly, compared to Caco-2 monolayers, its efficacy in these models was equal to C₁₀.

Instillations of solutions of uC₁₁ and C₁₀ to colonic loops yielded >2-fold increase in FD4 relative BA compared to jejunal, but again, this is in the context of a higher basal FD4 BA across jejunal loops compared to colonic, probably due to the larger microvilli surface area. The regional difference in MCFA enhancement action may also be due to differences in epithelial membrane structure between the upper and lower GI tract, with the apical membrane of the small intestinal epithelia designed to better withstand surfactant perturbation than the colon due to periodic jejunal exposure to 30 mM bile salts in the fed state (30).

Fig. 4. HCA of uC₁₁ on Caco-2 cells exposed for *black bar* 1 h, *gray bar* 8 h and *white bar* 24 h. **a** intracellular calcium (IC), **b** plasma membrane potential (PMP), **c** nuclear area (NA), **d** nuclear intensity (NI), **e** mitochondrial membrane potential (MMP) and **f** cell number (CN)

Fig. 5. TEER and Papp changes across isolated rat colonic mucosae following MCFA exposure. **a** TEER: uC₁₁, **b** TEER: C₁₁, **c** Papp [¹⁴C]-mannitol and **d** Papp FD-4. Ten mM C₁₀ data is shown for comparison as a positive control. *N*=3–6 per group. **p*<0.05; ***p*<0.01; ****p*<0.001 versus controls

Fig. 6. H & E of rat colonic mucosae following 2 h of incubations in chambers. **a** untreated control, **b** 10 mM C₁₀, **c** 10 mM uC₁₁ and **d** 10 mM C₁₁. Bar=100 μm. **e** ΔIsc to CCh in mucosae exposed to MCFAs (10 mM, *n*=3)

Increased PE effects of C₁₀ and other surfactants in the colon compared to small intestine has been widely reported (31–33). If a formulation containing surfactant-type PEs can be designed to reach the colon, it may have potential for significant systemic delivery of normally impermeable payloads. Concerns over colonic co-absorption of bystander pathogens, including bacteria, bacterial toxins and viruses in the presence of PEs have thus far not been vindicated, but studies have been largely restricted to *in vitro* models (34). Of interest is that similar C₁₀ concentrations required for PE *in vivo* inhibited adhesion of *Salmonella typhimurium* in isolated rat intestinal mucosae and indeed were

bactericidal against *S. typhimurium* and several gram-positive and -negative bacteria (35). Overall, it would be preferable for MCFAs to have no effect on the intestinal microbiota, either toxic, innocuous or probiotic species.

uC₁₁ and C₁₀ were incorporated into mini-tablets to assess if a solid dosage form could enable permeation enhancement. MCFA lipophilicity and capacity to perturb biological membranes increases with alkyl chain length, and this is associated with reduced aqueous solubility, which prolongs the time required for the MCFA dissolve out from tablets. This means that there is greater intestinal absorption MCFA from solutions than tablets *in vivo* and therefore that assumptions cannot be made on designating one PE as superior to another on the basis of ad-mixed solutions. Since increasing the alkyl chain length results in a lower CMC, as was experimentally detected, it will also reduce the maximum free monomer concentration in solution before forming micelles. Importantly, it is the monomer that is thought to be the species that perturbs membranes (15). Adsorption to epithelial membranes also reduces monomer concentration. Thus, MCFA concentration in the GI lumen from a tablet formulation is dissolution-rate limited. A high level of MCFA (60% w/w) was used in the mini-tablet formulation to ensure sufficient local concentrations were achieved *in vivo* in the intestinal lumen to perturb mucosal surfaces. This high MCFA concentration in the tablet imparts a low surface porosity. The use of Avicel®, an insoluble microcrystalline cellulose binder, ensured that mini-tablet disintegration was predominantly controlled by the intrinsic dissolution rate of the MCFA, allowing for subsequent study of its effect on permeation enhancement. Tablets therefore disintegrated by dissolution and erosion of MCFA at their surface, forming a matrix by which MCFA and FD4 release were subsequently controlled by MCFA dissolution.

Comparing C₁₀ solution and tablet colonic instillations suggested approximately 60% relative BA from the tablet formulation compared to solution. PE from the tablets may be due to altered fluid osmolality and mucosal dehydration in addition to surfactant action. It should be noted that the dehydration of mucosal surfaces may be more pronounced in rat loop studies than in Ussing chambers. The average diameters of the rat jejunal and colonic lumen are 4–5 mm and 3–10 mm, respectively, approximately one tenth that of human (36). Instillation of a 3.8-mm mini-tablet to rat intestinal region segments permits intimate contact of hygroscopic tablet components with the entire circumference of the mucosal surface. Dissolution of the 50 mg powder components also alters fluid osmolality and the effect of hyper-osmolality on C₁₀ interaction with the rectal GI epithelium has also been demonstrated in a suppository of ampicillin (37). Overall, data generated suggests that uC₁₁ may be able to strike the dissolution-perturbation balance in a tablet formulation more effectively than C₁₀ since the aqueous solubility and CMC of uC₁₁ lies between that of C₁₀ and C₁₁. Intestinal instillations revealed major differences between C₁₀ and uC₁₁ mini-tablet capacities to increase FD4 BA: the C₁₀ formulation did not increase FD4 serum levels following jejunal instillation, whereas uC₁₁ mini-tablets did. The greatest difference between the two MCFAs was found in the colon, and may be due to the prolonged perturbation resulting from slower dissolution of uC₁₁, leading to its superior efficacy. The uC₁₁ mini-tablets also had a much longer *T*_{max} (84 min) in

Fig. 7. **a** Serum FD-4 (jejunum), **b** FD4 AUC (jejunum), **c** serum FD-4 (colon) and **d** FD4 AUC (colon) following 3 h of instillations of FD-4 (40 mg kg^{-1}) solutions with or without 100 mM uC₁₁ or C₁₀. *white circle* control, *filled circle* uC₁₁, and *white square* C₁₀. $N=6$ per group. $*p<0.02$; $**p<0.001$. H&E stained sections of rat colon following 3 h of instillations of **e** FD4 control, **f** 100 mM uC₁₁ and **g** 100 mM C₁₀. Bar = 50 μm

the colon compared to C₁₀ (45 min). Still, a higher level of initial mucosal perturbation induced by uC₁₁ compared to C₁₀ cannot be ruled out as the cause of increased FD4 bioavailability. The novel application of uC₁₁ as a jejunal and colonic permeation enhancer suitable for a solid dosage form warrants further investigation.

The solution and mini-tablet studies using MCFAs on colonic loops demonstrated a concomitant increase in drug flux associated with mucosal perturbation. This was also observed by other investigators studying amphiphilic PEs (38) and was a likely finding consistent with HCA assessment. While isolated mucosae from Ussing chamber studies showed

Table I. PK of FD-4 (40 mg kg^{-1}) Solutions in Instillations with 100 mM uC₁₁ or C₁₀

	I.V.	Control—jejunum	C ₁₀ —jejunum	uC ₁₁ —jejunum
C_{max} ($\mu\text{g ml}^{-1}$)	123.9 $\pm$ 22.2	3.2 $\pm$ 1.2	14.1 $\pm$ 11.1	12.3 $\pm$ 4.8
T_{max} (min)	5.0 $\pm$ 0.1	30.0 $\pm$ 15.0	19.2 $\pm$ 15.6	30.8 $\pm$ 17.4
AUC ₀₋₁₈₀	6,250 $\pm$ 462	134 $\pm$ 81	1,399 $\pm$ 1,311	1,360 $\pm$ 680
BA (%)	—	2.1	22.4	21.8
Enhancement ratio	—	1	10.5	10.2
		Control—colon	C ₁₀ —colon	uC ₁₁ —colon
C_{max} ($\mu\text{g ml}^{-1}$)		2.7 $\pm$ 1.2	31.3 $\pm$ 13.0	30.1 $\pm$ 5.7
T_{max} (min)		60 $\pm$ 53	24 $\pm$ 11	38 $\pm$ 28
AUC ₀₋₁₈₀		107 $\pm$ 62	3,078 $\pm$ 1,445	3,111 $\pm$ 924
BA (%)		1.7	49.2	49.8
Enhancement ratio		1	28.9	29.2

Fig. 8. **a** Serum FD-4 (jejunum), **b** FD4 AUC (jejunum), **c** serum FD-4 (colon) and **d** FD4 AUC (colon) following 3 h of instillations of FD-4 (40 mg kg^{-1}) mini-tablets with or without 100 mM uC₁₁ or C₁₀, each at 60% w/w white circle control, filled circle uC₁₁ and white square C₁₀. $N=6$ per group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. H&E stained sections of rat colon following 3 h of instillations of **e** FD4 control, **f** 100 mM uC₁₁ and **g** 100 mM C₁₀. Bar=50 μm

Table II. PK of FD-4/uC₁₁ or FD-4/C₁₀ Mini-Tablets in Instillations

	Control tablets	FD-4/C ₁₀	FD-4/uC ₁₁
	Jejunum	Jejunum	Jejunum
C_{max} ($\mu\text{g ml}^{-1}$)	5.0 ± 3.2	16.6 ± 6.6	20.4 ± 10.2
T_{max} (min)	180 ± 0	132 ± 66	78 ± 51
AUC_{0-180}	343 ± 213	$1,645 \pm 1,135$	$2,072 \pm 651$
BA (%)	6.7	26.3	40.2
Enhancement ratio	1	4.8	6.0
	Colon	Colon	Colon
C_{max} ($\mu\text{g ml}^{-1}$)	2.5 ± 1.0	12.4 ± 2.5	32.2 ± 7.0
T_{max} (min)	105 ± 57	45 ± 21	84 ± 25
AUC_{0-180}	115 ± 64	$1,562 \pm 281$	$4,213 \pm 767$
BA (%)	2.2	30.3	67.4
Enhancement ratio	1	13.6	36.6

evidence of irreversible epithelial perturbation following 2-h exposure to 10 mM uC₁₁ and C₁₀, this was not observed in colonic instillations following 3-h exposure to 100 mM of both agents, in line with other instillation studies with surfactant challenge (39,40). This would suggest that a degree of membrane damage is inevitable with all effective PEs: screening should be on assessing whether adequate efficacy is achieved with an acceptable degree of reversible perturbation in concentration ranges that can be formulated in solid dosage forms for co-release with payload. This study confirms that this is the case for uC₁₁ and C₁₀, but not C₁₁. In conclusion, an alternative efficacious PE to C₁₀ has been discovered which may have some advantages over it, including its history of oral use in man with alternative medicines. Further studies with peptide cargoes (as against marker molecules) will elucidate the potential of uC₁₁ in solid dose formulations and in nanoparticle and conjugated formats. As to the issue of separating efficacy and toxicity of MCFAs, human studies to date with C₁₀ suggest that efficacy for this class can be achieved without significant reports of toxicity, likely due to rapid epithelial restoration (6,8). This does not address repeat dose administration risks nor does it reveal in depth analysis of the potential for lipopolysaccharide fragments to permeate compromised intestine to stimulate production of Type 1 cytokines (41). *In vitro* toxicity studies encompassing tissue histology, cytotoxicity assays and HCA in parallel with permeability assays will allow a mechanistic basis for rank ordering within and between classes of PE in respect of selection for *in vivo* study.

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