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RESEARCH PAPER

CriticalSorb™ Promotes Permeation of Flux Markers Across Isolated Rat Intestinal Mucosae and Caco-2 Monolayers

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ABSTRACT

Purpose CriticalSorb™ is a novel absorption enhancer based on Solutol® HS15, one that has been found to enhance the nasal transport. It is in clinical trials for nasal delivery of human growth hormone. The hypothesis was that permeating enhancement effects of the Solutol®HS15 component would translate to the intestine.

Methods Rat colonic mucosae were mounted in Ussing chambers and Papp values of [^{14}C]-mannitol, [^{14}C]-antipyrine, FITC-dextran 4000 (FD-4), and TEER values were calculated in the presence of CriticalSorb™. Tissues were fixed for H & E staining. Caco-2 monolayers were grown on Transwells™ for similar experiments.

Results CriticalSorb™(0.01%v/v) significantly increased the Papp of [^{14}C]-mannitol, FD-4 [^{14}C]-antipyrine across ileal and colonic mucosae, accompanied by a decrease in TEER. In Caco-2 monolayers, it also increased the Papp of [^{14}C]-mannitol FD-4 and [^{14}C]-antipyrine over 120 min. In both monolayers and tissues, it acted as a moderately effective P-glycoprotein inhibitor. There was no evidence of cytotoxicity in Caco-2 at concentrations of 0.01% for up to 24 h and histology of tissues showed intact epithelia at 120 min.

Conclusions Solutol® HS15 is the key component in CriticalSorb™ that enables non-cytotoxic *in vitro* intestinal permeation and its mechanism of action is a combination of increased paracellular and transcellular flux.

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INTRODUCTION

The use of the nasal cavity to achieve systemic delivery of relatively impermeable molecules has led to approval of a number of peptides including salmon calcitonin (Miacalcin®, Novartis), the gonadotropin releasing agonist, nafarelin (Synarel®, Pfizer) and desmopressin (Stimate®, CSL Behring) (1). These particular peptides are highly potent and do not require a bioavailability above 2–3% for efficacy. They are commercially viable due to the acceptable cost of production; further justification for viability is the need for regular dosing by patients over many years. The importance of having a wide safety margin in the presence of low and variable bioavailability is a feature of marketed nasal peptides. The advantage of delivering peptides nasally includes a potential concentration of the drug at the local application site, an ample blood supply to the mucosa, a large surface area for permeation of 150 cm^2, avoidance of first-pass metabolism, rapid onset of action, and high patient compliance despite the potential for some minor irritation from some drugs or delivery systems (2,3). In general, while the low systemic bioavailability for nasal peptide delivery is generally sub-optimal, it still surpasses that of oral delivery, for which only two structurally atypical peptides, desmopressin (small, stable) and cyclosporine (cyclic, hydrophobic), have been approved in products by that route. The low systemic bioavailability of even the currently approved nasally-delivered peptides means that a wide range of established injectable peptides and proteins (e.g. insulin, PTH, GLP-1 and hGH) as well as investigational ones (e.g. ghrelin, endostatin and growth factors) are not yet enabled by existing technologies, hence the rationale for investigating the potential inclusion of epithelial permeation enhancers in

novel nasal solution or powder formulations comprising also bioadhesive agents (4).

In selecting appropriate formulation-compatible nasal enhancers, the challenge is to significantly increase bioavailability while avoiding mucosal irritation and the potential for allergy. Bile salts, non-ionic surfactants, cyclodextrins, cell-penetrating peptides, nitric oxide donors and cationic polymers have been studied as nasal enhancers in preclinical studies (5) with some being more toxic than others. Of the less toxic, azones including cyclopentadecalactone (CPE-215, CPEX Pharma, USA), (6), alkyl maltosides (Intravail®, Aegis Therapeutics, USA) (7) and chitosan (ChiSys™, Archimedes, UK) (8) are examples of enhancers that are being incorporated in formulations for nasal peptide clinical trials. Importantly, there are no approved nasal peptides products that specifically include established mucosal enhancers, although the precise role of certain preservatives and stabilizing excipients including benzalkonium chloride and Tween®-20 may be open to some debate. Encouragingly, recent data suggests that, similar to the intestine, the nasal epithelium is also quite resilient to mild mucosal damage from non-ionic surfactants (9).

CriticalSorb™ (Critical Pharmaceuticals Ltd., UK) is a novel mucosal absorption enhancer system, the major component of which is Solutol® HS15 (macrogol 15 hydroxystearate; BASF, UK; Fig. 1). Solutol® HS15 is currently used to increase aqueous solubility of Biopharmaceutical Classification System (BCS) Class 2 and 4 lipophilic molecules (8,10,11). Solutol® HS15 is composed of a mixture of polyglycol mono- and di-esters of 12-hydroxystearate (70%) and free polyethylene glycol (PEG; 30%), comprising a molar ratio of 1:15 for 12-hydroxystearic acid: ethylene oxide (12,13). A small part of the 12-hydroxy group can be etherified with PEG. Due to the presence of some impurities in the starting materials, the resulting mixture also contains esters of 18 other fatty acids, including stearic and oleic acid (10). Solutol® HS15 was the first excipient to be reviewed under the IPEC

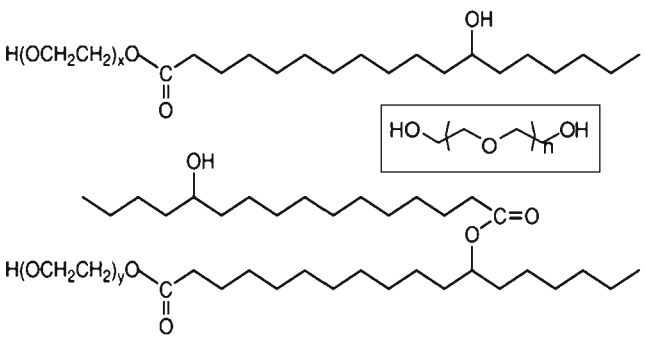

Fig. 1 Mono- and di-esters of 12- hydroxystearate. Inset shows PEG component of Solutol® HS-15.

Novel Excipient Safety Evaluation Procedure which, following FDA review and submission to the US Pharmacopeia (USP) led to the publication of its National Formulary (NF) monograph in 2009 (14). The importance of using excipients from the USP-NF such as Solutol® HS15 is that the regulatory pathway for approval by FDA is less onerous and less costly since it has already been approved as an excipient. CriticalSorb™ is the commercial term for a transmucosal drug delivery system that comprises a range of different excipients for various functions with suitable concentrations of Solutol® HS15. CriticalSorb™ formulations can be in the form of powder formulations or simple buffered solutions, as is the case here. Importantly, Solutol® HS15 is the key excipient in all CriticalSorb™ formulations. There are no additional excipients in the CriticalSorb™ formulation used in this study and hence indications of concentrations of CriticalSorb™ are equivalent to Solutol® HS15.

A nasal CriticalSorb™ formulation of hGH (CP024) is currently in Phase I trials and initial data have revealed comparable efficacy to sub-cutaneous administration, as indicated by induction of the biomarker, insulin growth factor-1 (15). Although CriticalSorb™ increases bioavailability of poorly absorbed drugs in the nasal cavity (16), its effects on intestinal epithelial permeability of hydrophilic agents have not yet been investigated. This is an important area of research, as the most clinically-advanced oral peptide delivery systems currently rely on inclusion of permeating-enhancing surfactants including acyl carnitines, sodium caprate and 5-CNAC {8-(N-2-hydroxy-5-chlorobenzoyl)-amino-caprylic acid)} (reviewed in 17). Although their safety profile has not raised substantial issues in clinical trials to date, these agents do not have official GRAS status and their capacity to significantly increase oral bioavailability by intestinal permeation enhancement is restricted primarily to low molecular weight molecules. We therefore examined the capacity of the well-known and accepted solubilizing excipient, Solutol® HS15, to promote permeation of paracellular and transcellular flux markers across Caco-2 monolayers, as well as across isolated rat ileal and colonic mucosae *in vitro* as there is a need for more effective and reliable enhancers. Since positive effects of enhancers do not always translate from intestinal epithelial cell culture epithelial models, it was important to confirm data isolated tissue mucosae as part of a reductionist approach. In addition, as many approved surfactants demonstrate P-glycoprotein (P-gp) inhibition (18), we further examined CriticalSorb™ in intestinal epithelial tissue since Solutol®HS-15 inhibits P-gp in other cell types (19). Finally, we assessed if Solutol® HS15 resulted in cytotoxic effects in the intestinal epithelia using the methylthiazolyldiphenyl-tetrazolium bromide conversion (MTT) and the lactate dehydrogenase (LDH) assays along with tissue histology. The data show that Solutol® HS15 is a combined intestinal paracellular and transcellular permeation

enhancer, that it partially inhibits P-gp with low potency and has minimal cytotoxicity. This study provides evidence upon which to base an assessment of whether to further pursue the development of a CriticalSorb™ based oral delivery system to achieve therapeutically-interesting oral products of peptides and proteins

MATERIALS AND METHODS

Materials/Reagents

Solutol ® HS15 was supplied by BASF (Germany). [^{14}C]-antipyrine and [^{14}C]-mannitol were supplied American Radiolabeled Chemicals, USA. Tissue culture reagents were from Gibco (Ireland).

Preparation of Solutol ® HS15 Solutions

Solutol® HS15 was melted at approximately 45°C. To insure a homogenous mixture, the vial was inverted 20 times, before the melt was cooled to room temperature. A 100 mM Solutol® HS15 solution was prepared bi-weekly in PBS. The solution was stored in the fridge at 4°C and used within 2 weeks. Dilutions (1 μM-1 mM) of the Solutol® HS15 preparation (100 mM) were prepared in PBS.

Intestinal Tissue Preparation for Ussing Chamber Studies

Male Wistar rats (250–300 g; Charles River, UK) were housed in the Biomedical Facility in University College Dublin (UCD) under controlled environmental conditions with 12:12 light: dark cycles, as well as with access to tap water and standard laboratory chow *ad lib*. The animals were euthanased by stunning followed by cervical dislocation. Studies were in accordance with the UCD Animal Research Ethics Committee policy, Regarding the Use of Post-Mortem Tissue from Animals in Research and Teaching at UCD (2007) (www.ucd.ie/researchethics/pdf/arec_post_mortem_tissue_policy.pdf) as well as in adherence with the "Principles of Laboratory Animal Care," (NIH Publication #85-23, revised in 1985). Ileal or colonic mucosae were removed and placed into freshly oxygenated Krebs-Henseleit buffer (KH). Excised intestinal tissue was opened with scissors along the mesenteric line. Faecal matter was washed from the mucosal side with fresh KH and the intestine was turned over onto the serosal side to expose the external muscle layer. Circular and longitudinal muscle layers were removed by blunt dissection using fine-tipped watchmaker forceps (#5), as described previously (20). The mucosae were pinned between pre-equilibrated Ussing chamber halves (World Precision Instruments, WPI, UK) with circular diameters of 0.63 cm^2. 5 mL KH was added bilaterally and chamber fluids were oxygenated using a gas-lift system with 95% O_2/5% CO_2 at 37°C. Each chamber half had voltage and current electrodes connected to a voltage clamp apparatus (EVC4000; WPI, UK) via a pre-amplifier. Silver/silver chloride (Ag/AgCl) electrodes were prepared by heating 3 M KCl with 3% agar and cooled in plastic casing. The potential difference (PD; mV) was measured across the mucosa in an open circuit configuration. When the voltage was clamped to 0 mV, the short circuit current (Isc; μA/cm^2) was determined and transepithelial electrical resistance (TEER; Ω.cm^2) was determined by Ohm's Law.

Caco-2 Monolayers

Caco-2 cells (Passage 55-60) were obtained from ECACC and grown in 75 cm^2 flasks containing DMEM supplemented with 10%v/v heat-inactivated fetal calf serum, 1% v/v penicillin/streptomycin solution, 1%v/v non-essential amino acids and 1%v/v L-glutamine in a humidified incubator gassed with 95% O_2/5% CO_2, at 37°C. Viability was determined by the exclusion of trypan blue with the Vi-CELL™ Series Cell Viability Analyzer (Beckman Coulter). Caco-2 cells were seeded at a density of 500,000 cells/well and grown for 21 days on 12 mm polycarbonate Transwell® supports with a pore size of 0.4 μm, and were fed with fresh media every 2 days (21). Transepithelial electrical resistance (TEER; Ω.cm^2) was determined throughout the experiment using an EVOM® voltohmmeter with chopstick-type electrodes (WPI, UK). Percentage reduction in TEER was measured relative to TEER at the start of the experiment. Monolayer fluxes were measured in HBSS supplemented with HEPES, (25 mM) and glucose (11 mM).

Permeability of Flux Markers across Rat Intestinal Mucosae and Caco-2 Monolayers

Following 20 min equilibration, dilutions of 100 mM CriticalSorb™ stock were made to give final concentrations equivalent to 0.01–1 mM in the 5 mL apical-side chamber of mucosae in the presence of the paracellular markers, [^{14}C]-mannitol (0.1 μCi/mL) and FITC-4000 Da (FD-4; 2.5 mg/mL) (20). For Caco-2 monolayers, a 10x solution was prepared and the same range of concentrations were added to the 0.5 mL apical bath. The flux of the transcellular marker, [^{14}C]-antipyrine (10 mM, 0.1 mM 'hot': 9.9 mM 'cold'), was also assessed (22). Basolateral samples (200 μL) were withdrawn at 20 min intervals for 120 min and replaced with buffer. Samples were mixed with 3 mL of scintillation fluid and analyzed on a liquid scintillation analyzer (Packard Tricarb 2900 TR). The apparent permeability coefficient (Papp,

cm/s) was determined by:

$$P_{app} = \frac{dQ}{dt} \cdot \left(\frac{1}{A \cdot C_0}\right)$$

where dQ/dt is the transport rate (mol/s); A is the surface area (0.63 cm^2 for mucosae; 1.1 cm^2 for Caco-2 monolayers,); C_0 is the initial concentration in the donor compartment (mol/mL).

Transport of Rhodamine-123 in Caco-2 Monolayers and Rat Colonic Mucosae

The flux of the P-gp substrate Rhodamine-123 (Rh-123) was determined in the absorptive (A to B) and secretory direction (B to A) in Caco-2 monolayers and in rat colonic mucosae (23). Rh-123 was dissolved in 70% ethanol, protected from light and stored at 4°C. Rh-123 (10 μM) was added to the donor side at T=0 and receiver samples (200 μL) were removed every 20 min for 120 min and placed into a light protected 96-well plate. Receiver-side samples were replaced with fresh buffer (200 μL; HBSS for Caco-2 or KH for mucosae). CriticalSorb™ (1 μM-1 mM) was added either apically or bilaterally at T=0. Verapamil (10 μM) was used as a positive control for inhibiting Rh-123 secretion via apical membrane-mediated P-gp efflux (24). At 120 min, fluorescence was measured using a spectrofluorimeter with an excitation wavelength of 485 nm and emission wavelength of 530 nm and the Papp (cm/s) determined.

Cytotoxicity Assays in Caco-2 Cells and Mucosae

The viability of Caco-2 cells treated with CriticalSorb™ was determined by both LDH (25) and MTT assays (26) . Cells were seeded at a density of 1×10^5 cells/mL in 96-well plates for 24 h. Fresh DMEM (phenol red-free DMEM for LDH) was applied and allowed to equilibrate before CriticalSorb™ (1 μM–1 mM) or Triton X-100 (0.1%v/v) were added. Plates were then placed into a humidified incubator with 95% O_2/5% CO_2, at 37°C for a 60 min exposure. For the LDH assay, test media (75 μL) was removed (in duplicate) and mixed with assay substrate solution (150 μL) for 30 min (TOX-7). The reaction was stopped using 0.1 M HCL and the formation of the tetrazolium dye was measured spectrophotometrically at 490 nm in a multi-well plate reader. Percent LDH release from cells was measured relative to Triton X-100 (0.1%v/v). The LDH assay was also carried out for CriticalSorb™-treated rat mucosae as previously described (27). Ileal and colonic mucosae were mounted in Ussing chambers for 120 min and samples of apical bathing solution (200 μL) was removed at 0, 60 and 120 min and replaced with fresh KH. Samples were mixed with 200 μL TOX-7 for 30 min and assayed as above. Percent LDH release from mucosae was measured relative to Triton X-100 (10%v/v). For MTT assay, following incubation with CriticalSorb™ or Triton X-100, cells were washed and incubated with fresh medium with MTT (0.5 mg/mL in PBS). Cells were incubated for a further 3 h. The remaining media was removed and DMSO (100 μL) was added to each well and shaken at 250 rpm for 2 min to dissolve the formazan crystals. The resulting absorbance was read at 550 nm on a multi-well plate reader and viability was determined and compared to untreated controls.

Confocal and Light Microscopy

Filter-grown Caco-2 epithelial cell monolayers were treated with CriticalSorb™ (1 mM) for 20 min, washed 3 times with phosphate buffered saline (PBS) and fixed with paraformaldehyde (4%w/v) in PBS for 20 min. The monolayers were then washed 3 times with PBS and permeabilized with Triton X-100 (0.1%v/v) in 5%w/v normal goat serum (NGS) in PBS for 60 min. After an additional 3 washes with PBS, monolayers were stained with primary antibody of mouse anti-claudin-2 (1:200; Zymed Laboratories, USA) in 5% NGS for 60 min, before being washed a further 3 times with PBS. The monolayers were stained with a secondary antibody of Alexa Fluor® 568 anti-mouse IgG (1:200; Zymed Laboratories) and FITC-phalloidin in 5% NGS for 60 min before being washed 3 times with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000) for 10 min. The monolayers were washed with fresh PBS to remove any extra stain. The monolayers were then excised from the Transwell® inserts and mounted onto glass slides with Vectrashield® mounting medium (Vector Laboratories Ltd., UK) and the edges were sealed with clear nail varnish and stored at −20°C. Confocal microscopy was preformed on a Zeiss LSM 510 Meta using LSM5 acquisition software (Carl Zeiss Inc.) with a 543 nm filter (for rhodamine detection), a 488 nm filter (for FITC detection)and a 364 nm filter (for DAPI detection). For light microscopy, rat ileal and rat colonic mucosae were treated apically with CriticalSorb™(10 μM-1 mM) for 120 min. The mucosae were removed from the chambers and placed into 10%v/v buffered formalin for at least 48 h and subsequently embedded in paraffin wax. Tissue sections (5 μm) were cut on a microtome (Leitz 1512; GMI, USA), mounted on adhesive coated slides, and stained with hematoxylin and eosin (H & E) or alcian blue 8GX. The slides were visualized under a light microscope (Labophot-2A; Nikon, Japan) and images taken with a high-resolution camera (Micropublisher 3.3 RTV; QImaging, Canada) and Image-Pro® Plus version 6.3 (Media Cybernetics Inc., USA) acquisition software.

Statistical Analysis

Statistical analysis was carried out using Prism-5® software (GraphPad, USA). Unpaired Student's t-tests and ANOVA were used for single and group comparisons respectively. Results are given as mean ± SEM. A significant difference was considered to be present if $P<0.05$.

RESULTS

CriticalSorb™ Decreases TEER in Caco-2 Cell Monolayers and Intestinal Mucosae

The baseline TEER across Caco-2 cell monolayers was $1900\pm27\ \Omega.cm^2$ ($n=6$) in accordance with literature values (28,29). The apical addition of CriticalSorb™ (1 mM) significantly decreased monolayer TEER over 120 min (Fig. 2a) whereas lower concentrations of CriticalSorb™ did not. Basal rat ileal and colonic mucosal TEER was $46\pm1\ \Omega.cm^2$ ($n=13$) and $139\pm3\ \Omega.cm^2$ ($n=32$) respectively, in accordance with the literature (30,31). Apical CriticalSorb™ even at low concentrations (10 μM–1 mM) also caused significant decreases in TEER across both tissues (Fig. 2b,c).

Criticalsorb™ Increases Papp of [^{14}C]-Mannitol and FD-4 in Caco-2 Cell Monolayers and Intestinal Mucosae

The basal P_{app} of [^{14}C]-mannitol and FD-4 across Caco-2 cell monolayers was $7.6\pm0.2\times10^{-8}$ cm/s ($n=6$) and $1.1\pm0.1\times10^{-8}$ cm/s ($n=12$), respectively, (Fig. 3a,b), in accordance with reported values (28,29). Apical addition of 1 mM CriticalSorb™ significantly increased the permeability of both flux markers, matching the concentration that reduced TEER (Fig. 3a,b). There was little evidence however, of a concentration-dependent effect on fluxes in Caco- 2 cell monolayers. The basal P_{app} of [^{14}C]-mannitol and FD-4 across rat ileal mucosae was $1.2\pm0.1\times10^{-6}$ cm/s ($n=6$) and $1.3\pm0.1\times10^{-6}$ cm/s ($n=8$), respectively (Fig. 3c,d), while respective values across colon were $5.7\pm0.6\times10^{-7}$ cm/s ($n=9$) and $1.2\pm0.2\times10^{-7}$ cm/s ($n=4$) (Fig. 3e,f), in line with reported values (30,31). An apical addition of CriticalSorb™ (10 μM–1 mM) to ileal and colonic mucosae caused a significant increase in the P_{app} of [^{14}C]-mannitol and FD-4 at concentrations of 10 μM and higher (Fig. 3c–f).

Criticalsorb™ Induces F-Actin and Claudin-2 Localization in Caco-2 Cell Monolayers

Caco-2 monolayers exhibited strong FITC staining of F-actin between cells, as well as strong DAPI staining of nuclei

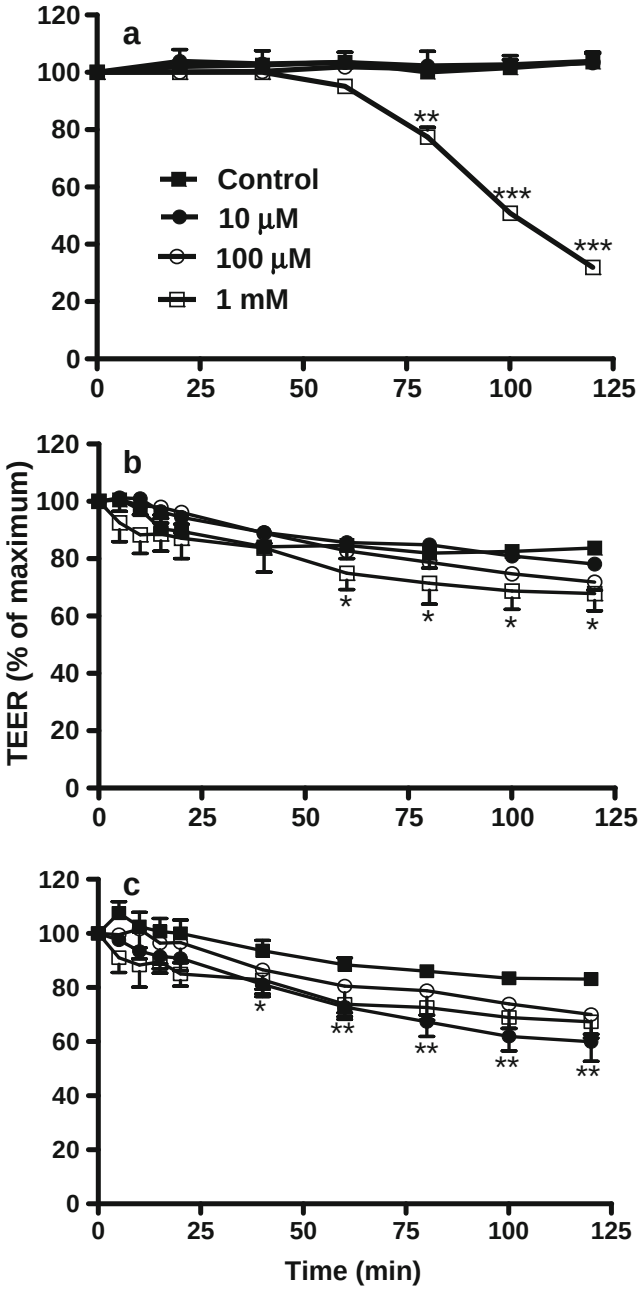

Fig. 2 Reduction in TEER after apical-side addition of CriticalSorb™ (10 μM–1 mM) to (**a**) Caco-2 monolayers, (**b**) rat ileum and (**c**) rat colonic mucosae. *$P<0.05$; **$P<0.01$, ***$P<0.001$, compared to controls ($n=4$, each group).

(Fig. 4a). There was no claudin-2 expression between cells in untreated monolayers (Fig. 4c). Apical CriticalSorb™ (1 mM) caused a change in expression of F-actin between cells, diverting it from the periphery to the cytosol. There were no changes evident in the nuclei (Fig. 4b). CriticalSorb™ caused an increased expression of claudin-2 between some adjoining cells, as denoted by red staining around the circumference (Fig. 4d). This suggests that the expression of tight junction

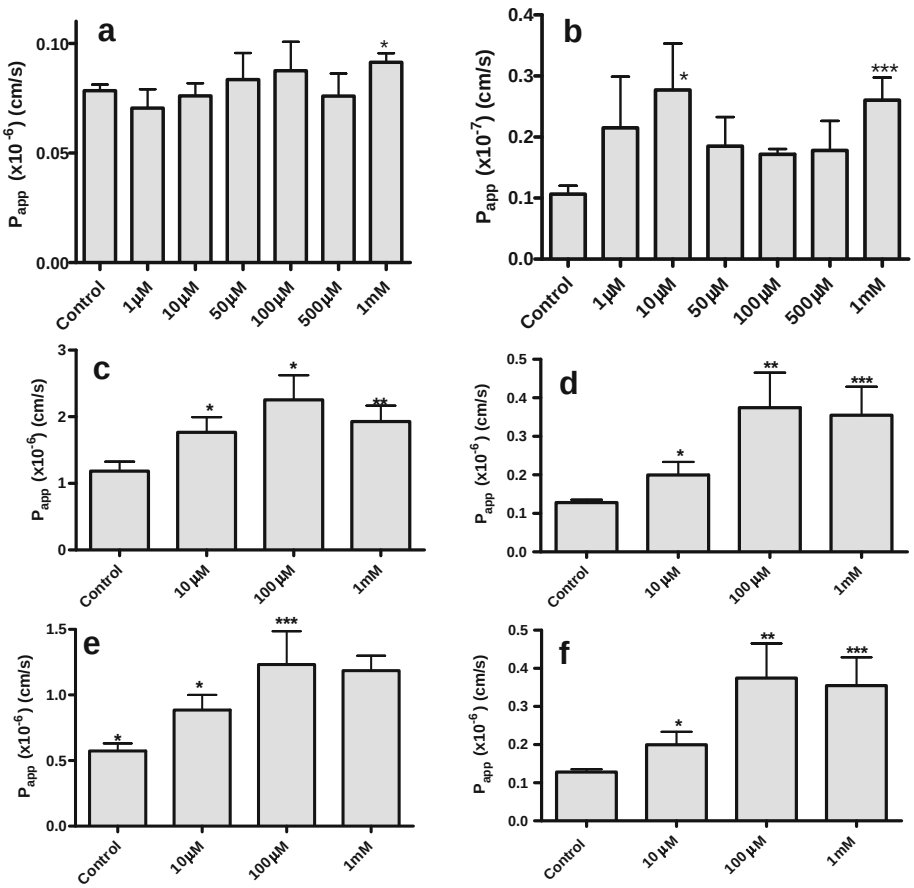

Fig. 3 CriticalSorb™ increases the apical-to-basolateral P_{app} of [^{14}C]-mannitol and FD-4 across (**a**, **b**) Caco-2 monolayers, (**c**, **d**) rat ileum and (**e**, **f**) rat colonic mucosae respectively. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared to untreated controls (n=12 for mannitol and n=6 for FD-4 in Caco-2 respectively; n=4, all other groups).

protein, claudin-2, as well as F-actin in Caco-2 cell monolayers were altered by CriticalSorb™, consistent with a paracellular permeability increase.

CriticalSorb™ Increases Papp of [^{14}C]-Antipyrine in Caco-2 Monolayers and Intestinal Mucosae

The basal P_{app} of the BCS Class I transcellular marker, [^{14}C]-antipyrine across Caco-2 monolayers was $3.6\pm0.1\times10^{-5}$ cm/s (n=4) (Fig. 5a), within the range of previous literature (32). Apical-side addition of CriticalSorb™ (100 µM- 1 mM) increased the P_{app} of [^{14}C]-antipyrine significantly (Fig. 5a), but not to the same extent as that induced by Triton X-100 (0.1%v/v). In the rat ileal and colonic mucosae, the basal P_{app} of [^{14}C]-antipyrine was $2.8\pm0.1\times10^{-5}$ cm/s (n=5) and $3.9\pm0.1\times10^{-5}$ cm/s (n=10) (Fig. 5b,c), respectively, in line with previous literature (33). Apical-side addition of CriticalSorb™ (100 µM–1 mM) caused a significant increase in P_{app} of [^{14}C]-antipyrine across rat ileal (Fig. 5b) and colonic mucosae (Fig. 5c). However, the increases were again significantly less than that induced by Triton X-100 (10%v/v). Triton X-100 elicits a complete loss of the epithelial barrier due to membrane fluidization, therefore the fact that the flux in the presence of CriticalSorb™ was less than that induced by Triton-X-100 would suggest that CriticalSorb™ is not as abrasive, and this was corroborated by histology and cytotoxicity assays (below).

CriticalSorb™ Decreases the Efflux of Rh-123 across Caco-2 Cell Monolayers and Rat Colonic Mucosae

Inhibition of Rh-123 secretory efflux from the basolateral-to-apical side of intestinal epithelia was previously used as a test for P-gp inhibition (34). Data for Caco-2 cell monolayers are shown in Fig. 6a. In summary, the P_{app} of Rh-123 was polarized and indicated the presence of functioning P-gp: $0.1\pm0.1\times10^{-6}$ cm/s, A to B direction; $6.9\pm0.1\times10^{-6}$ cm/s, B to A direction. Bilateral addition of the gold standard P-gp substrate, verapamil (10 µM), caused a small but significant increase in Rh-123 flux in the A to B direction, and a large and significant decrease in flux in the B to A direction. Similar to the effects of verapamil, bilateral addition of CriticalSorb™ (100 µM) significantly increased the P_{app} of Rh-123 in the A to B direction and decreased P_{app} of Rh-123 in the B to A direction, typical of a putative P-gp inhibitor. Data for the colonic tissue is shown in Fig. 6b. In summary, the Rh-123 flux over 120 min in the A to B direction across colonic mucosae was $1.5\pm0.2\times10^{-6}$ cm/s

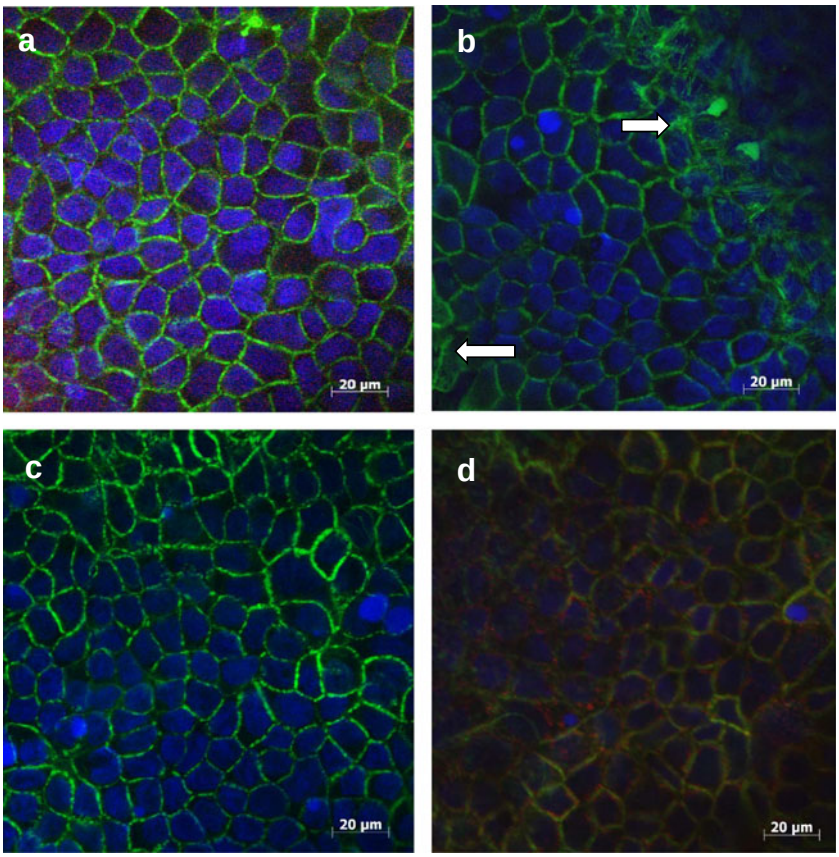

Fig. 4 Confocal microscopy of Caco-2 monolayers. (**a**) Control: F-actin (*green*) and DAPI staining (*blue*); (**b**) CriticalSorb™ (1 mM): F-actin (*green*) and DAPI staining (*blue*)]; *arrows* depict F-actin moving from the periphery to cytosol (**c**) Control: claudin-2 (*red*), F-actin (*green*) and DAPI staining (*blue*); (**d**) CriticalSorb™ (1 mM): claudin-2 (*red*), F-actin (*green*) and DAPI staining (*blue*).

(n=4) and $15.2 \pm 0.2 \times 10^{-6}$ cm/s in the B to A direction, evidence of polarization and functioning P-gp. Addition of verapamil caused a significant decrease in the flux of Rh-123 in the B to A direction, but was without effect on A to B flux. Similarly, the addition of CriticalSorb™ (1 mM) caused a significant decrease in Rh-123 flux in the B to A direction, but had no effect on the A to B flux. In both systems therefore, CriticalSorb™ acts as a P-gp inhibitor, however it is not as potent or efficacious as verapamil. Maximal reduction in B-to-A fluxes in both systems was 50-66% with CriticalSorb™ compared to over 80–90% for verapamil, with the former requiring mM concentrations to do so, compared to low µM concentrations for verapamil.

CriticalSorb™ is Not Cytotoxic to Caco-2 Cells or Rat Intestinal Mucosae: LDH, MTT and Histology

In the MTT assay on Caco-2 cells, viability following a 60 min exposure to CriticalSorb™ (1 µM–1 mM) was above 90%, whereas Triton-X-100 (0.1%v/v) left only 15% alive (Fig. 7a). Even when CriticalSorb™ was exposed to cells for 24 h, a time point of questionable relevance for an oral system, viability to 100 µM was still above 90%, although higher concentrations left only 20% alive (Fig. 7b). In the LDH assay on Caco-2 cells over 60 min, concentrations from 1 to 100 µM were not significantly different from control, and 1 mM only increased release to 32% compared to 20% for controls (Fig. 7c), data that were similar at 24 h (Fig. 7d). In rat ileal and colonic mucosae, while apically-applied CriticalSorb™ (1 mM) showed a slight increase in damage at 60 and 120 min in the LDH assay (Fig. 8a,b), there were no effects on cell death at 10 and 100 µM, the same concentrations which enhanced permeation. These data were confirmed by histological analysis of ileal and colonic mucosae using H & E and alcian blue staining following 120 min in Ussing chambers. Untreated rat ileal mucosae after 120 min showed an intact epithelium and actively secreting goblet cells (Fig. 9a, b). Increasing concentrations of apical CriticalSorb™ (10 µM-1 mM) induced formation of some cellular debris and edema, as well as increased amount of mucus secretion, although overall structures were still well defined (Fig. 9c–h). Untreated rat colonic mucosae, mounted in Ussing chambers for a 120 min, showed an intact epithelium (Fig. 9i, j). Apical CriticalSorb™-treated rat colonic mucosae also caused some edema and mucus secretion, but there was no loss to the epithelial barrier (Fig. 9l–p). This indicates that CriticalSorb™ concentrations up to 1 mM which enhance paracellular and

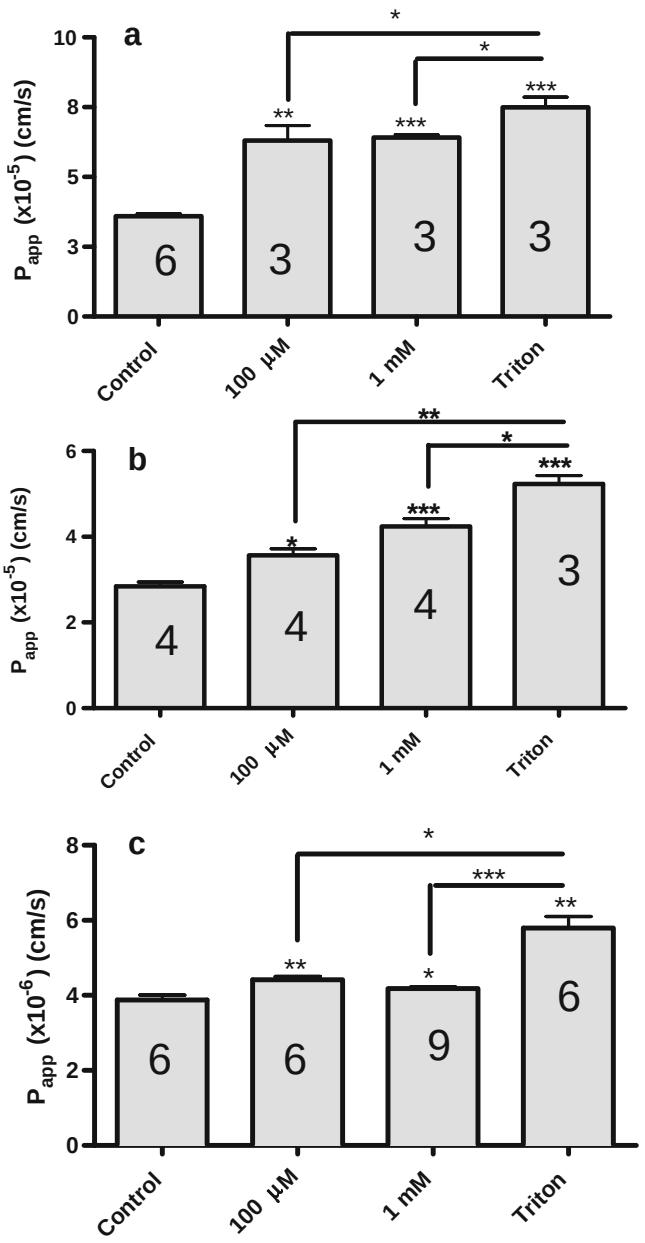

Fig. 5 CriticalSorb™ increases the apical-to-basolateral P_{app} of [^{14}C]-antipyrine across (**a**) Caco-2 monolayers, (**b**) rat ileum and (**c**) rat colonic mucosae. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$, compared to both control (astrices directly above error bars) and to Triton (Triton X-100, 0.1%v/v). N numbers are on bar graph.

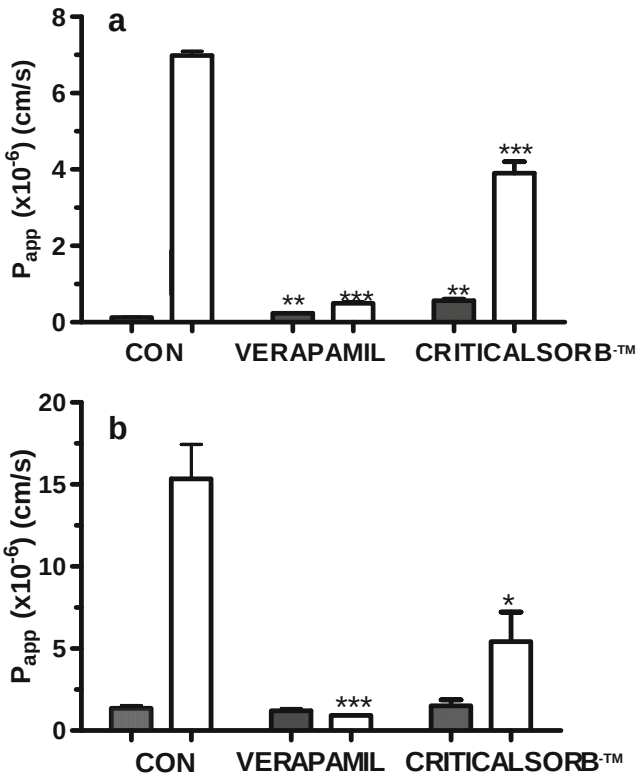

Fig. 6 The P_{app} of Rh-123 in the A-to-B (■) and B-to-A (□) directions across Caco-2 monolayers and rat colonic mucosae. (**a**) Caco-2 with verapamil (10 μM) or CriticalSorb™ (100 μM). *** $P < 0.001$ compared to Rh-123 control flux in B-to-A direction; ** $P < 0.01$ compared to Rh-123 control flux in A-to-B direction. (**b**) Colon: with verapamil or CriticalSorb™. ***P < 0.001, *P < 0.05 compared to Rh-123 control flux in B-to-A direction. (n=3-4, per group).

transcellular permeability do not cause major substantial damage in either Caco-2 monolayers, rat ileal or colonic mucosae after 1–2 h exposures.

DISCUSSION

Solutol®HS15 is a non-ionic amphiphilic surfactant excipient and emulsifier used primarily as a solubiliser for poorly soluble drugs. It is associated with very low toxicity and is a component of several marketed parenteral products outside the US. Its original development by BASF was an attempt to reduce the generation of sensitizing levels of histamine seen in dog studies with Cremophor® EL and RH 40; thus the castor oil was replaced with 12- hydroxystearic acid and combined with polyethylene glycol 30 to yield the new excipient. A drug master file for Solutol®HS15 was filed with the FDA, resulting in a USP monograph (35). Recent preclinical nasal studies in conscious rats with the CriticalSorb™ formulations containing significant levels of Solutol®H15 resulted in dramatically increased nasal bioavailabilities of 100% (0–1 h) and 50% (0–2 h) with respect to sub-cutaneous administration of insulin and hGH respectively. The increase in bioavailability was not associated with histological changes or damage to rodent nares (16). The discovery of permeation enhancing effects on nasal epithelia led to a recent Phase I trial by Critical Pharmaceuticals for a nasal hGH formulation, for which positive interim efficacy and tolerability data was recently reported (15). Here, we investigated if the effects seen after application to the nasal epithelia for CriticalSorb™ could be repeated for

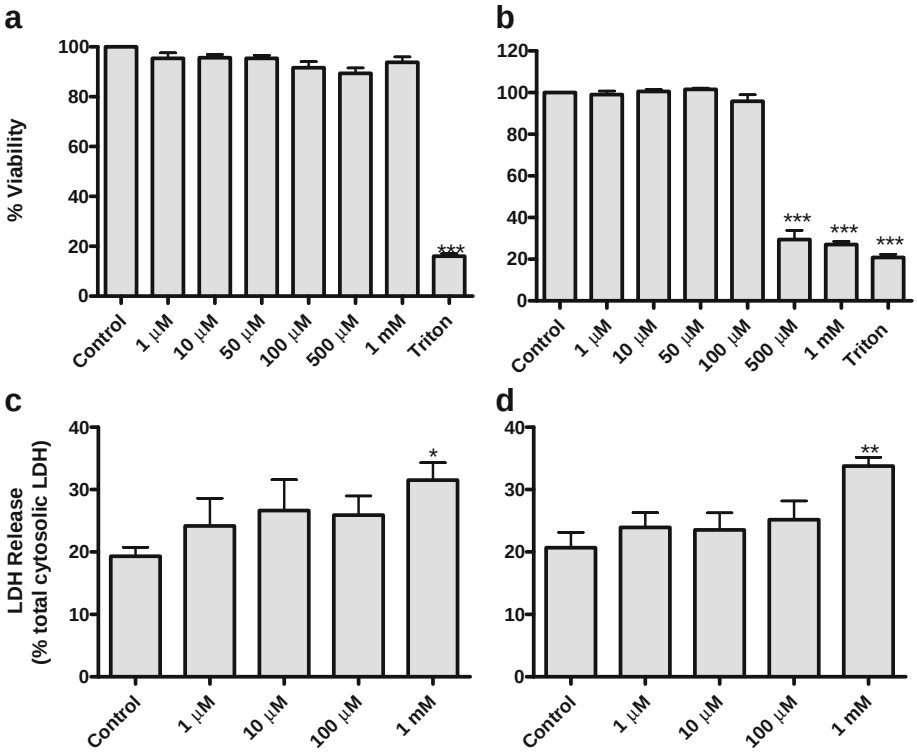

Fig. 7 Effect of CriticalSorb™ on cell viability by MTT and LDH in Caco-2 cells. (**a**) MTT, 60 min and (**b**) MTT, 24 h; 24 h. Positive control: Triton-X-100 (0.1%v/v); (**c**) LDH, 60 min and (**d**) LDH, 24 h; values are expressed as a percentage of the 100% release induced by Triton X-100. *$P<0.05$; **$P<0.01$, ***$P<0.001$, compared to medium control; $N=3$–4 in each group.

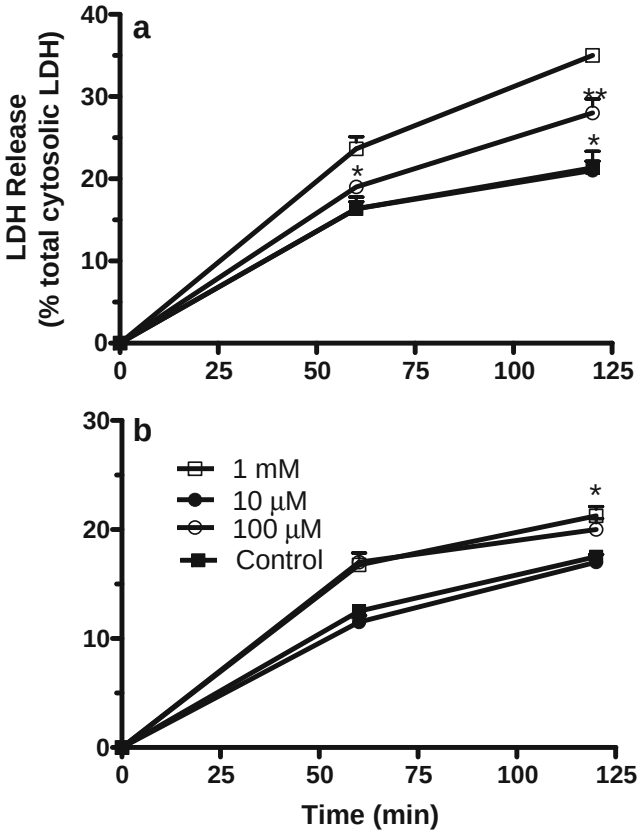

Fig. 8 LDH release from rat mucosae following 120 min incubation with apical CriticalSorb™. (**a**) Ileal and (**b**) colon mucosae. Values are expressed as the percentage of the 100% release induced by Triton X-100 (0.1%v/v). *$P<0.05$; **$P<0.01$, compared to control; $N=3$–4 in each group.

application to intestinal tissue, since there is particular interest in enabling permeation of peptides and proteins.

The current study showed that the key Solutol® HS15 component of CriticalSorb™ acts as a permeation enhancer across intestinal epithelia in the absence of drug solubilization. Using a selection of hydrophilic and hydrophobic markers for different transport routes across three different types of intestinal epithelia (Caco-2 cell monolayers, isolated rat intestinal ileum and colonic mucosae), the present data convincingly show that CriticalSorb™ reduced TEER and increased fluxes of the paracellular markers, FD-4 and mannitol, as well as the transcellular marker, antipyrine. Effects on fluxes were present in the absence of cytotoxicity and, moreover, specific induction of expression of the tight junction associated protein, claudin-2, was detected in Caco-2 monolayers. In addition, in common with a number of established oral excipients including Cremophor®-EL (19), Tween-80 and PEG-400 (36), we confirmed in three different intestinal models that CriticalSorb™ is a rather weak inhibitor of intestinal P-gp.

To date, Solutol®HS15 has been typically used as an oil-phase surfactant in oral self-emulsifying drug delivery systems (SEDDS) formulations for poorly soluble drugs, typical examples being Coenzyme Q (37) and dipyridamole (38). Solutol®HS15 was also incorporated as the key component in a self-despersing oral formulation of cyclosporine, which gave a 2-fold higher bioavailability as compared to micronized cyclosporine powder in rats (39). In this instance the major factor for improving CsA bioavailability was again an

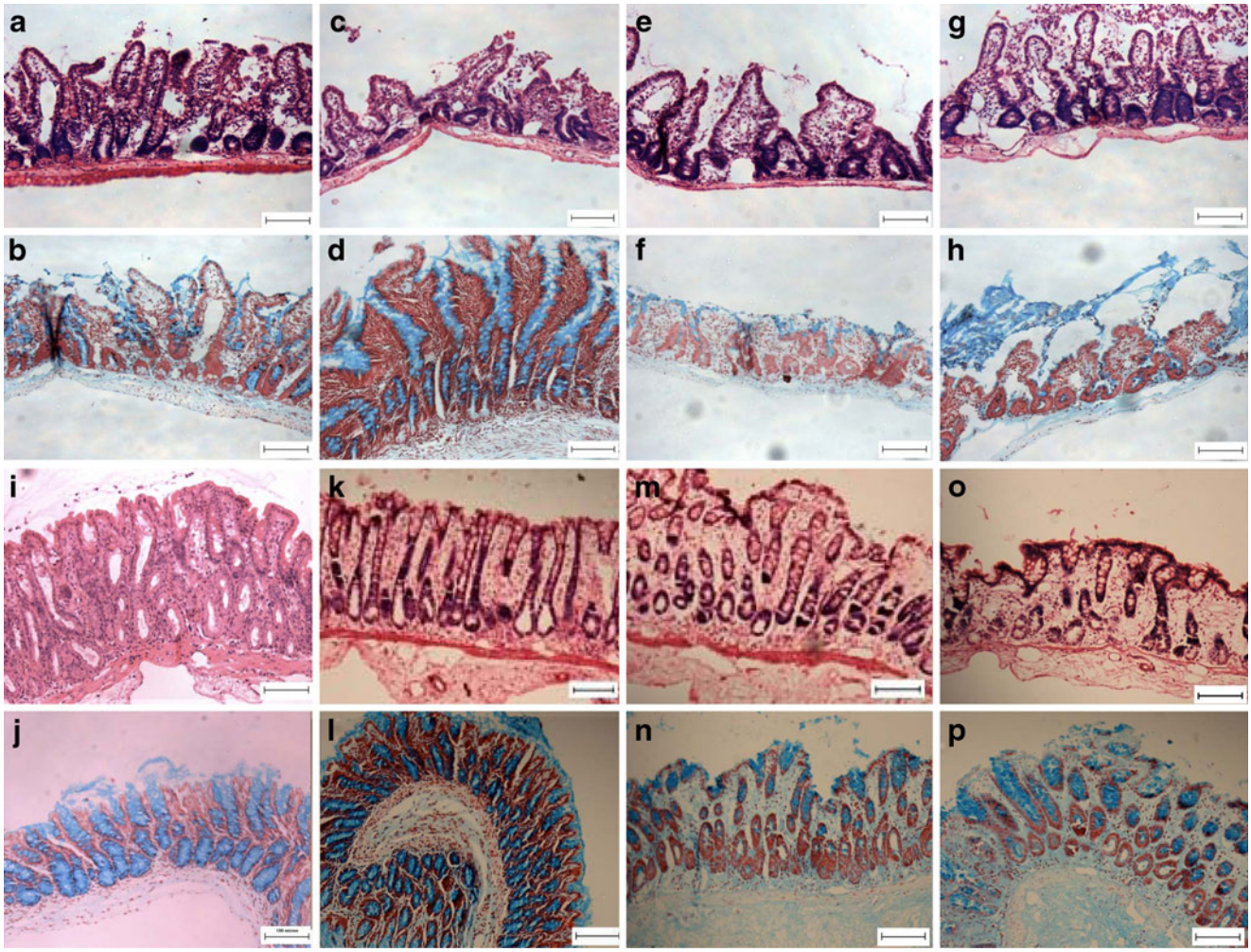

Fig. 9 H & E and alcian blue-stained light micrographs of rat intestinal mucosae mounted in Ussing chambers and exposed to CriticalSorb™ for 120 min. (**a**, **b**) Ileum control; (**c**, **d**) 10 μM, ileum; (**e**, **f**) 100 μM, ileum; (**g**, **h**) 1 mM, ileum. (**h**, **i**) Colon control; (**j**, **k**) 10 μM, colon; (**l**, **m**) 100 μM, colon; (**o**, **p**) 1 mM, colon. Horizontal bars denote 10μm.

improvement in its aqueous solubility since the peptide is a BCS Class II molecule. Recent data suggests however that microemulsions incorporating Solutol®HS15 as one of the surfactants can also be mixed with a poorly permeable BCS Class III peptide, PTH (1–34), to form a clear homogenous solution which protects the peptide from intestinal enzymatic degradation and increases oral bioavailability from a very low baseline up to an impressive 12% in rats (40). As Solutol®HS15 was one of the components in the microemulsion that also included the established inactive excipients, Labrasol® and D-α-tocopheryl acetate, our data would support the thesis that it is likely that some proportion of the increased PTH (1–34) permeation seen in that study was due to effects of Solutol®HS15 on both paracellular and transcellular pathways. Amphiphilic surfactants that increase paracellular transport tend to have a number of features in common including polyethylene glycol esterification, high hydrophilic-lipophilic balance and the presence of long chain carbons. Other studies also show effects of the agent on permeability as well as on aqueous solubility: Solutol ®HS15 (1.5 mM) increased permeability of both paclitaxel and mannitol across Caco-2 cell monolayers in the absence of LDH release and accompanied by some inhibition of P-gp efflux in relation to the former; however effects on absorptive fluxes were modest (<2 fold) and comparatively less than other surfactants tested in parallel (11). Our current data therefore concurs with that in respect of increases in mannitol flux across Caco-2 cell monolayers and then extends these findings using isolated rat intestinal tissue.

It is important to assess CriticalSorb™ in comparison to gold standard intestinal permeation enhancers so that it can be benchmarked appropriately. In similar studies using isolated rat intestinal mucosae, top ranked enhancers, sodium caprate (10 mM) and tetradecyl maltoside (0.1%) yielded 7–10 fold increases in fluxes of mannitol and FD-4 (20,41). Unlike CriticalSorb™, these substantial permeability increases were associated with temporary mild membrane perturbations likely caused

by micelle-induced plasma membrane lipid extraction. From a toxicity perspective, the MTT, LDH and tissue histology data support the body of extensive toxicology in animals showing the CriticalSorb™ is amongst the mildest of non-ionic surfactants. Permeability effects of CriticalSorb™ were therefore clearly dissociated from membrane damage, although it should be emphasized that this was at the level of single as against repeated intestinal mucosal exposures to the excipient.

CriticalSorb™ does not cause dramatic increases in intestinal permeation *in vitro* and levels of induced permeation seem to be on a par with moderately effective enhancers such as melittin (20). In view of the exceptional efficacy of CriticalSorb™ as a nasal enhancer *in vivo*, it is reasonable to conclude that it is somewhat less effective in the intestine than in the nasal cavity. Since Solutol®HS15 is quite stable and is used widely as an excipient for poorly soluble small molecules, this is a potential advantage compared to less robust enhancer candidates based on peptide motifs. Definitive conclusions can only be made however, when studies are carried out using rat intestinal instillations and perfusions of peptides and CriticalSorb™ instead of model drugs.

In relation to P-gp inhibition, CriticalSorb™ reversed multidrug resistance in human tumour cells *in vitro* in μM concentrations with similar potency to verapamil (12,42). CriticalSorb™ blocked the efflux of Rh-123 across Caco-2 and rat colon, tissues known to express substantial quantities of P-gp resulting in polarized fluxes. We note that CriticalSorb™ and verapamil also increased *absorptive* Rh-123 flux in Caco-2 monolayers, but not in rat colon mucosae. While the reason for the difference is not apparent, it has been argued that while secretory efflux of Rh-123 is specifically governed by apical membrane-located P-gp in intestinal epithelia, absorptive flux of Rh-123 is via tight junctions (43). Hence the key evidence for a candidate P-gp inhibitor is prevention of efflux in intestinal epithelia and this was found in both tissue types. In contrast to previous studies on skin tumour cells however, CriticalSorb™ was not as potent or efficacious as verapamil in intestinal epithelia and required mM concentrations to block the efflux. Part of the discrepancy may be due to heterogeneity of active fraction compositions between different mixtures of Solutol®HS15 (19) or, more likely, different sensitivities to Solutol®HS15 in different tissues presented by different levels of reductionism. In summary, the data does not suggest that CriticalSorb™ is a potent P-gp inhibitor in the intestine and this conclusion is supported by studies of SolutolHS15 -loaded indinavir nanocapsules where the increased distribution of the active across the blood brain barrier and testes of mice was ascribed to mechanisms other than P-gp blockade by Solutol®HS15 (44).

CONCLUSIONS

In addition to its well-known solubilizing actions, Solutol®HS15 in the format of CriticalSorb™ has the capacity to increase paracellular and transcellular flux across intestinal epithelia in the absence of *in vitro* cytotoxicity. It may therefore have potential as an excipient for oral formulations of impermeable molecules in addition to its current status in which substantial permeation of hGH has been demonstrated from a CriticalSorb™ nasal formulation. The data support the hypothesis that, similar to many non-ionic surfactants, permeability enhancement across a range of epithelia is a feature of this class of agents. The innocuous actions of CriticalSorb™ on intestinal epithelia are further supported by rather weak P-gp Inhibition.

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