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Safety and efficacy of sodium caprate in promoting oral drug absorption: from *in vitro* to the clinic

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Abstract

A major challenge in oral drug delivery is the development of novel dosage forms to promote absorption of poorly permeable Class III drugs across the intestinal epithelium. To date, no absorption promoter has been approved in a formulation specifically designed for oral delivery of Class III molecules. Promoters that are designated safe for human consumption have been licensed for use in a recently approved buccal insulin spray delivery system and also for many years as part of an ampicillin rectal suppository. Unlike buccal and rectal delivery, oral formulations containing absorption promoters have the additional technical hurdle whereby the promoter and payload must be co-released in high concentrations at the small intestinal epithelium in order to generate significant but rapidly reversible increases in permeability. The most advanced promoter in the clinic is the medium chain fatty acid (MCFA), sodium caprate (C_{10}) , a compound already approved as a direct food additive. We discuss how it has evolved to a matrix tablet format suitable for administration to humans under the headings of mechanism of action at the cellular and tissue level and *in vitro* and *in vivo* efficacy and safety studies. In specific clinical examples, we review how C_{10}-based formulations are being tested for oral delivery of bisphosphonates using Gastro Intestinal Permeation Enhancement Technology, GIPET® (Merrion Pharmaceuticals, Ireland) and in a related solid dose format for anti-sense oligonucleotides (ISIS Pharmaceuticals, USA).

Keywords: Oral drug delivery, Sodium caprate (C_{10}), absorption promoter, drug delivery platforms, clinical trials, oral formulation, drug delivery systems.

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[1] Introduction

The number of drugs emerging from R & D programmes as lead candidates that are poorly absorbed following oral administration is increasing, the majority of which are delivered by injection at considerable patient inconvenience. Biotech drugs represent a growing proportion of drugs in preclinical development and these have inherently low oral bioavailability (F) [1]. The delivery route has a significant impact on the commercial success of therapeutics for long-term indications and the potential market of selected biotech drugs may not have been maximized due to the requirement for repeated injections [2, 3]. Nasal formulations have largely superseded subcutaneous (s.c.) injections for the peptide calcitonin, but there are still issues of rhinitis and local tolerance that reduce patient compliance and hence an oral delivery system would be preferable [4]. In addition, the first pulmonary formulation of insulin (Exubera®, Pfizer Ltd, USA) was withdrawn due to poor patient uptake, possible side-effects, an unattractive device and a concomitant requirement for insulin injections [5]. One of the major challenges in biopharmaceutical development therefore continues to be the need for effective oral delivery systems.

The biopharmaceutics classification system (BSC) categorizes soluble drugs with poor intestinal permeability as Class III drugs [6]. Candidates comprise peptides, proteins, nucleic acid therapeutics and polysaccharides as well as some conventional organic molecules. Unlike most lipophilic agents, hydrophilic molecules are generally not passively absorbed across intestinal epithelia, largely due to restricted permeation across the brush border and the basolateral membranes (Fig. 1). Absorptive flux of small

hydrophilic molecules including valacyclovir, bestatin and cephalexin may occur to some extent either via carrier-mediated transporters on epithelial cell apical membranes (primarily hPEPT1 [7]) or alternatively by paracellular flux via tight junctions (TJs) [8]. TJs form a barrier to the uncontrolled absorption of noxious luminal antigens (gate function), and maintain epithelial polarity (fence function) [8, 9]. In general, the TJ consists of a restrictive pathway (shunt) with a sharp molecular size cut off, and a second unrestrictive pathway (small pore) that permits paracellular permeation of molecules of radii <4.0 Å [10, 11]. Depending on the intestinal region, TJ pore sizes range from 6-22 Å, sufficient to permit mannitol (6.7 Å) and EDTA (10.8 Å) to permeate to an extent, whereas the passage of inulin (30-40 Å) and fluorescent-dextran 4kDa (FD-4, 26 Å) is essentially impeded [12-16].

A number of approaches have been used to promote oral delivery of Class III drugs (Fig. 1). One of the simplest technical approaches to increasing oral bioavailability is the use of intestinal absorption promoters. Study of absorption promoters began in the 1960s when EDTA was shown to increase absorption of heparin in rats and dogs [17]. Since then, there are numerous reports of epithelial-permeating activity by a number of dietary agents, surfactants and polymers, some of which have 'generally recognized as safe' (GRAS) status as food additives. Recently, more sophisticated TJ modulators have emerged in *in vitro* and preclinical studies arising from a greater understanding of the structure and function of TJs [18-20]. The candidate molecules that are to be delivered orally using absorption promoters should have a sufficiently wide therapeutic index in order to cater for the increased variance in F between individual subjects normally seen in

clinical studies. It also helps if the drug is relatively inexpensive and is of high potency since oral absorption will be significantly reduced when co-formulated with even the most promising delivery technology. To date however, there are only a selected number of intestinal promoters licensed for use as excipients in the delivery of poorly-absorbed drugs, particularly for Class IV drugs. While most of these approved excipients were designed to improve solubility, some increase transcellular permeability (e.g. macrogol-8 glyceride (Labrasol®, Gattefosse Corp., France [21]). Registration of products containing enhancers has however, not occurred to the extent that one might expect, given the many convincing *in vitro* and preclinical reports. Some valid concerns about the development of enhancer-containing products relate to the known direct intestinal epithelial toxicity induced by many promoters (e.g. surfactants, ethylenediaminetetraacetic acid (EDTA), bacterial-derived toxins and unknown chemical entities), while unresolved issues pertain to the potential for by-stander pathogen and toxin absorption through the reversibly weakening of the gut barrier on a repeated basis.

The medium chain fatty acid (MCFA) promoter, sodium caprate (C_{10}), is a both a food additive and a component of a rectal suppository formerly marketed in Sweden (Doktacillin®, Meda, Solna, formerly marketed by AstraZeneca, Södertälje [22]) and in Japan (Kyoto Pharmaceutical Industries, Ltd, Kyoto [23]). It is currently in clinical trials as a key component of several proprietary oral formulations [24-26]. The nature of its mechanism of action, efficacy, and the possibility of inducing toxicity are of primary interest in commercialization of formulations based on this technology. Here, we focus on the development and current status of C_{10} in formulations designed to increase oral

bioavailability (F) in the context of other absorption- promoting technologies and alternative approaches.

[1.1] Alternative approaches to delivery of poorly permeable drugs

There are currently two peptides licensed for use by the oral route. These include cyclosporin (Neoral®, Novartis, Switzerland), which is delivered in a solubilising micro-emulsion. Its oral F of approximately 30% can be explained in part by the unique physicochemical characteristics of the cyclic undecapeptide [27]. Desmopressin (DDAVP®, Sanofi-Aventis, France) is a potent vasopressin analogue that is delivered orally, despite its very low F (0.1%) [28]. Successful approaches to overcoming poor intestinal permeability have also focused on prodrugs, inactive drug precursors with greater permeability across the intestinal epithelium than the active [7, 29-31]. Once absorbed across the intestinal epithelium the prodrug is hydrolytically or enzymatically converted to active drug. The most common prodrugs have a moiety that increase drug lipophilicity thereby promoting passive transcellular diffusion (e.g. enalapril, pivampicillin) or a recognition ligand that enables the drug to be shuttled across the epithelium on an epithelial transporter (e.g. hPEPT1 for valaciclovir and midodrine) (Fig. 1). Although prodrugs have been effective for small organic drugs and some short chain peptides, it has been less successful for macromolecules and longer chain peptides.

Conjugating biotech drugs to polymers that increase transmucosal permeability and stability is also a useful approach. Fatty acid conjugates of calcitonin have been synthesised and they demonstrate greater absorption and stability [32]. Furthermore,

direct site-specific PEGylation to the lysine-18 of salmon calcitonin (sCT) increased peptide stability and led to decreased serum calcium levels upon intra-duodenal instillation in rats [33]. Biotinylation of sCT has been shown to increase transmucosal flux across Caco-2 monolayers through targeting of apical membrane biotin receptors [34]. An alkylated PEGylated amphiphilic insulin conjugate (HIM-2, Biocon Corp., India) increased oral F of insulin in dogs [35] and has reached Phase II clinical studies [36]. An oral amphiphilic calcitonin conjugate is also under investigation using similar technology [37]. Conjugation of insulin to vitamin B_{12} for receptor-mediated delivery can also lead to increased absorption of insulin in diabetic rats [38], although there are receptor capacity-related issues that may ultimately limit efficacy in man.

Despite encouraging data from peptide conjugations, direct chemical modification is molecule-specific. An attractive alternative is the use of oral drug delivery platforms that do not involve new chemical entities and which can be fine-tuned to apply to a range of impermeable drugs. Mucoadhesive polymers that prolong the contact time between drug and the intestinal epithelium can create a steep concentration gradient to drive passive absorption [39, 40]. Thiomers are an interesting group of mucoadhesives that have shown promise in animal models. For example, improved oral F of low molecular weight heparin and insulin has been achieved in rodents with thiolated polycarbophil formulations [41, 42]. Use of mucoadhesives in the gastrointestinal (GI) tract may however, be problematic because of the high rate of mucus turnover and the large amount of competing mucus in the intestinal lumen [40, 43]. In contrast, mucoadhesion is employed in successful buccal delivery systems including glyceryl trinitrate (Suscard®,

Forest Laboratories, USA) and miconazole (Lauriad®, BioAlliance Pharma, France) [44]. Alternatively, the use of nanoparticles comprising biocompatible polymers (e.g. chitosan, polylactide-co-glycolide, starch and glucans) can protect cargoes from GI proteases, increase GI retention and promote absorption across gut associated lymphoid tissue (GALT) and to a lesser extent, enterocytes [45-47]. Most data on nanoparticle absorption from rodent models suggest that M cells in the follicle-associated epithelium of Peyer's patches (PP) are the favored site of uptake for particles of diameter 500nm-1000nm [45]. Given the paucity of M cells in the GI tract of adults, the relevance of PP uptake remains controversial and, for example, convincing oral vaccine data using nanoparticles in man is lacking [48, 49]. Recent *in vitro* data suggests that particle uptake by enterocytes can be increased if interaction with the mucous layer can be overcome using a coating of low molecular weight 'non-stick' poly ethylene glycol (PEG) [50], the opposite to mucoadhesion.

Targeted nanoparticles can be created by attaching surface ligands to stimulate receptor-mediated transport of particle-entrapped payload and, unlike the direct 'payload conjugation-to-ligand' approach, this may have greater potential to deliver a greater ratio of drug per transporter to compensate for lower receptor numbers or transport capacity [51]. Targeted particle systems for oral delivery unfortunately require complex synthetic and manufacturing processes and rely on unpredictable translation of data from rodent models to man in respect of differences in GI physiology and variable receptor expression. Simpler mixing and blending nanoparticulate drug formats have, however, been very successful in oral delivery of insoluble Class II drugs (e.g. NanoCrystal®,

Elan, Ireland) [52], where permeability is not the issue. It is possible that this technology may also be adapted for poorly permeable peptides since there are surfactants can be adapted into the process [53].

[1.2] Intestinal absorption promoters

A large number of well-known substances have been shown to alter intestinal permeability and these range from spices and fatty foods [54, 55], alcohol [56] and drugs [57] to bacterial toxins [58]. Increased intestinal permeability is also associated with inflammatory bowel disease [59] and even strenuous exercise [60]. The majority of absorption promoters tested in cultured intestinal epithelial models have not been tested in man due to inherent toxicity. In any case, only a very small number of drug delivery platforms (of which oral absorption promoters are a subset) have advanced to clinical evaluation [61]. Amongst initial pre-clinical investigations of agents that did not proceed to the clinic are the macrocyclic fungal metabolites, cytochalasins, which increase paracellular permeability through contraction of the perijunctional ring of actin and myosin II (PAMR) causing displacement of TJ proteins [62]. The calcium chelator, ethylene glycol tetraacetic acid (EGTA), increases gut permeability via myosin light chain kinase (MLCK)-dependent dilation of the PAMR [63]. Similarly, some detergent surfactants including sodium dodecyl sulphate (SDS) and Triton-X-100 increase transmucosal drug absorption by destruction of the mucosal surface and exfoliation of epithelia [64].

Microbial toxins also increase paracellular permeability across intestinal epithelial TJs, although they are unlikely candidates for oral drug delivery technology in their native form. Examples include *Zonula occludens* toxin (Zot), a virulence factor in diarrhoea associated with strains of *Vibrio cholera* [58] and *Clostridium perfringens* enterotoxin (CPE), which can cause necrosis and desquamation of the epithelial surface of human ileal mucosae [65]. Structural analogues of Zot and CPE are members of a new generation of promoters that target TJ proteins [18-20, 66-68]. A review of the patent literature reveals a vast number of peptide based promoters that target the paracellular pathway [20]. These promoters offer greater specificity for the paracellular pathway and may offer reduced cytotoxicity compared to many surfactants, but their safety and efficacy in man has yet to be established. It is not yet clear whether transiently-modulating TJs to increase drug absorption (in the absence of effects on transcellular pathways) will increase oral F to an acceptable level in man, since the paracellular pathway comprises only 0.1% of the surface area of the intestinal epithelium, but it may still be a relevant permeation route for selected potent low molecular weight molecules.

One of the most advanced carrier technologies in clinical trials based on absorption promotion is Eligen® (Emisphere Technologies, New Jersey, USA). The proposed mechanism for these delivery agents is that they increase transcellular drug absorption via non-covalent linkage to the carrier [69], although there is ongoing controversy over the thermodynamic aspects of the interaction. Given the structural attributes, it is still likely they act as mild surfactants on the epithelium. An acetylated amino acid carrier, N-[8]-(2-hydroxybenzoyl)amino] caprylate (SNAC), increased oral F of a range of poorly

permeable cargoes in human studies [2, 69-74]. Issues for this and other absorption promoter technologies that must be addressed are large intra-subject variability in efficacy, the large ratio of carrier: active, and the high dosing frequency that would be required. A number of oral peptide proprietary formulations are also in the clinic based on enteric-coated capsules containing promoters that are GRAS excipients (Axcess^{-TM} Technology, Bone Medical, Australia [75]). This platform has been used to deliver insulin (Capsulin®) and calcitonin (Capsitonin®) in Phase II clinical studies. Hydroance Technology^{TM} by Lipocine Inc (USA) has constituents including a controlled release system with bile acid/salt and a mixture of hydrophilic and hydrophobic surfactants [76]. Pre-clinical studies in rat, porcine and primate models demonstrated increased absorption of both low molecular weight heparin (LMWH) (5kDa) and a peptide hormone (3.5 kDa). On the polymer side, soluble trimethylated chitosan appears to be a promising absorption promoter and/or vaccine adjuvant in preclinical research as it could offer peptide protection and to aid permeation when presented in a particle format (reviewed [42, 77-79]). Approved in some markets, albeit for buccal delivery, is Oralin® (Generex Biotech, Canada), a formulation for the delivery of insulin which promotes absorption via a microfine mixed micelle spray containing GRAS surfactants and bile salts (RapidMist®, Generex Biotech, Canada) [80].

[2] C_{10}

C_{10} is the sodium salt of the aliphatic saturated 10-carbon MCFA, capric acid, also known as sodium decanoate (or the sodium salt of decanoic acid). Capric acid is present in dairy products, particularly milk, where it constitutes a significant proportion of the fatty acid

content. Percentage levels of the total fatty acid content in mammalian milk are: trace amounts in rats, 1-3% in humans and cows, 9% in sheep, 8% in goats and 20% in rabbits [81, 82]. The approximate concentration of capric acid in human and cow milk can therefore be estimated to be as high as 0.2mM [82, 83]. Importantly, this concentration is still 50-to-1000 fold lower than that required to increase drug permeability. Capric acid is also present in a number of oils including coconut oil (4.5-9.7%), palm kernel oil (7-14%), bay tree oil (37%) and elm seed oil (50%) [81]. The LD_{50} of capric acid following acute oral gavage to rats was 3.7g/kg [84, 85]. Importantly, long term dietary exposure of rats to capric acid added to rice (100g /kg rice) with an approximate daily intake of 500mg /kg rat weight for 150 days resulted in no observable changes in stomach morphology [86]. C_{10} is approved by the FDA as a direct food additive for human consumption [87, 88]. Furthermore, when reviewed by the FAO/WHO Joint Expert Committee on Food Additives, C_{10} was not limited to a specific allowable daily intake because it was judged that the presence in food would have no impact on human health [87, 88].

[2.1] Intestinal absorption-promoting capacity of C_{10}: cultured human intestinal epithelial monolayers, isolated intestinal mucosae and animal models

The ability of C_{10} to facilitate rectal absorption was first discovered over 25 years ago [89-91]. Rectal formulations containing C_{10} increased the absorption of a range of β-lactam antibiotics in rodent, dog and human studies [89, 92]. Since the initial studies of rectally-administered C_{10} in 1982, the promoter has since been assessed extensively with a wide range of co-administered poorly permeable drugs in every accepted small- and

large intestinal delivery screening system. These include intestinal epithelial cell monolayers (Table I), isolated animal and human intestinal mucosae (Table II), *in situ* gut perfusions and intestinal instillations (Table III), and extensive animal (Table III) and human studies (Table IV).The increase in drug absorption one observes with C_{10} is dependent on the animal species and on the model used. For example, delivery of the same test solution in three rat models demonstrated an enhancement in the following order: jejunal closed loop > anesthetized instillation >catheter intubation to conscious rats (Personal communication, Tillman L.G., ISIS Pharmaceuticals, USA). *In vivo* model-specific variables include the type of surgery, extent of tissue damage, the damage/repair cascade, the type and rate of delivery of anesthetic and its effect on water absorption and secretion. Therefore, it is important to consider the limitations of the models used to evaluate C_{10} in order to make an informed assessment.

C_{10} increases the flux of many different types of poorly permeable agents across intestinal epithelia *in vitro*, including antibiotics [93], heparin [94] and recombinant EGF [95]. The concentration of C_{10} required to increase the flux of paracellular markers across Caco-2 monolayers is 10-13mM, close to its reported critical micelle concentration (CMC) in HBSS [96, 97]. In parallel, it causes a rapid reversible concentration-dependent reduction in transepithelial electrical resistance (TEER) values across Caco-2 monolayers (e.g. [96, 98, 99] and Table I). The TEER values of Caco-2 monolayers do not recover after extended exposure periods or from higher concentrations of C_{10} [96, 98, 100, 101]. Still, the relevance of exposing monolayers to C_{10} for long exposure times is questionable, since it is rapidly absorbed *in vivo*. Isolated intestinal mucosa mounted in

Ussing chambers permit comparison between effects of permeation enhancers on different regions of the intestine, thus TEER and flux changes similar to that seen in Caco-2 were noted in jejunal, ileal and colonic mucosae from a range of species upon exposure to C_{10}. In tissue mucosae, C_{10} decreased TEER with a concomitant increase in flux of poorly permeable markers including phenol red [102], poly-sucrose [103] and a range of FITC-dextrans [104] (Table II). While high concentrations of C_{10} (>13mM) invariably lead to greater enhancement of fluxes of paracellular markers in Caco-2 monolayers and isolated intestinal mucosae (Table I and II), conclusions on mechanisms of action and of the presence cytotoxicity become rather irrelevant. Similar to Caco-2 results, the reduction in TEER caused by 10-15mM C_{10} in *human* colonic mucosae was recoverable upon washout [105], as were the promoting effects on paracellular flux [11].

Despite significantly increasing permeability across *in vitro* and *ex vivo* intestinal models, it is worth noting that the capacity of C_{10} to increase the Papp using these models does not always permit the conclusion that there will be a significantly absorbed fraction *in vivo*. For example, the promoter increased the flux of FD70 across isolated rat colonic mucosae by 44-fold at a concentration of 10mM, but the actual resulting Papp value of 10^{-8} cm/s was still very low [104]. In colonic *in situ* instillations however, C_{10} did not increase the absorption of FD70 at all, even at a concentration of 100mM [106]. Likewise, in Caco-2 monolayers, the degree of enhancement with C_{10} (10-13mM) increased in proportion to molecular weight (MW) of the associated drug [107]. For example, the Papp of [^{14}C]-PEG (MW 326 Da) was increased by just 5-fold over basal compared with that of [^{14}C]-PEG (MW 546 Da, 17-fold). For solutes above a MW of

1200 Da, increased Papp values upon exposure to C_{10} in Caco-2 monolayers were not considered large enough to translate to an increased fraction of absorbed drug *in vivo*. Thus, for larger MW payloads, while the enhancement ratio in the presence of C_{10} may be higher *in vitro* because the basal flux is lower compared to molecules of lower MW, a large MW drug will still have poor oral F *in vivo* unless the concentration of C_{10} is increased significantly [99]. The proportion of *in vivo* studies that used concentrations of C_{10} above the CMC are higher than those used *in vitro* and *ex vivo*. In 15% of studies using cell culture models, concentrations $\geq$ 20 mM C_{10} were used to increase permeation of larger solutes (Table I). In *ex vivo* intestinal tissue models, 37% of studies used concentrations $\geq$ 20mM (Table II) and in those animal studies where the small intestinal luminal concentration can actually be estimated, the percentage increased to 85% (Table III and IV). The average concentration of C_{10} used *in vivo* (where it could be calculated) was 100 mM (Table III, IV), whereas it was 15mM and 20mM in monolayers (Table I) and isolated tissue (Table II), respectively.

Table III shows the effects of C_{10} on intestinal absorption of Class III molecules in a wide range of different types of animal studies. In some studies, the local concentration at sites along the GI tract could not be calculated because the promoter is part of a solid dosage form. In other studies where the absorption of the candidate drug was assumed to be zero in the absence of promoters, the enhancement ratio could not be determined. Increased absorption has been measured after instillation of C_{10} with an array of associated drugs (e.g. [108-112]) to different intestinal regions of rats (Table III). In general, the promoting action of C_{10} differs significantly, depending on the drug delivery model,

particularly when comparing *in situ* models to *in vivo* oral or to rectal delivery. In summary, C_{10} was effective in promoting drug absorption from oral- and rectally-delivered dosage forms in mice, rats, rabbits, dogs, pigs and cattle (Table III and IV).

There are a number of factors that may affect the absorption promoting activity of C_{10}. The majority of successful *in situ* intestinal studies in rodents demonstrate the effectiveness of co-administering solutions of C_{10} and payload to specific intestinal regions, and the data consequently would suggest that solid-dose formulations that ensure contemporaneous co-release are desirable [113, 114]. Differential rates of release from a solid dosage form could result in the promoter quickly reaching the intestinal epithelium, increasing permeability in a transient reversible fashion, but ahead of the arrival of the payload; this is especially relevant for C_{10}. Unlike SDS and EDTA, enhancement seen with C_{10} is rapid and reversible *in vivo* [21, 98, 104-115]. This is possibly because it is rapidly absorbed with a Tmax of < 10 min [115-117].

We examined the significance of keeping C_{10} and FD4 together at the gut wall in an *in situ* colonic instillation study in rats. Pretreatment with C_{10} for 15 min (followed by removal) did not increase FD4 absorption when the flux marker was administered 15 min later, in marked contrast to the significant absorption promotion seen when both agents were administered together or when FD4 was administered within 10 min (Wang X, PhD Thesis, NUI Dublin 2009). While another promoter, SDS, increased phenol red absorption in rat intestine, its effects took longer than C_{10} to dissipate [118]. In another rat perfusion study, co-administration of either SDS, EDTA, or C_{10} with cefoxitin increased

absorption of the antibiotic [113]; upon removal of the promoter, cefoxitin plasma levels continued to increase with SDS and EDTA, but not in the case of C_{10}. In the same study, co-administration of 50mM C_{10} with cefoxitin for 30 min was more effective at delivery than co-administration of 100mM C_{10} with the agent for 15 min, suggesting that co-presentation for a sustained period is more important than having a higher concentration of absorption promoter for a shorter time [113]. In a study assessing the effectiveness of C_{10} –based solid dosage forms, absorption of sulpiride in the presence of C_{10} was increased by ensuring their contemporaneous co-release from hydroxypropyl methyl cellulose (HPMC) matrix formulations compared to non-synchronous matrices [119], and moreover, matching the promoter dissolution to that of the drug is therefore essential, otherwise they may never reach the intestinal mucosa together. This is particularly true in the small intestine where, in addition to rapid absorption of the promoter, the fluid volumes (fasted 105ml, fed 45ml) have a considerable diluting effect compared with those of the large intestine (fasted 13ml, fed 11ml) and rectum (3ml), as well as a quicker transit time which could also prevent the optimal promoter/drug concentration at the small intestinal epithelium [120-122]. Solid dosage forms based on the contemporaneous release of C_{10} with payload have been used in man for the delivery of oligonucleotides, bisphosphonates and LMWH [24, 26, 123, 124]. It is worth mentioning that not all formulations designed for the controlled intestinal release of drug with C_{10} have had a positive effect on oral absorption. For example, an enteric-coated formulation of C_{10} with DMP 728 was absorbed to similar levels as controls [125].

The argument for synchronous delivery of C_{10} and payload to the small intestine refers to studies that were carried out primarily in rodents. However, this may not apply in higher species where dilution in the small intestine becomes more important. There is also a case to be made for presentation of payload at its highest concentration when the dosage form disintegrates, while achieving and then *maintaining* high luminal concentrations of C_{10} through additional pulsed release mechanisms [26, 126]. In the case of oligonucleotides, their absorption seems to follow first order kinetics and therefore reductions in the luminal concentration might reduce the absorption rate. The importance of the contemporaneous presence of the promoting agent and cargo shown in animal models has also been confirmed with C_{10} in human studies [24]. Intra-jejunal administration of C_{10} to human subjects increased sugar absorption up to 20 min after C_{10} administration, but not at 40-60 min as measured by the lactulose: mannitol urinary excretion ratio. This dataset confirmed that the window for promoting action is narrow and that oral formulations containing C_{10} should be designed to maintain the concentration of C_{10} at the intestinal epithelium. It is clear that the concentrations of C_{10} and candidate drug released over a set period must be optimized; however, assumptions cannot be made that dosage forms designed for rats will translate effectively to man.

The effect of other excipients on the absorption-promoting action of C_{10} could have a significant impact on efficacy. Removal of the jejunal mucus layer should, in theory, permit the direct contact between the promoter/cargo and the intestinal epithelium. Pre-treatment with the mucolytic, N-acetyl cysteine (NAC) with C_{10} led to more rapid absorption of sCT in a rat jejunal instillation as measured by serum calcium reduction

(Wang and Brayden, unpublished data), although this was not the case when both agents were co-administered [127]. A rectal formulation of 5-fluorouracil with C_{10} and Witepsol H-15 had a rectal F of 25.5%, however when Witepsol H-15 was replaced with PEG_{2000} F increased to 64.4% [128]. In an *in situ* rectal perfusion in rats, C_{10} only increased the absorption of hEGF when presented with sodium carboxymethylcellulose (CMC Na) [23]. F increased from 0 % with C_{10} (100mM) alone to 68 % when combined with CMC Na (1 % w/v). These reports indicate the potential effects of other excipients in maximizing C_{10}'s capacity for enhancement. Such additional actions of co-administered excipients could permit use of C_{10} with larger solutes and/or permit use of lower concentrations of the promoter, provided the excipients do not damage the intestinal epithelium.

The most effective region of the GI tract to target with intestinal absorption promoters remains unclear. The barrier properties of the mucosal surface changes in different regions of the intestine due to altered numbers of TJs, thicker mucous secretions [129], as well as different distribution of active transporters. Although the small intestine has a larger surface area for absorption compared to the colon, it has variable fluid volume and composition, short transit time and a higher concentrations of proteases [43, 120, 121, 130-132]. Transit time in the human small intestine is reasonably constant at 4-5 hours, while colonic transit can range from 20-30 hours to over two days, and this may permit longer residence time for co-releasing permeation enhancers and payloads in the colon [120-122, 132]. We examined the effect of jejunal flow on the promoting activity of C_{10} in a single pass rat perfusion model [106]. At a constant flow rate of 0.2ml/min, C_{10}

increased the Papp of [^{14}C]-mannitol by 2-fold, upon lowering the flow rate to 0.1ml/min however, mannitol permeability increased by 9-fold, suggesting that slowing the flow rate is beneficial. Enhancement at the colonic mucosa where the flow rate is naturally lower may therefore have potential in the delivery of peptides [120-122, 132]. It is possible therefore that use of anti-motility agents in the presence of C_{10} could lead to a further increase in F.

A potential advantage of both the colonic and rectal mucosae is their sensitivity to drug enhancement by a large number of promoters including MCFAs [114, 133, 134]. C_{10} increased the absorption of ebiratide [135], phenol red [102], and insulin [136] across rat colonic, but not jejunal epithelial mucosae (Table II). C_{10} also increased paracellular permeability of a number of solutes in the colon but not in the small intestinal mucosae of rats and rabbits [137]. The promoting activity of C_{10} on insulin absorption in the rat *in situ* closed loop intestinal model was ranked in order of colon>ileum> jejunum>duodenum, similar to the rank order obtained with EDTA and glycocholate [138]. Enhancement of fosfomycin [109] and carboxyfluorescein [139] absorption by C_{10} in rats was also greater in rat colon than jejunum. In an *in situ* instillation study in rats, the increase in FD-4 bioavailability conferred by C_{10} was greater in the colon than the jejunum. Although, since basal F was greater in the colon than the small intestine, the enhancement ratio was similar in both regions at 33-fold [106]. The reason for greater colonic sensitivity to absorption promoters at least in rats is not fully understood [140]. One of the reasons suggested for this is that there might be a limit to the capacity of a paracellular promoter to further loosen TJs in leaky small intestinal epithelia. Other

possible reasons include differences in residence time and net water fluxes [12, 133, 141]. Not all promoters however, exhibit greater activity in the colon. Unlike C_{10}, which acts in all GI tract regions, the C-terminal of *Clostridium perfringens* enterotoxin (C-CPE) [67], Zot [14], and bile salts [109] have greater promoting activity in the upper GI tract, likely due to their enriched target receptor expression in that region.

[2.2] Mechanism of C_{10} permeability enhancement across intestinal epithelia

While the mode of action of C_{10} in humans is not yet fully understood, *in vitro* and *in vivo* studies suggest that the promoter acts on both the transcellular and paracellular pathways (Fig. 2a). Concentrations of C_{10} that are effective in cell cultures (10-13mM) are thought to promote paracellular permeability through modulation of TJs. The higher concentrations of the promoter that are required in animal and human studies potentially relate to the promoter's additional mild surfactant properties. These result in destabilization and solubilization of enterocyte membranes, which impact on the contribution of the transcellular permeation pathway. Another potential aspect to the mechanism of action of C_{10} is the non covalent interaction of either monomeric, micellar or vesiclar C_{10} with the candidate drug (e.g. GnRH antagonists) altering its physicochemical properties thereby improving drug absorption across the epithelium in a fashion not too dissimilar to the eligen technology. However, this aspect to C10's mode of action has not been widely studied.

[2.2.1] Paracellular mode of action studies

C_{10} caused dilation of 42 % of TJs in Caco-2 monolayers (Fig. 2b) [142], which was accompanied by contraction, redistribution and disbandment of perijunctional actin and also alteration in the localization of zonula occludens-1 (ZO-1), occluden and claudin-1 [96, 97, 143-145]. C_{10} also disassembled F-actin at the TJ in human ileal mucosae [146] and dilated TJs in 34% and 37% of isolated rat [103] and human [146] ileal mucosae, respectively. In addition to intestinal epithelia, the involvement of specific tight junction proteins has also been described in airway and kidney epithelia and in skin epidermis. In human airway epithelial (HAE) monolayers, C_{10} caused redistribution of F-actin and reorganization of claudin-1, claudin-4, β-catenin, junctional adhesion molecule (JAM), but not ZO-1 [147, 148]. In Madin-Darby canine kidney (MDCK) epithelial monolayers C_{10} displaced claudin-4, -5 and occluden from lipid rafts into soluble microdomains with greater fluidity [149]. Although the study showed that C_{10} acted on specific tight junction proteins, it is not clear whether the displacement results from activation of a signal transduction pathway or whether the transcellular surfactant activity of C_{10} has the ability to destabilize the TJ by extracting loosely held TJ proteins from the low fluidity region of the lipid raft microdomain. In human epidermal keratinocytes, C_{10} induced reversible redistribution of the tight junction proteins, claudin and occluden, as well as deterioration of TJ strands [150].

A proposed mechanism that describes how C_{10} increases epithelial paracellular permeability is based on data generated from exposure of Caco-2 monolayers to C10. Based on these studies, the data suggest that activation of phospholipase C (PLC) causes cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP3)

and diacylglycerol (DAG) [97, 151]. The activation of PLC can also be linked to surfactant actions of the promoter. Low concentrations of surfactants can affect membrane-bound enzyme activity by either activation or inhibition with some enzyme activity increasing as much as ten fold [152]. IP_3 then mobilizes intracellular Ca^{2+}, which complexes with calmodulin (CaM) to alter its structural conformation. The Ca^{2+}/CaM complex subsequently activates CaM-dependent protein kinases, CaMKII and MLCK. The latter then phosphorylates the regulatory light chain of myosin II (MLC) leading to contraction of perijunctional ring of actin and myosin II (PAMR), which opens the tight junction [153]. In support of this model, the absorption promoting activity of C_{10} was attenuated with PLC inhibitors in Caco-2 monolayers [97, 144, 151]. C_{10} also mobilized intracellular Ca^{2+} from stores in Caco-2 cells (Fig. 2c) [144, 151, 154], while pre-treatment with the intracellular Ca^{2+} chelator, BAPTA-AM, attenuated C10's permeation-enhancing activity [97, 143, 145] and its effects on F-actin reorganization [145]. Pharmacological inhibition of CaM and CaMKII prevented C_{10} from increasing paracellular permeability across Caco-2 monolayers, suggesting that calcium-induced activation of CaM is a downstream event in the mechanism [97, 145, 155, 156], data that were confirmed in isolated rat and human colonic mucosae [157]. Further along the pathway, pharmacological inhibition of MLCK in Caco-2 cells and isolated rat and mouse colonic mucosa [158] also decreased the permeation-enhancement activity [97, 143, 158]. In the latter study, C_{10} phosphorylated the MLC of both rodent models (Fig. 2d), an effect that was attenuated by inhibition of MLCK [158]. Inhibition of CaM with high concentrations of the antagonist, W7, also reduced the absorption-promoting action of C_{10} from a rectal suppository in rats [159]. Inhibition of the other PIP_2 cleavage

product, DAG, (a PKC agonist), also inhibited C_{10}'s effects on permeability [97]. DAG's action on permeability may be independent of action on PKC since inhibition of the kinase increased the permeation-enhancement action of C_{10} in Caco-2 [97, 151, 155]. Consistent with these Caco-2 data, pretreatment with a PKC antagonist did not reduce the absorption-promoting activity of C_{10} from a rectal suppository nor did it block the reduction in TEER caused by the promoter [159]. In summary, C_{10} has a permeation-enhancing effect *in vitro* that appears to be mediated in sequence through PLC, calmodulin and MLCK activation, whereas the role of DAG and the involvement of PKC have yet to be fully deciphered.

Another lesser known mechanism that has been proposed for C_{10} is that the presence of the promoter leads to a reduction in intracellular ATP [97], which can lead to increased paracellular permeability [156]. The decrease in ATP induced by C_{10} in hamster colonic cells was comparable to that seen with three other promoters (tartaric acid, lauroylcarnitine and palmitoylcarnitine) [97, 156]. C_{10} also depleted ATP and increased the energy charge potential (a marker of accessible energy supply) in human ileal epithelial mucosae [146]. It also depleted ATP in airway epithelia, but this did not seem to contribute to the acute effects of the promoter [147]. The decrease in ATP induced by low concentrations of C_{10} used *in vitro* might not however, be sufficient to account for the permeation enhancement activity of C_{10} [97], but the higher concentrations of C_{10} used *in vivo* could result in significant ATP depletion. In Caco-2, large increases in permeability induced by C_{10} were also correlated with reduced mitochondrial dehydrogenase activity [160]. Ultrastructural studies on monolayers and isolated mucosae

revealed swelling of the mitochondria in the presence of C_{10} [96, 97, 104, 105, 146]. Taken together, these data suggest that the effects of C_{10} on cellular respiration could be an indirect part of the mechanism of action, particularly at the higher concentrations that pertain to *in vivo* studies. However, to-date there have been no studies carried out *in vivo* that report the involvement of cellular respiration in the promoting action of C_{10}.

[2.2.2] Physicochemical properties of C_{10} in solution

Before we can address the surfactant activity and transcellular mode of action of C_{10}, it is important to outline how the promoter behaves in solution. In aqueous solutions, surfactants self-assemble by forming a range of structures. Sodium and potassium salts of fatty acids are water soluble and form an intermediate liquid crystalline phase before reaching a narrow concentration range when micelles are formed [161]. Subsequent addition of fatty acid leads to the formation of large colloidal structures. The presence of monomeric fatty acids and one or more colloid structures may impact on the concentration of fatty acid presented at the intestinal absorption site. The concentration range at which a surfactant starts to form micelles and when the free monomer is at its highest concentration is defined as the critical micelle concentration (CMC) (Table V). Below the CMC no micelles are detected, and above the CMC, all additional surfactant forms micelles or vesicles [162]. A unique CMC value cannot be defined because it depends much on test conditions including temperature, pH, ionic strength, concomitant surfactants (e.g. phospholipids, bile salts), as well as methods of measurement [162-164]. The CMC of C_{10} depends especially on the ionic strength of the buffer and on pH because close packing of the carboxyl head groups is strongly opposed by electrostatic repulsion

unless this is offset by sufficient salt (counter ion) or acid protonation [152, 162, 165, 166]. In addition to the CMC value, a critical concentration for vesicle formation (CVC) has been proposed. C_{10} in aqueous solution exists in monomeric form in equilibrium with a number of different phases, including micelles and/or vesicles (Fig. 3). While it forms micelles in alkaline conditions, vesicles are generated when the pH is lowered to ~6.5-8, with possible co-formation of micelles and vesicles at a pH of 7.5 [163]. It is therefore advisable to be cautious if attempting to correlate permeability data to an exact CMC value. The reported CMC values of C_{10} vary considerably, ranging between ~10-100 mM (see Table V and see also [163]). C_{10} forms heterogenic-sized vesicles at a pH of 6.4-7.8; CVC values ranged from 8-26 mM (pH 6.6-7.6 [163]) and from 14-29 mM (pH 6.8-7.4 [166]). The poly-disperse particulate structures of C_{10} formed at a concentration of 200 mM in saline are shown in Fig. 3 and can be viewed in the Supporting Information (motion of particles recorded by Nanosight LM20, NanoSight Ltd, Amesbury, UK). The effects of these large vesicular nano-particulate structures on the promoting action and cytotoxicity of C_{10} have not yet been defined.

The pKa of capric acid can affect its solubility and it's CMC; it can impact on its promoting action and in turn on its own absorption. Although the pKa of C_{10} has long been thought to be similar to that of short chain fatty acids (pH 5), one study showed that due to the closer packing of C_{10} molecules at the interface, there is an increase in pKa in fatty acids that follows chain length [167]. The pKa of C_{10} may therefore be closer to pH 7 [163, 167], which underscores the likelihood of the molecule to convert to the unionised acid form at the pH of the small intestine and consequently to be rapidly

absorbed or to precipitate out as an inactive oil. This is an important aspect to consider in designing an oral formulation.

[2.2.3] Transcellular mode of action studies

Transcellular enhancement by surfactants results from detergent-like perturbation of enterocyte plasma membranes, which results in leakage of drug across the intestinal epithelium. The effect of a number of promoters on transcellular permeability has been reviewed [133, 168, 169]. The behavior of a large number of absorption promoters tend to be concentration-dependent with greater transcellular enhancement and cytotoxicity at higher concentrations [170]. C_{10} causes plasma membrane aberrations based on release of carboxyfluorescein from jejunal and colonic brush border membrane vesicles (BBMV) [108, 137]. In studies in artificial lipid bilayers, effects of C_{10} were associated with changes to the hydrophobic and polar domains leading to increased fluidity [168]. It also caused a concentration- and time-dependent decrease in the fluorescence polarization of lipid- and water-soluble probes in jejunal and colonic BBMV, suggesting that the promoter induces transcellular packing reorganization in both the lipid and protein domains [108, 137, 171]. It seems that C_{10} therefore partitions into lipid bilayers, disrupting intermolecular forces between membrane phospholipids to decrease resistance to transcellular permeation. Similar data has been presented for mixed micelles of oleic acid and azone [172-174]. High concentrations of fatty acids also effect the interior of phosphatidylcholine-rich liposomes leading to bilayer destabilization, and this is likely to be similar to actions at the plasma membrane [134, 175, 176].

The interaction of surfactants with biological membranes has been eloquently reviewed elsewhere [152, 162, 165, 177, 178]. In general, surfactant monomers partition in the plasma membrane where they form defects leading to subtle alterations in permeability [152, 162, 169]. Low concentrations of surfactant monomer penetrate the lipid bilayer and act as wedges, thereby increasing the surface area and alter the osmotic equilibrium to increase the permeability of drug substances [152]. Increasing the surfactant concentration also leads to increased fluidity, perturbation and destabilization of the plasma membrane, which can lead to cell lysis [133, 152, 177]. The lytic process can be divided into stages: adsorption of the surfactant monomer to the plasma membrane, penetration into the membrane, change in the molecular architecture, alteration in permeability and osmotic equilibrium, and leakage of intracellular content [152]. After lysis, the final actions of a surface active agent are solubilisation of the lamellar bilayer structure into mixed micelles [152, 178]. Solubilisation results in a mixture of protein surfactant complexes, mixed micelles and surfactant micelles that are in equilibrium with free surfactant monomers at the CMC [152, 177]. Typically, the concentration of the surfactant required to solubilise the plasma membrane tends to increases with the CMC value. The higher the CMC value, the lower the hydrophobicity of the fat soluble moiety and the less effective the surface active agent is at penetrating lipid bilayers. To emphasise, while there is no evidence of the full solublisation cascade occurring when C_{10} promotes transcellular absorption in a rapid reversible fashion, this is the general principle for surfactants.

The CMC was initially considered a good criterion for identifying the threshold concentration of capric acid and other MCFAs to enhance absorption [160]. However, it is now clear that the CMC alone is not the overriding factor in determining the effectiveness of MCFAs in increasing transmucosal drug flux [179]. In general, both very non-polar and highly polar surfactants are poor promoters; the optimum is in the mid-range [169]. Another criterion used to assess the interaction of surfactants with biological membranes and the formation of micelles is the hydrophilic-lipophilic balance (HLB), a measure of the solubility of aqueous and lipidic moieties of the molecule. The HLB values of capric acid and C10 are 4.8 and 21, respectively [180], which further emphasizes that the pH and counter ion concentration of the luminal fluid are important factors in the promoter's surfactant properties.

Surfactants can increase bilayer permeability below the CMC [169, 181]. For example, *in vitro* and *in vivo* studies that show C_{10} alters the integrity of the intestinal epithelium leading to release of intracellular mediators ([182]), or to internalization of normally excluded dyes (propidium iodide [96, 182] and trypan blue [100]). This is not unique to C_{10} as other surfactants, including endogenous bile salts also cause lysis at concentrations below their CMC [152]. Concentrations of C_{10} that promote drug absorption in cultured epithelial monolayers were also found to damage the integrity of erythrocyte membranes leading to release of haemoglobin (data not shown). However, erythrocytes are highly susceptible to changes in tonicity and are far removed from more sophisticated models used to assess cell membrane perturbation. While the above solubilisation model has been proposed for many surfactants, it has not yet been conclusively demonstrated with

C_{10} and the exact nature of the promoter's interaction with the intestinal mucosa remains unclear.

The physicochemical properties of C_{10} presented at the intestinal mucosae can also be influenced by formulation excipients, luminal fluid composition and pH, the region of the GI tract and dietary composition. The importance of an appropriate medium for the use of C_{10} as a delivery agent is highlighted by studies examining the role of osmolality on the interaction of the promoter with the intestinal epithelium. In Caco-2, anisotonic solutions of C_{10} increased the apical-to-basolateral flux of mannitol compared to isotonic solutions, which was also accompanied by greater cytotoxicity [142]. Clinical trials with Doktacillin® suppositories containing C_{10} suggest that the hyper-osmolality of the formulation contributed significantly to the absorption enhancement and to temporary mucosal damage [22]. It is therefore important to examine the absorption promoting activity of C_{10} in both fasted-state-simulating intestinal fluid (FaSSIF) and fed-state-simulating intestinal fluid (FeSSIF), a media dominated by high concentrations of deoxycholate, salt and lecithin. In Caco-2 monolayers, FaSSIF did not significantly influence the permeating-enhancement effects of C_{10}. However, it did reduce the effectiveness of palmitoylcarnitine chloride by over 15-fold [164]. The formation of mixed micelles between C_{10} and bile salts or other dietary surfactants in the upper GI tract could affect the promoter's CMC and could hasten solubilisation of the enterocyte membrane [169]. For example, a mixture of capric acid with bile acids enhanced the membrane lytic activity of the promoter [183]. In contrast to bile salts and mixed micelles, CMC of the fatty acids alone does not appear to correlate with the lytic effect.

The consensus however, is that dissolved monomeric fatty acids perturb the enterocyte membrane whereas mixed micelles are not absorbed *per se* (for review see [184]). The rate of uptake of fatty acids depends on the concentration and permeability coefficient of monomeric fatty acids, where the latter reflects the partition between the aqueous milieu and the lipidic enterocyte membrane. The amount of monomeric decanoic acid available for penetration into the enterocyte therefore depends on the equilibrium between the amount of dissolved monomeric decanoic acid, mixed micelles (where the decanoic acid preferentially is solubilised), and on whether the pH microclimate favours formation of decanoate/decanoic acid vesicles. The pH microclimate determines the ratio of C_{10} to capric acid while the amount of free monomeric unionized capric acid ready for penetration into the enterocyte depends on the equilibrium between the dissolved free monomer, pure or mixed surfactant micelles or vesicles and the amount of capric acid already bound the plasma membrane.

[2.3] Preclinical safety data for C_{10}

The effect of C_{10} on cell viability and morphology has been studied in intestinal epithelial cell cultures and isolated intestinal mucosae (Table VI-VII). Assessment is far more complex and germane *in vivo,* where the (usually higher) concentrations of the promoter fluctuate considerably due to variable residence time caused by dilution of intestine fluid volume [120], individual variation in gastrointestinal transit [132] and absorption of the promoter itself [116]. Data from intestinal monolayer cultures indicate incubation with C_{10} can perturb the mucosal membrane of enterocytes, leading to release of intracellular content and/or uptake of dyes normally excluded from the cell (e.g. [101, 185, 186] and s

Table VI). In these *in vitro* models the effects on viability were typically concentration- and time-dependent and reversible. In some studies C_{10} led to a concentration-dependent reduction in cell viability in MTT assay (e.g. [96, 98, 185] and see Table VI). Light microscopy revealed that morphological deterioration was measured only at high concentrations (> 15mM) [99]. These higher concentrations led to membrane solubilisation, cell extrusion, and cell death [96, 99]. In anisotonic conditions, C_{10} caused focal damage to enterocyte microvilli [142]. Some *in vitro* studies suggest that there is a relationship between cytotoxicity and promoting action [187]. Damage caused by C_{10} partially recovered over 2-5 days depending on the concentration and initial exposure time [98] and the epithelial insult was hypothesized to primarily be due to a temporary reduction of oxidative phosphorylation rather than to cell death [64, 98].

Damage also appeared to be caused by C_{10} in isolated intestinal tissues (Table VII). In isolated rat and human colonic mucosae C_{10} perturbs the mucosal surface leading to the release of intracellular mediators [104, 171]. The effect of the promoter on the release of LDH from mucosae was however, significantly lower than the detergent, Triton X-100 [104]. Acute exposure of human colonic mucosae to C_{10} led to abrasion of the apical surface and there was evidence of early oedema in the sub-mucosa [104]. Similarly, C_{10} uncoupled oxidative phosphorylation leading to a drop in intracellular ATP in isolated human intestinal mucosae [146]. These results were not corroborated in other *in vitro* studies however, where there was no toxicity associated with the promoter. For example, in the rat everted intestinal sac model there was an increase in the aqueous pore radius to paracellular markers, but no evidence of mucosal damage [188].

Although *in vitro* models may be useful tools in the preliminary assessment of mucosal cytotoxicity, they lack contextual aspects of *in vivo* studies (e.g. transit time, dilution, histological changes and food effects). Toxicity of C_{10} has been comprehensively studied in animal models (Table VIII). In studies where the local intestinal concentration can be estimated, 10-fold higher concentrations of C_{10} are often required *in situ* and *in vivo* to promote drug absorption and therefore one might expect to detect increased mucosal toxicity. A number of *in situ* intestinal studies show that C_{10} perturbs the mucosal surface causing release of membrane phospholipids, protein and intracellular protein (Table VIII). Similar to *in vitro* toxicity assays, the rate of release of LDH into the small intestinal lumen was however significantly lower for C_{10} than for SDS [189]. Regional delivery of C_{10} (100 mM) led to mild abrasion of the epithelial surface in rats [104]. Concentrations of the promoter between 200-400mM also increased the histological damage score and caused rectal contraction in rats [67, 190, 191]. While region-specific promotion by C_{10} suggests the actions of the promoter are not related to general membrane damage [137], the duodenum and jejunum are designed to be able to cope with mild surfactants due to their continuous exposure to high concentrations of bile salts.

It is possible for mucosal erosion to progress to ulceration so it is important to understand the effect of C_{10} on the intestinal epithelium *in vivo*, especially upon repeated administration [192]. Repeated oral administration of capric acid (500mg/kg) to rats for 150 days was well tolerated and did not lead to gross morphological damage in either forestomach or glandular stomach [86]. Oral delivery of an enteric-coated oligonucleotide formulation containing promoting concentrations of C_{10} did not induce

intestinal histopathology after 13 weeks of repeated administration in rats or dogs [193]. Oral delivery of high doses of C_{10} also showed no effect on histology of the intestinal mucosae of dogs or pigs, nor did it lead to abnormalities in clinical chemistry [24, 115, 194]. Other than producing lipid-rich stools, dogs dosed with up to 1 g of C_{10} for 7 days displayed no adverse effects [24]. Furthermore, the oral delivery to dogs of the GIPET® formulation containing C_{10} and C_{12} (1:2) at a dose of 0.9g/kg/day did not lead to adverse findings in ECG, clinical chemistry or histology measurements [24]. A few dogs showed weight loss and vomited, which was thought to be more associated with the intake of up to 18 capsules per day.

In vitro measurements of cytotoxicity are often unrelated to *in vivo* toxicity and are unlikely to be predictive [24, 133, 192]. For example, erythrocyte haemolysis (a highly sensitive assay) is widely used to test the effect of drugs on membrane integrity. C_{10} (10mM) causes 100% haemolysis, but significantly higher concentrations of the promoter in animal models do not result in any appreciable toxicity (Table VIII). Gross morphological damage to Caco-2 monolayers by C_{10} (50mM) was not reflected in noteworthy histological damage in rat single pass jejunal perfusion at the same concentration, nor in rat rectal instillations at even a ten-fold higher concentration [99, 106]. In a combined *in situ* and *in vitro* rat colonic loop and isolated tissue mucosae study, washout completely reversed C_{10} 's effects on permeation enhancement of mannitol flux, which suggests that any morphological damage was not very relevant [11] and implies a primary mechanism of mixed micelle enhancement in this model. Furthermore, mucosal aberration was less apparent in a number of *in situ* and *in vivo* drug

delivery models compared to *in vitro* models (Table VIII). There are a number of potential reasons why C_{10} does not lead to comparable toxicity *in vivo* compared with *in vitro*. Firstly, C_{10} may have restricted access to the mucosal surface due to infoldings in the intestinal lumen. Furthermore, the presence of a thick mucous layer, glycoproteins (e.g. glycocalyx), and the phospholipid composition of the lipid bilayer can reduce the solubilising action of detergents on the cell membrane [152]. Secondly, the rate of absorption of C10 is rapid, and the time course of exposure is extremely short relative to in vitro experiments. Thirdly, the small intestinal mucosa has a rapid cell turnover where an estimated 100 million cells are sloughed in to the lumen per minute and the entire intestinal surface is replenished over 2-6 days, so damaged mucosae are rapidly regenerated [130, 192]. Injured cells extend membranous lamellipodia projections to cover the breached epithelial barrier to assist epithelial restitution [195, 196]. Many studies describe the ability of the intestinal epithelium to be repaired (for review see [133]). For example, damage to human colonic mucosa was estimated at 95% after a 5 min exposure to HCl (100mM); there was restitution after 15 min and recovery to a level of less than 20 % damage after 3 hours [197]. Focal epithelial denudation induced by Triton X-100 was repaired over 2 hours in isolated ileal mucosae of guinea pigs [198]. Mucosal erosion caused by the strong surfactants, TDC and NP-POE-10.5 was comparable to control levels after just 2-3 hours in the rat perfusion model [199]. In rat rectal perfusions using sodium lauryl sulfate, sodium deoxycholate and EDTA, epithelial recovery was seen within two hours [200]. Physiological concentrations of dietary surface active lipids can compromise the mucosal epithelium, but the epithelium is rapidly repaired [55]. In a rat perfusion study, oleic acid emulsified in rat hepatic bile

caused rapid mucosal damage to the jejunal epithelium which recovered after 50 min [201]. Indeed, even in the most extreme cases of epithelial damage (including almost total loss of the epithelium) induced by absorption promoters in rats, the damage has been interpreted as reversible by qualified veterinary pathologists [114]. Finally, the concentrations of bile salts and mixed micelles found in the duodenum and jejunum can lead to mucosal damage, yet repair is the typical outcome [169]. Rapid mucosal repair and regeneration are therefore normal physiological processes and hence the mucosal perturbation caused by dietary substances, endogenous secretions and absorption promoters can be tolerated.

Understanding the kinetics of epithelial damage and recovery at the selected dosage frequency is important. In an *in situ* rat colonic instillation model, C_{10} caused mild mucosal damage when the tissue was histologically-assessed at the Tmax for absorption promotion [106]. A number of other *in situ* and *in vivo* rat studies show barely discernable C_{10}-induced mucosal damage at later time points, likely masked by the intestinal epithelium's ability to recover as the agent is absorbed. In addition to examining the kinetics of epithelial recovery, any damage caused must be examined objectively and compared to that of other xenobiotics. To put it in context, many other dietary substances also cause mild intestinal epithelial aberrations during the absorption process and this is normal. For example, ethanol can cause mucosal damage in rats and dogs at concentrations commonly found in typical alcoholic beers (~4% v/v) [202], and a number of marketed rectal suppositories have been shown to reversibly damage the rectal epithelium [122, 203].

The premature progression of epithelial cells from the crypts to the villi surface during epithelial restitution could compromise nutrient absorption since these immature cells might not yet adequately express all of the proteins required for nutrient absorption and fluid homeostasis [192, 204]. C_{10} inhibits Ca^{2+}- and cAMP- dependent electrogenic chloride secretion in rat and human colonic mucosae [104, 205]. The effect of the promoter on reducing the chloride secretion induced by the adenylate cyclase activator, forskolin, and muscarinic agonists was reversed at 24 hours [156]. C_{10} also decreases the basal short circuit current (Isc, $\mu A.cm^2$) in rat and human colonic mucosae [105, 156]. In a rat rectal perfusion, C_{10} was also shown to increase water absorption [206]. Inhibition of electrogenic chloride and alteration to water absorption/secretion processes, if present *in vivo*, could affect the hydration of the mucosal surface. This could potentially affect the intestinal relationship with commensal bacteria, the flushing away of pathogens, as well as impacting on the efficiency of nutrient absorption.

One of the key safety concerns with transiently-modulating gut permeability is that other xenobiotics, antigens, toxins and bacteria present in the lumen could be co-absorbed leading to inflammation of the sub-mucosa and/or septicaemia [14]. Capric acid and C_{10} have established antibacterial- and antiviral properties at similar concentrations to those required for absorption-promotion [207-209]. We found that C_{10} does not increase the translocation of *E. coli* across Caco-2 monolayers, while the detergent, Triton X-100, significantly increased *E. coli* flux at concentrations that modulate permeability (Fig. 4a). This could be related to the antimicrobial activity of C_{10} against the test organism at

concentrations that promote solute absorption (Fig. 4b), while *E. coli* are resistant to the surfactant actions of Triton X-100 [210] (Fig. 4c). Further studies with bacteria that are resistant to high concentrations of C_{10} and/or micro-particles that mimic bacteria could help to understand whether the promoter has the propensity to increase bacterial translocation. This could be difficult because a wide range of bacteria are susceptible to capric acid and $C_{10,}$ including *E. coli*, *S. typhimurium,* and *L monocytogenes, C. jejuni, C. perfringens* and *S. sonnei* [207, 208]. Capric acid also has antimicrobial activity against a panel of bacteria that are frequently found in clinical microbiology [211]. In a screen of 13 bacterial isolates, C_{10} inhibited growth and biofilm formation at concentrations that are used to promote absorption in cell cultures and *in vivo* models (Unpublished data, Rawlinson L. and Brayden DJ). In addition to antimicrobial activity, C_{10} has been shown to reduce adhesion of *S. typhimurium* to isolated ileal mucosa of rats [207]. Capric acid has antiviral activity against a number of enveloped viruses [209], antifungal activity [211] as well as anti-protozoal activity against the common intestinal parasite, *Giardia duodenalis* [212]. In cultured monolayers of human airway epithelial cells, pre-treatment with C_{10} increased the translocation of adenovirus (Ad*lac*Z and Ad-*CFTR*) vectors [148]. C_{10} pre-treatment has also been shown to effectively increase viral gene transfer *in vivo* in both murine nasal [213] and murine tracheal [214] models. However, co-administration of AdlacZ adenovirus with concentrations of C_{10} above the CMC (13mM) completely abolished gene transfection because of the interaction of C_{10} with the virus [213].

The initial bacterial and viral data reported above suggests that the high luminal concentration of C_{10} required to enhance epithelial permeability, could prevent

infiltration of micro-organisms. The corollary is that its anti-bacterial activity could neutralize local commensal bacteria in the gastrointestinal lumen, but this requires investigation. Perhaps the most convincing data that C_{10} does not lead to inflammation of the rectum or distal colon, at least in short term use, is the presence of the promoter at high concentrations in Doktacillin®. While the presence of the ampicillin in the rectal preparation could confound this aspect of the safety data, intestinal inflammation or septicemia was not reported in any of the human trials. Finally to put the issue in context, C_{10} significantly enhances permeability of drugs with a typical Mw cut off of approximately 3-10 kDa with a molecular radii of 10-20 A. Lipopolysaccharides, enterobacterial toxins, bacteria and viruses have MW values and radii far in excess of those values.

[3] Promoting activity and safety of C_{10} in man

Care must also be taken when extrapolating permeability enhancement data from intestinal cell cultures and isolated mucosal tissues to effects in humans [170, 215]. Promoters that are effective in rats may have little or no promoting activity in larger animals and humans [140, 203]. Similarly, *in vitro* and *in situ* mechanistic studies may have little or no relevance to the human oral absorption. The efficacy and safety of C_{10} can only be established in well-designed clinical studies. The promoting activity of C_{10} in man has been assessed thoroughly with rectal formulations of antibiotics carried out almost 25 years ago (Table IV). C_{10} (10%, 100mg) increased the proportion of ampicillin excreted in urine over six hours from 1.7 % to 38.1% from a suppository [89]. Suppositories of ceftizoxime formulated with C_{10} (3-5%, 15-25mg) had a Tmax of 30

min and the Cmax was 10-12µg/ml, although the extent of enhancement was not examined because all of the formulations contained C_{10} [216]. Ceftizoxime suppositories containing C_{10} (3%, 15mg) were also assessed in pediatric patients, where the Tmax was also 30 min and the Cmax was 8.9µg/ml [216]. Rectal antibiotic suppositories containing both ampicillin, Witepsol H5 and C_{10} (2% w/w) or ceftizoxime with 3% w/w C_{10} were licensed in pediatric patients in Japan in the mid-1980s (Kyoto Pharma Industries and Sumitomo Pharma Co, Japan).

The efficacy and safety of these antibiotic suppositories in pediatric patients has been reviewed in a number of clinical studies [217-220]. 543 patients received 3 to 4 ampicillin suppositories per day to yield a bacterial eradication rate of over 80 % [219, 220]. In an equivalent study with ceftizoxime, 248 patients with a daily frequency of 3 to 4 suppositories also had a bacterial eradication rate of over 80 %. Although these studies did not specifically identify the promoting action of C_{10} in the suppositories, data from related animal studies would indicate that the promoter does have considerable enhancing effects following rectal administration (e.g. [218] and see Table III). C_{10} was also formulated as part of a rectal suppository with glycyrrhizin for the treatment of chronic hepatitis [221]. Patients receiving the suppository twice daily over 12 weeks showed a significant drop in alanine aminotransferase levels with the effects comparable to an i.v. formulation. In contrast, C_{10} (18mM) was ineffective in the delivery of antipyrine and phenoxymethylpenicillin when delivered as a solution in a rectal perfusion device [215]. Apart from the low dose of C_{10} used compared to most other human rectal studies, the

relatively high rectal perfusion flow rate (2.5ml/min) could have a significant bearing on the lack of effect [106].

Safety assessment of rectal formulations containing C_{10} shows the promoter can lead to irritation of the rectal mucosa in a concentration-dependent fashion (Table IX). Suppositories containing 40-60µg of C_{10} with glycyrrhizin did not irritate the rectal mucosa [221]. However, the higher dose of 80µg caused severe irritation to the rectal mucous membrane. This could be related to synergy between C_{10} and glycyrrhizin [154]. In ceftizoxime suppositories containing C_{10} (5%, 25mg) that were administered 3 times a day for 5 days, there was a slight increase in the feeling of a foreign body and a propensity for defecation, but no burning sensation or pain [216]. In the same study, no irritation was noted in pediatric patients that received the single dose ceftizoxime suppository containing C_{10} (3%, 15mg) [216]. In an ampicillin suppository administered 3 to 4 times daily to 642 pediatric patients, only 17 patients had diarrhea (3.6%), six patients had soft stools (1.3%), 1 patient had abdominal pain(0.2%) and 4 had periproctal redness (0.8%) [219, 220]. Abdominal clinical symptoms were similar in pediatric patients receiving a ceftizoxime suppository, although a higher percentage of patients had diarrhea (8.6%) [219, 220]. Further to this study, suppositories containing 80mg of C_{10}, (where the concentration of C_{10} could be over 100 mM assuming complete dissolution), there was a slightly increased sensation of defecation over 30 min, yet scores of pain, burning or itch were similar to control formulations [191]. It is worth stating that there is not enough evidence to conclude that there is a relationship of these rare adverse events to direct effects of C_{10} in the suppositories.

The importance of the contemporaneous delivery of the promoting agent and drug cargo shown in animal models has also been demonstrated with C_{10} in human studies [24]. Intra-jejunal administration of C_{10} (500mg) to healthy human subjects increased sugar absorption up to 20 min after C_{10} administration, but not at 40-60 min as measured by the lactulose: mannitol urinary excretion ratio. These data confirmed that the window for promoting action is narrow and that oral formulations containing C_{10} should be designed to synchronize the release of C_{10} and payload.

[3.1] Case study I: Rectal delivery of ampicillin using C_{10}

The major success of C_{10} to date was the approval in Sweden of Doktacillin® in 1992 [142, 222]. The composition of the Doktacillin® suppository was similar to the formulation licensed for use in pediatric patients in Japan in 1985 with only minor differences in the suppository base [219, 220]. Doktacillin® suppositories (125 or 250 mg ampicillin, 15 or 25mg sodium caprate) administered to 421 pediatric patients for between 3-10 days cured 37% and improved the condition of 52 % of patients with *Otitis media*, with mean plasma concentrations of 5.9 or 8.5µg/ml for the low and high dose formulation respectively [223]. Gastrointestinal disturbances including diarrhea, loose stools and perianal pain were reported in 28% of patients. However, the study was not placebo-controlled, thus hampering direct assessment of safety and efficacy of C_{10} in the formulation. The most comprehensive clinical assessment of Doktacillin® suppositories in the public domain was carried out by Lindmark *et al* (1997) [22]. C_{10} formulated with ampicillin and Pharmasol B-105 increased urinary recovery, plasma Cmax and AUC of

ampicillin by 1.8-, 2.6- and 2.3- fold, respectively compared to the triglyceride base alone. Local rectal irritation was examined by histological scoring following a rectal biopsy. The triglyceride base alone led to a loss of epithelial cells and damaged the basal lamina. The authors suggest that the reversible damage detected was due to the solubilising concentrations (≤86mM) of the promoter released from the suppository [96, 97]. The damage may also have been due to a synergistic effect of the combination of C_{10} with the triglyceride base, along with the influence of high osmolality of rectal fluid [142]. The efficacy and safety of C_{10} in this rectal formulation has therefore never been accurately investigated, stripped of confounding factors. However, these studies do indicate that mild reversible mucosal damage caused by agents that promote rectal absorption by transcellular perturbation in humans are acceptable for short term treatments.

[3.2] Case study II: Oral delivery of oligonucleotides using C_{10}

ISIS Pharmaceuticals (California, USA) first disclosed formulations for the oral delivery of oligonucleotides with C_{10} and other adjuvants [224]. Pulsatile formulations containing high concentrations of C_{10} were effective in promoting the oral delivery of antisense oligonucleotides in a Phase I study [26]. Mini-tablets containing ISIS 104838 (antisense to TNF-α) and C_{10} were coated with varying concentrations of Eudragit® RS30D to create a mixture of immediate- and delayed-release pellets. The mini-tablets were encapsulated in an enteric-coated capsule with Eudragit® L30 D55. The pulsatile formulations were designed to cause release of C_{10} and oligonucleotide at high concentrations followed by a sustained release of the promoter, which itself is rapidly

absorbed; this might maintain the enhancement window while there are still high concentrations of the oligonucleotide present in the intestinal lumen. The final weight of C_{10} in each capsule was 660mg, over 25-fold greater than that found in Doktacillin® suppositories [22, 26]. The maximal oral F of ISIS 104838 was 12% in fasted patients and 12.9% in patients fed a high fat meal prior to dosing [26]. An assumption is made that the oral F of ISIS 104838 in the absence of C_{10} in the capsule would be close to zero. The formulations were well tolerated with no adverse effects seen on physical examination or clinical chemistry. This was consistent with safety studies with C_{10} in rats and dogs with another ISIS oligonucleotide (ISIS 301012 [225], an antisense inhibitor of apolipoprotein B (ApoB) [193]. In another Phase I clinical study, a once-daily dosage of ISIS 301012 in a formulation containing C_{10} for up to 90 days significantly reduced serum LDL-cholesterol and apoB [123, 124]. A mean $F_{0\text{-}24h}$ of 6 % was observed upon oral administration and the optimal PK responses for both apoB and LDL-cholesterol were achieved on days 55 and 69 (see Fig. 5). Again, while there was no treatment group without C_{10}, it can be assumed that the unenhanced oral F of such a large hydrophilic molecule is negligible. In support of this, the oral F of ISIS 2302, (antisense to ICAM-1) following rat jejunal instillation was ~0 % without an absorption promoter, while in the presence of a combination of C_{10} and C_{12} , the oral F was significantly increased to 8% [224]. In this study, the oral formulation containing C_{10} was generally well tolerated with only mild, intermittent GI symptoms reported.

[3.3] Case study III: GIPET®, Oral formulations of poorly permeable drugs with C_{10}

GIPET® formulations (Merrion Pharmaceuticals, Dublin, Ireland) are a group of oral solid dosage forms designed to promote absorption of poorly permeable drugs. The typical GIPET®-I preparation contains a poorly-absorbed drug with C_{10} as the promoter in an enteric-coated tablet. The absorption-promoting actions of constituents of GIPET® have been disclosed in preclinical and clinical studies [226]. One such pilot study assessed the effect of intra-jejunal administration of high concentrations of C_{10} (550mg-1650mg) on the absorption of low molecular weight heparin (LMWH) in healthy human volunteers [226]. The promoter-drug mixture was delivered in 15mls of water and F was measured relative to a s.c. reference. The concentration of the promoter in this volume ranged between 185-566mM (Table III). C_{10} increase the oral F for LMWH to 5-9 % compared to sub-cutaneous bioavailability. The amount of C_{10} used in this oral human study was up to 66 times greater than that of the Doktacillin® suppository, even though orally-administered C_{10} is diluted in a larger small intestinal volume than the rectum, the concentration of C_{10} at the jejunal mucosa should be considerably higher than the latter. The clinical efficacy and safety of GIPET® formulations in oral delivery of a range of unrelated poorly permeable drugs has recently been reviewed [24, 25]. Briefly, GIPET® improved the oral F of LMWH to between 3.9-7.6% relative to s.c. administration [24]. Oral F of LMWH in humans and dogs following intra-jejunal intubation of GIPET-LMWH® was approximately 10 % and 20 %, respectively [25]. It is worth pointing out that these estimates of oral F for LMWH are based on pharmacodynamic measurements and are therefore based on indirect metrics.

The impact of GIPET® on oral absorption of the gonadotropin-release hormone antagonist, acyline (Mer-104), was first demonstrated in a preclinical *in situ* duodenal instillations in beagle dogs [227].The formulation increased F of acyline versus the un-enhanced formulation by 5-16 fold. In a subsequent human trial, single dose administration of acyline in a GIPET® formulation suppressed serum gonadotropin and testosterone biosynthesis in normal men [228]. In a Phase I study, GIPET® also improved the oral F of the bisphosphonate, alendronate, 12-fold compared to alendronate sodium tablets (Fosamax®, Merck), to yield an oral F of 7.2 % based on urinary excretion data of the unchanged molecule [24, 229]. The absorption-promoting action of GIPET® in humans complemented studies in dogs where oral and intra-jejunal delivery of alendronate- GIPET® improved permitted an oral F of 2.3 % and 3.3 %, respectively [25].

Zoledronic acid (Zometa®, Norvartis, Switzerland) is a bisphosphonate drug that is used to reduce the potential for skeletal related events caused by the debilitating effects of bone metastases. Since it has very poor oral F, Zometa (4mg) is normally delivered once monthly by a 15 min i.v. infusion, which requires continuous involvement of healthcare professionals and bisphosphonate-naïve is inconvenient to patients. Nephrotoxicity is related to the Cmax of the drug and occurs at this dose and infusion rate in some patients. Preclinical studies in beagles demonstrated that intra-duodenal delivery of GIPET® improved the absolute oral F of zoledronic acid to 7-10%, as measured via urinary excretion [230]. In a recent subsequent multi-center Phase II study in hormone-refractory prostate cancer patients, once-weekly zoledronic acid (20mg) administered orally in a

GIPET® solid dosage form (Orazol®) had similar bioavailability as the marketed parenteral preparation delivered once-monthly [231]. In addition, there was no significant difference in the primary endpoints: blood and urine biomarkers of bone metabolism between Orazol® and the reference group. A study in healthy human volunteers established that the percent urinary excretion of zoledronic acid from Orazol® was comparable to that of the Zometa® infusion (Fig. 6).

In each of the Phase I and II studies using GIPET® the formulations were well tolerated (Table IX). Importantly, the alendronate-GIPET® formulation permits one twelfth of the dose of the bisphosphonate to be used compared to the marketed form of alendronate (Fosamax®, Merck, UK). The large reduction in the alendronate dose appeared to reduce gastric- and oesophageal-related toxicity. This formulation may eventually promote better compliance since patients were not required to take the medication with a full glass of water or remain upright for over 30 min after administration according to established dosing requirements [25]. In a total of 16 Phase I studies involving 300 volunteers, some 800 exposures to GIPET® did not induce measureable toxicity; indeed in some cases individual patients were safely dosed up to six times each [24]. In routine blood biochemical assessments, a single dose administration of GIPET® to healthy volunteers did not lead to abnormal haematology, clinical chemistry or hepatic function [228]. In the Phase II study of Orazol® referred to above, GIPET® was administered once weekly for 8 weeks and was well tolerated with no abnormalities in urinalysis, haematology or clinical chemistry. These data closely reflect the safety data for GIPET® observed in dogs (see Table VIII).

[4] Perspective on the safe and effective use of C_{10} in oral drug delivery

The promoter with the greatest efficacy *in vitro* or *in situ* is not necessarily the best candidate for clinical assessment [169]. Many additional factors impact on the ultimate potential of promoters, including safety, effectiveness and stability in the GI tract, and the kinetics of intestinal absorption of the promoter itself. Promoters that solely increase paracellular permeability through the transient opening of TJs might be considered to be potentially safer than ones with multiple mechanisms of action because they activate physiological-based processes that may not lead to mucosal epithelial damage (reviewed [18-20, 66, 232]). TJ-specific candidates include C-terminal of C-CPE [67], Zot [14], the rotavirus spike protein VP8 [233] as well as a number of engineered peptide-based promoters [20, 68]. However, the TJs of the small intestine are relatively leaky and there is a view that paracellular promoters might not be able to open TJs any further *in vivo* [137]. It is also likely that a degree of perturbation of the apical membrane of enterocytes is necessary to promote significant drug absorption across the small intestine in man and that TJ openings *per se* will not be adequate. Another concern with the use of some absorption promoters is their safety in the event of systemic absorption, in contrast to the well-accepted safety profile of well-absorbed dietary C_{10} [87, 88].

C_{10} has the capacity to cause superficial damage to the intestinal epithelium *in vivo*. Despite this, data from the initial clinical trials and from repeat-dosing studies in a range of species suggest that C_{10} should be safe for use in high concentrations in oral dosage forms that may need to be given repeatedly even on a long-term basis. However, it might not be advisable to administer C_{10} formulations contemporaneously with other drugs that

have the potential to elicit gastrointestinal side effects (e.g. NSAIDs, alcohol), nor to subjects with inflammatory bowel disease where basal intestinal permeability is already increased. In one human study where C_{10} was coadministered with glycyrrhizin in a rectal suppository, there was a higher degree of rectal irritation at a much lower concentration of C_{10} than in most of the studies reported for C_{10} alone ([221] and Table IX). It is notable that the repeat administration of C_{10}-ISIS 301012 oral tablets to human subjects for up to 90 days yielded just mild intermittent gastrointestinal symptoms [123].
Although epithelial repair after jejunal exposure of C_{10} is extremely rapid in healthy volunteers, this could potentially be offset by disease, other medications, and excessive bile secretion [181].

The current safety profile of C_{10} compares well with other dietary constituents and endogenous secretions, and is significantly better than agents including aspirin, paracetamol, alcohol and codeine [122, 203, 234, 235]. Importantly, the damage and repair cycle that occurs through continuous exposure to bile salts is a physiologically-normal phenomenon, so similar effects likely to be induced by C_{10} should be viewed with concern [169]. Indeed, in a study examining the effects of bile salts and dietary fatty acids on the mucosal surface, it was concluded that mucosal injury might be caused by the normal hydrolytic digestion of fat following the intake of a fatty meal [201]. Finally, it is noteworthy that many other formulation excipients that are well-established (e.g. Witepsols) could have greater toxicity compared to C_{10} [122, 203].

The co-absorption of antigens is a legitimate safety concern with the use of intestinal absorption promoters. In a vaccination study in the African catfish, orally-administered C_{10} increased *Vibrio anguillarum* O2 serum antigen- and antibody levels [236]. Similar effects were demonstrated with sodium salicylate and with the vitamin E analogue, TPGS. Alcohol consumption has been shown to increase plasma endotoxin levels in alcoholics, presumably by increasing intestinal permeability [202]. The absorption of bystander antigens is not necessarily a pathophysiological process, since antigens are routinely absorbed into the portal blood and sequestered by Kupffer cells in the liver [192]. However, since endotoxemia can progress to endotoxic shock, further studies are required to assess whether C_{10} causes an increase in plasma endotoxin levels. Studies in man would indicate this is not the case, since delivery of the promoter to the bacteria-rich rectum in suppositories has not been reported to increase plasma endotoxin levels, even though absorption by this route partially bypasses first pass metabolism. Another area that may warrant further study is the effect of the promoter on translocation of viral particles. Rectal administration of a non-ionic surfactant with the *Salmonella* 0-1 Felix phage in rabbits rapidly increased the serum viral load [237]. Overall, the intestinal histological data in relation to C_{10} would suggest that any induced reversible mucosal-damage would not be at the level induced by NSAIDs, alcohol or even many other formulation excipients. *In vivo* data in humans indicate that increases in intestinal permeability induced by C_{10} are a fraction of those seen with NSAIDs, both in magnitude as well as in respect of the time course. The increased epithelial permeability as defined by the lactulose:mannitol urinary excretion assay in healthy volunteers taking aspirin ranged from 146%-1967% [238-241] greater than control subjects, yet the increase with

GIPET® was 50% (data not shown). While aspirin is not considered to be a more effective intestinal absorption promoter than C_{10}, such studies suggest that the promoting actions of either monomeric, micellar or vesicular C_{10} could relate to its physical interaction with the candidate drug. A final consideration is that coincidental enhanced absorption of a second drug with a narrow therapeutic index could potentially result in over-exposure [169, 192]. Such potential drug interactions are unrealistic however, since it is unlikely that the co-administered marketed drug would be poorly absorbed *per se*, and it is even less likely that it would leave the stomach at the same time as the solid dose enteric-coated formulation. It would then require a similar GI tract transit time as the original payload so as to ensure presence at the same small intestinal epithelial region just as C_{10} was being released.

[5] Conclusions

Evidence from preclinical and clinical studies indicate that C_{10} in solid dosage forms, designed for the initial simultaneous release of high concentrations of promoter and cargo, can effectively increase the oral F of Class III drugs, including peptides, oligonucleotides and polysaccharides, as well as poorly-absorbed antibiotics and bisphosphonates. From the standpoint of efficacy, significant oral F has been achieved for selected low MW molecules with a relatively narrow intra-subject variation. Mode of action studies suggest the actions of the promoter *in vivo* using high concentrations pertain to a transient transcellular perturbation in addition to the well-studied paracellular pathway. It seems that damage caused to the intestinal epithelium of enterocytes may not be physiologically-relevant as it is transient, mild and reversible. Finally, although some of the safety concerns with the use of absorption promoters have been addressed in recent clinical studies and the marketed antibiotic suppositories, certain safety aspects may require additional study. These include the effect of repeat administration, as well as the effect of the promoter on the absorption of other drugs, luminal antigens and nutrients.

Acknowledgements and disclosure

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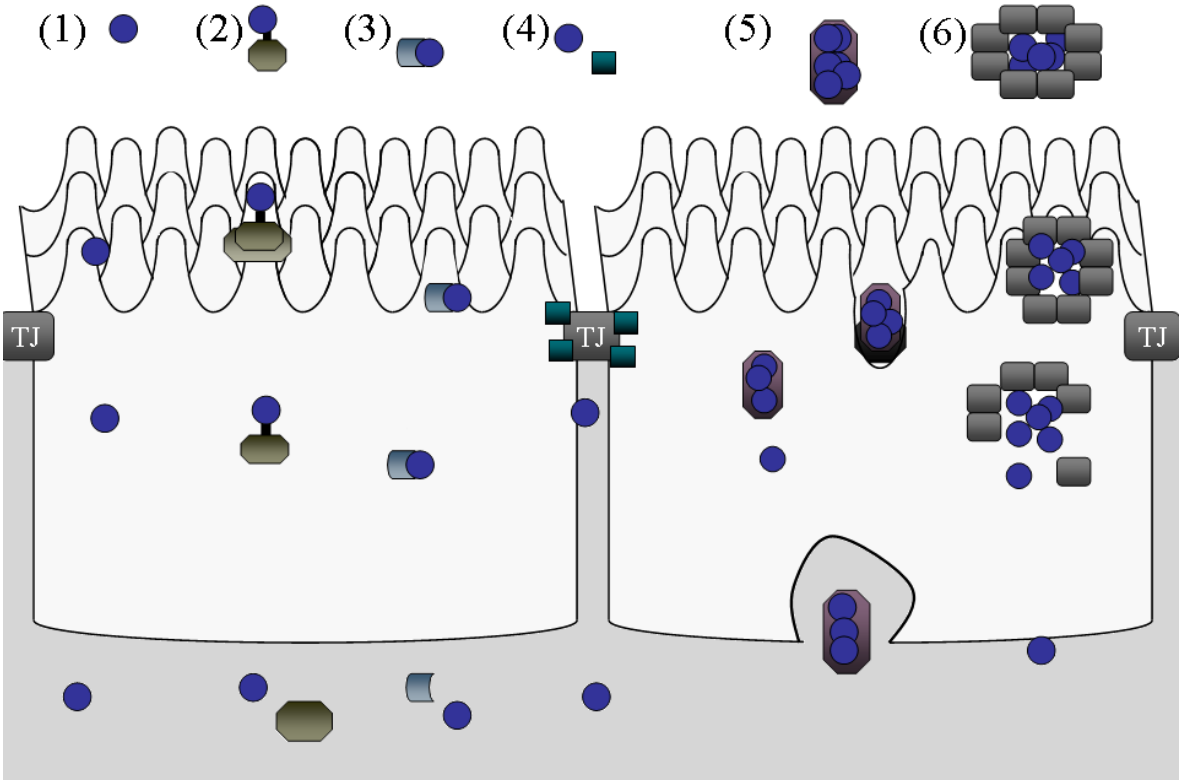
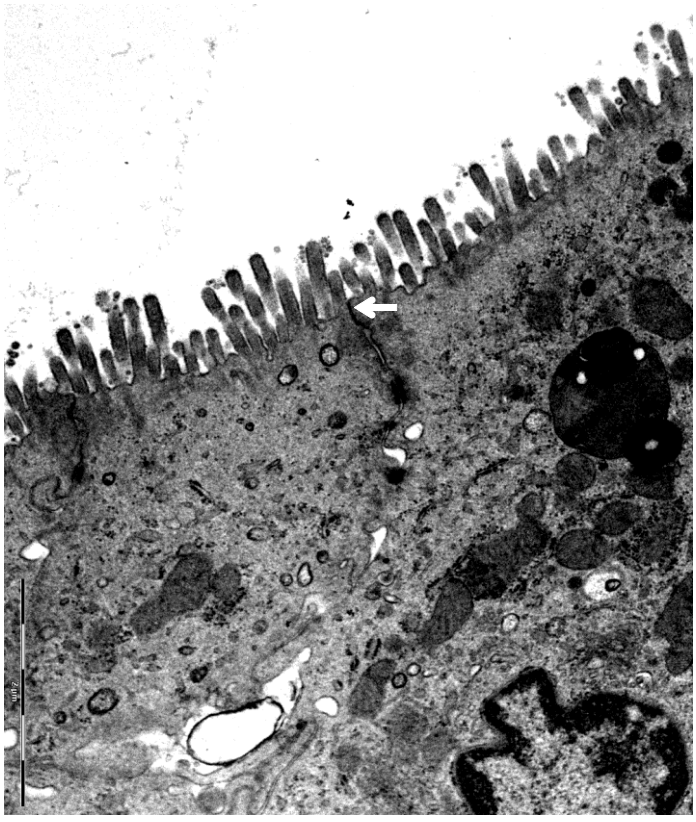
Fig. 1:

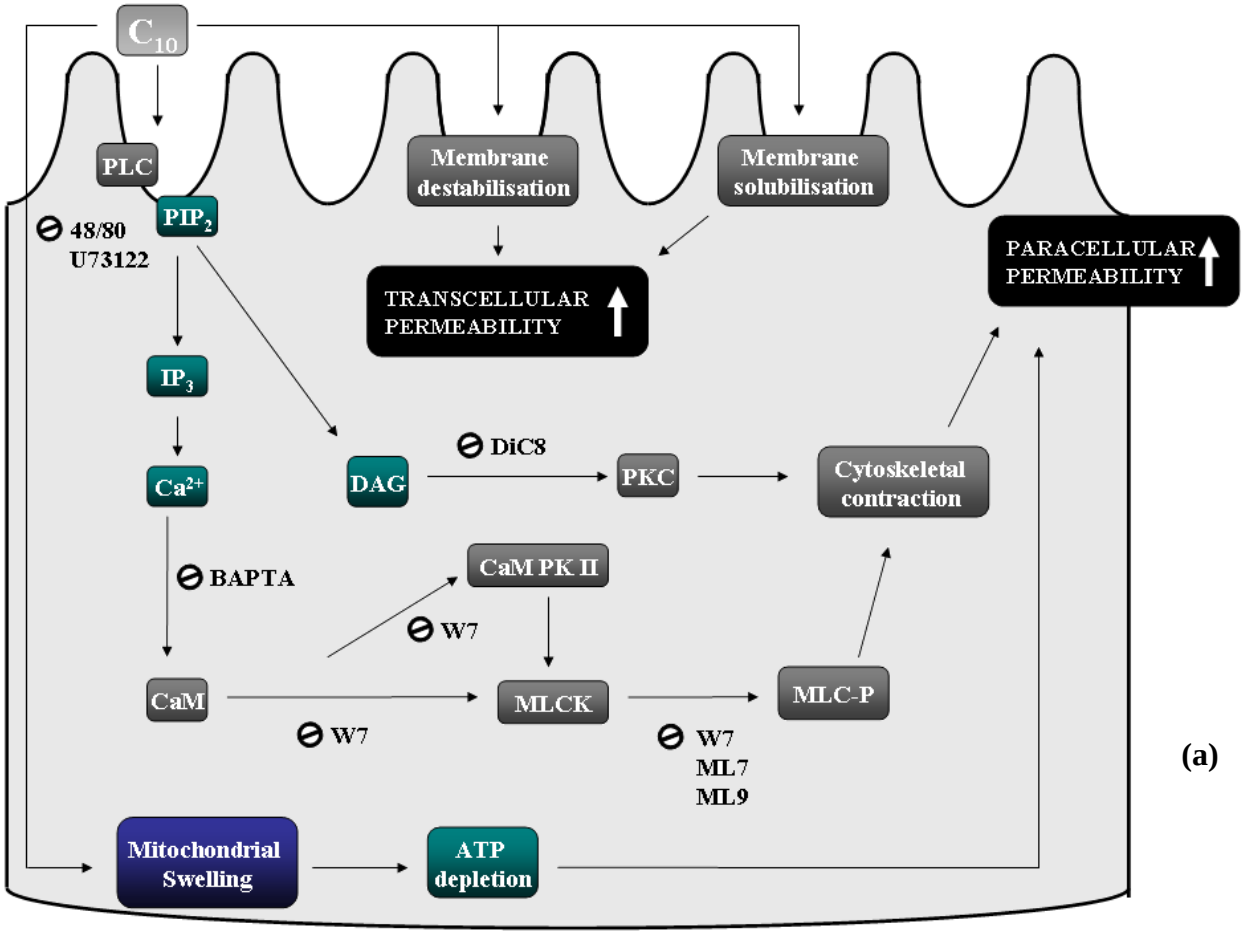


(a)



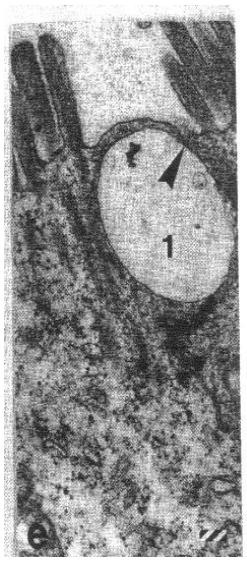
(b)

Fig. 2:

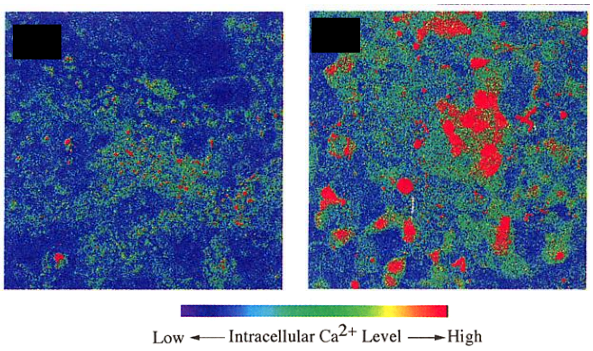

C10
PLC
PIP2
48/80
U73122
Membrane destabilisation
Membrane solubilisation
TRANSCELLULAR PERMEABILITY
PARACELLULAR PERMEABILITY
IP3
Ca2+
DAG
DiC8
PKC
Cytoskeletal contraction
BAPTA
CaM PK II
W7
CaM
MLCK
MLC-P
W7
W7
ML7
ML9
Mitochondrial Swelling
ATP depletion

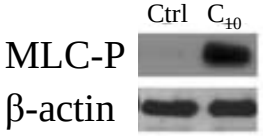
(a)

(b)



(c)


Low ← Intracellular Ca2+ Level → High


Ctrl
C10
MLC-P
β-actin

(d)

Fig. 3:

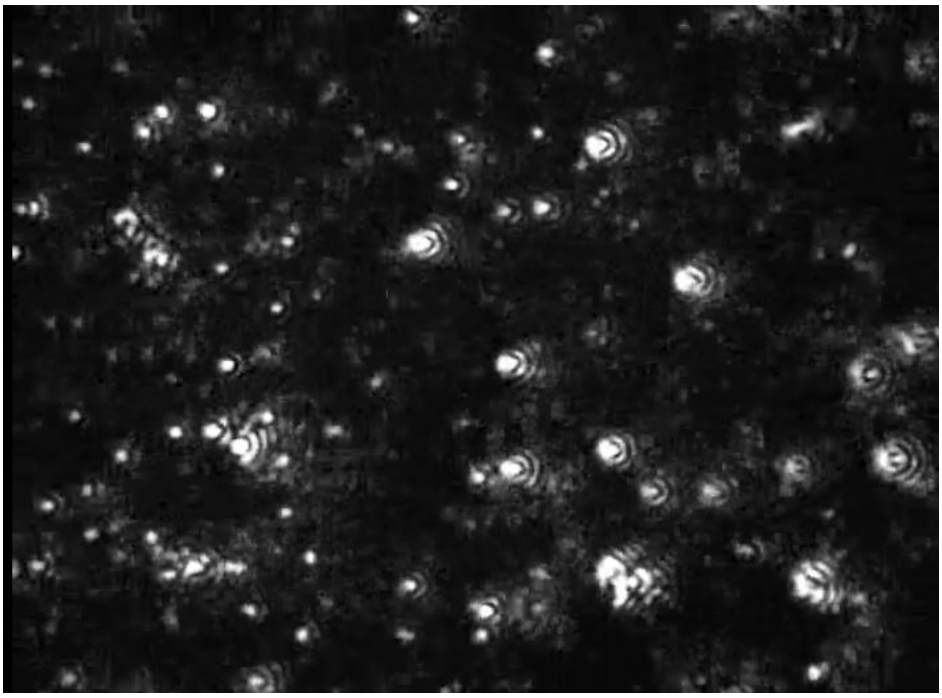


Fig. 4:

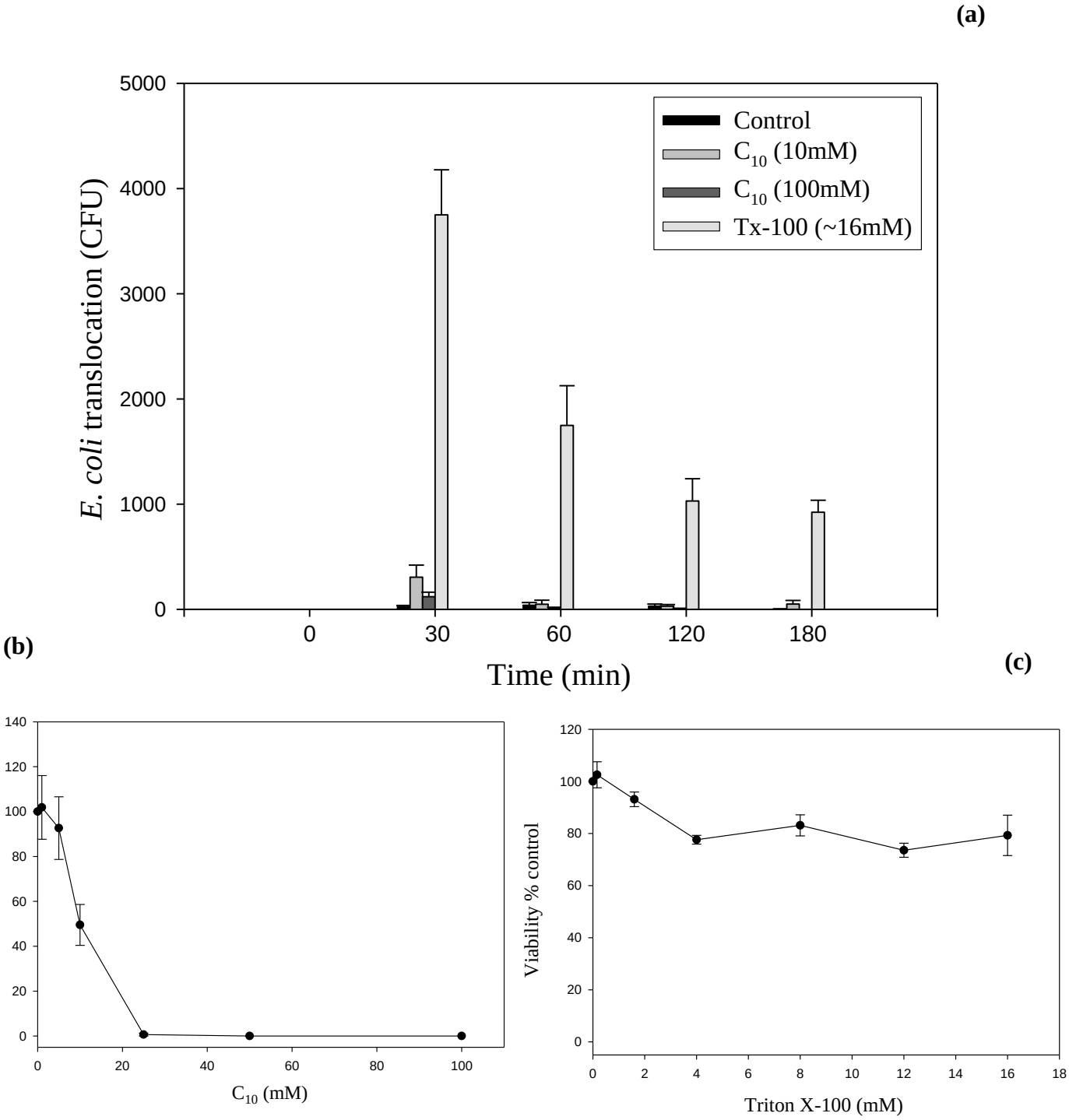

(a)
5000
4000
3000
2000
1000
0
E. coli translocation (CFU)
Control
C10 (10mM)
C10 (100mM)
Tx-100 (~16mM)
0
30
60
120
180
Time (min)
(b)
140
120
100
80
60
40
20
0
Viability % control
0
20
40
60
80
100
C10 (mM)
(c)
120
100
80
60
40
20
0
Viability % control
0
2
4
6
8
10
12
14
16
18
Triton X-100 (mM)

Fig. 5:

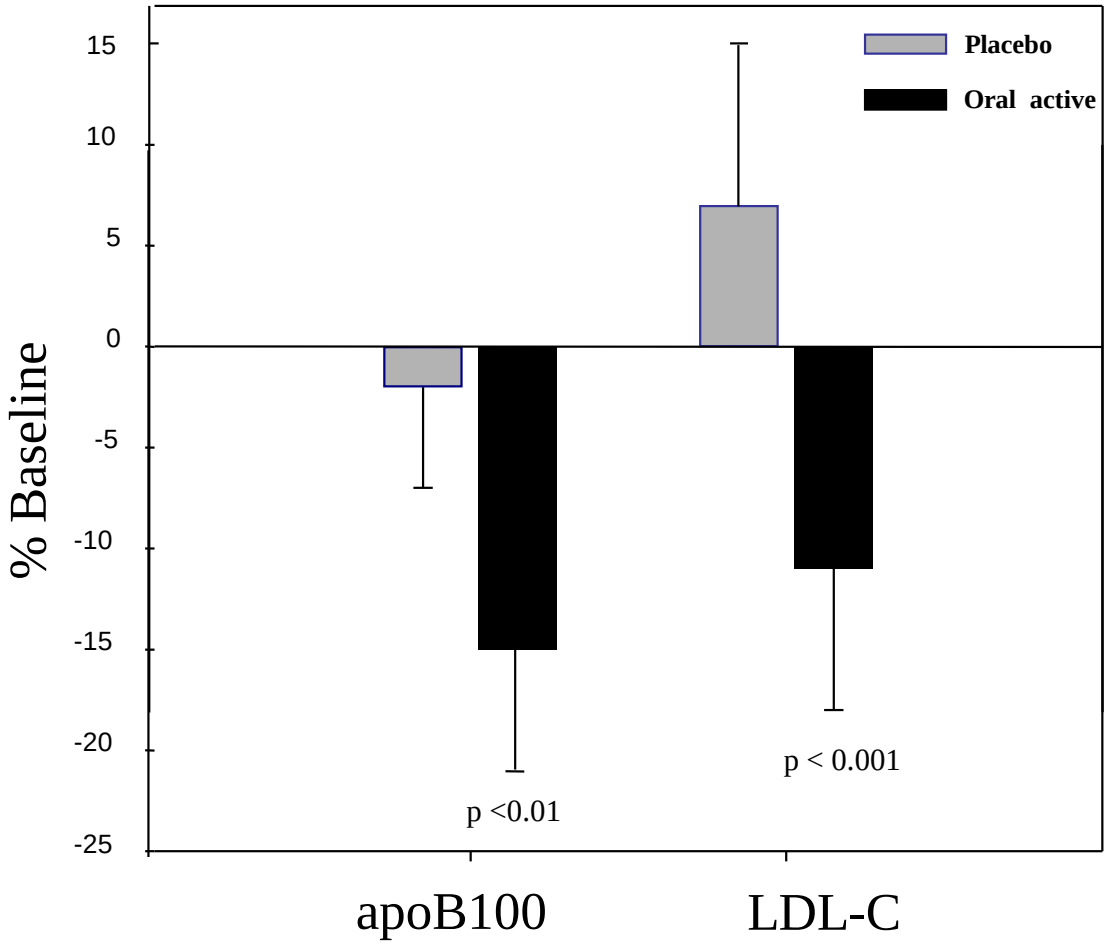

Placebo
Oral active
% Baseline
15
10
5
0
-5
-10
-15
-20
-25
p <0.01
p < 0.001
apoB100
LDL-C

Fig. 6:

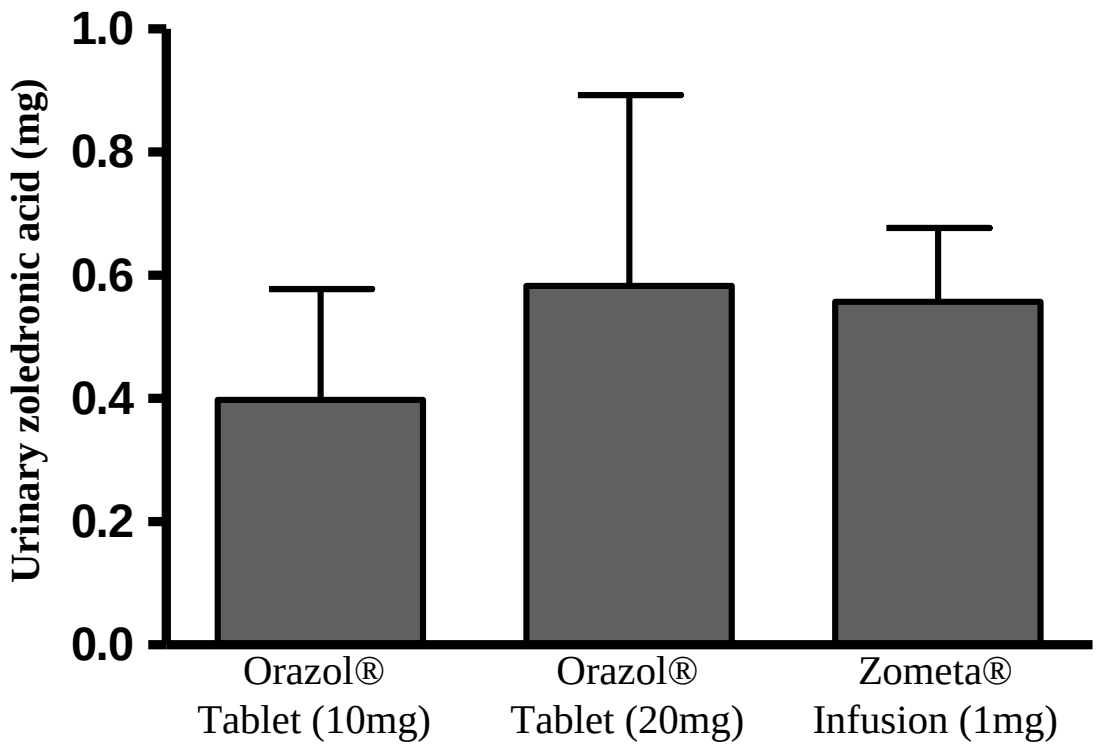

Urinary zoledronic acid (mg)
1.0
0.8
0.6
0.4
0.2
0.0
Orazol® Tablet (10mg)
Orazol® Tablet (20mg)
Zometa® Infusion (1mg)

Figure Legends

Fig. 1: (a) Schematic representation of drug delivery systems that have been shown to increase transmucosal drug permeability. (1) Most oral drugs with the desired solubility are normally absorbed passively by transcellular path without the need for a drug delivery system, (2) receptor mediated prodrug formulation (e.g. valaciclovir) (3) modified solubility prodrug formulation (e.g. enalapril), (4) Absorption promoters can increase permeability by paracellular permeability and/or by transcellular perturbation (not shown in this Fig.) (e.g. C_{10}) (5) Receptor mediated nanoparticle endocytosis (e.g. vitamin B12) and (6) Carrier-based drug delivery systems (e.g. Eligen®, Emisphere, USA) (b) EM of a cross section of the human colonic mucosa with intact transcellular and paracellular barriers. Single arrow head denotes tight junction. Vertical bar denotes 2µm.

Fig. 2: (a) Schematic representation of the mechanisms through which C_{10} is thought to promote drug absorption across the intestinal epithelium, (b) shows induction of TJ dilation by C_{10} (10mM) across Caco-2 monolayers, (c) increased intracellular calcium induced by C_{10} in Caco-2 monolayers (arrow head) and (d) C_{10} (10mM) induction of myosin light chain phosphorylation in isolated rat colonic mucosae mounted in Ussing chambers [96, 145, 158]**.**

Fig. 3: Polydisperse particulate structures of C_{10} formed in saline at 200mM. Motion of particles can be viewed in the Supporting Information (motion of particles recorded by Nanosight LM20, NanoSight Ltd, Amesbury, UK).

Fig. 4: Do promoters increase translocation of commensal bacteria across the intestinal epithelium as they promote absorption of poorly absorbed solutes? (a) Effect of C_{10} (10-100mM) and Triton-x-100 (16mM) on the translocation of *E. coli* (pHKT3 [285]) across Caco-2 monolayers over 3 hours, (b-c) Effect of C_{10} and Triton-x-100 on the viability of *E. coli* (pHKT3) measured with an antimicrobial assay from the Clinical and Laboratory Standards Institute (Maher S and Brayden DJ, unpublished data).

Fig. 5: Oral delivery of ISIS 103012, antisense to apolipoprotein B, compared to placebo control in human patients over 55 and 69 days [124]. The primary endpoint was a measure of the mean (trough) plasma LDL-cholesterol and apolipoprotein B. Oral ISIS 301012 was formulated in an enteric coated pulsatile formulation with C_{10}. The absorption of ISIS 301012 in the absence of the promoter is assumed to be negligible.

Fig. 6: Comparison of the urinary excretion of zoledronic acid following oral delivery in GIPET® (10-20mg Orazol® tablet, n=12) and i.v. administration (Zometa® infusion, n=11). Values are given as mean ± standard deviation from the mean.

Table I: Permeating-enhancement properties of C_{10} in Caco-2 monolayers

Marker/Drug	C_{10} (mM)	Enhancement ratio	Ref
Clodronate	10	<1†	[242]
Mannitol	0.75	1	[243]
Mannitol	13	15†	[96]
Mannitol	5	1.3†	[143]
Mannitol	10	1†	[242]
Mannitol	10	6.3†	[145]
Mannitol	13	9	[244]
Mannitol	13	12	[107]
Mannitol	10	8	[142]
Mannitol	16	7.7	[245]
Mannitol	10	5†	[164]
Mannitol	50	66†	[99]
Mannitol	50	64†	[95]
Decapeptide	25	3†	[99]
Atenolol	13	20†	[246]
Danshensu	13	>13†	[246]
Salvianolic acid B	13	>40†	[246]
Lucifer yellow	5	>10†	[143]
Ardeparin	13	7.3	[244]
rhEGF	50	10.6†	[95]
Fluorescein	5	1.4	[154]
Fluorescein	10	2.7†	[247]
Fluorescein	13	3†	[97]

FD4	5	0.9	[154]
FD4	5	>10†	[143]
FD4	1	1.7	[101]
FD4	10	16.5†	[247]
FD4	10	3†	[158]
FD4	10	6.5	[248]
FD4	10	10.6†	[182]
FD4	13	6†	[155]
FD4	13	5.5†	[151]
FD4	13	37	[107]
FD4	50	4.3†	[249]
FD10	5	>10†	[143]
FD20	5	>10†	[143]
FD20	13	56	[107]
FD40	5	>10†	[143]
Acamprosate	16	2.3†	[245]
Rhodamine	10	4.6†	[247]
Inulin	50	70†	[95]
PEG 900	25	>10†	[99]
Cyclopeptide	10	17†	[182]
Penicillin G	13-16	—	[96]
Penicillin V	10	>2	[187]
Penicillin V	10	8.5	[145]
Penicillin V	10	16	[93]
Cimetidine	50	20†	[95]
Heparin	10	2.3†	[94]

Vasopressin	13	10	[107]
Epirubicin	10	1.4†	[250]
PEG 326	10	5	[107]
PEG 546	10	17	[107]
Streptokinase	10-20	>50	[185]
PEG 4000	13	3.5	[251]

† Drop in TEER across monolayers treated with C_{10}

Table II: Permeating-enhancing properties of C_{10} using *ex vivo* models of the GI tract.

Species	Intestinal Region	Model	Marker/Drug	C_{10} (mM)	Enhancement Ratio	Ref
Mouse	Colon	Ussing	Mannitol	10	3†	[158]
Rat	Stomach	Ussing	Ardeparin	13	1.1	[244]
Rat	Duodenum	Ussing	Ardeparin	13	1.3	[244]
Rat	Jejunum	Ussing	Ardeparin	13	1.3	[244]
Rat	Jejunum	Ussing	Ebiratide	20	1.5	[135]
Rat	Jejunum	Ussing	Insulin	20	0.97	[136]
Rat	Jejunum	Ussing	Phenol red	20	1.1	[102]
Rat	Jejunum	Everted sac	Epirubicin	100	4.5	[250]
Rat	Jejunum	Everted sac	Cefotaxime	13	4.7	[171]
Rat	Jejunum	Everted sac	Ceftazidime	13	1.8	[171]
Rat	Ileum	Ussing	Ardeparin	13	1.3	[244]
Rat	Ileum	Ussing	Mannitol	30	80	[207]
Rat	Ileum	Ussing	EDTA	10	4.5†	[103]
Rat	Ileum	Ussing	Poly-sucrose	10	10†	[103]
Rat	Ileum	Everted sac	Epirubicin	100	2	[250]
Rat	Colon	Ussing	Ardeparin	13	1.6	[244]
Rat	Colon	Ussing	Insulin	20	2.5	[136]
Rat	Colon	Ussing	Phenol red	20	4	[102]
Rat	Colon	Ussing	Phenol red	—	7.6	[252]
Rat	Colon	Ussing	Ebiratide	20	3.8	[135]
Rat	Colon	Ussing	Mannitol	10	7†	[104]

Rat	Colon	Ussing	Mannitol	10	3.9†	[158]
Rat	Colon	Ussing	Mannitol	13	11†	[11]
Rat	Colon	Ussing	FD4	10	25†	[104]
Rat	Colon	Ussing	FD4	26	31†	[157]
Rat	Colon	Ussing	FD70	10	44†	[104]
Rat	Colon	Everted sac	Inulin	13	>5	[188]
Rat	Rectum	Franz cell	Propranolol	—	1.5	[253]
Rabbit	Jejunum	Ussing	Inulin	13	1	[137]
Rabbit	Jejunum	Ussing	Mannitol	13	1	[137]
Rabbit	Jejunum	Ussing	Thiourea	13	<1	[137]
Rabbit	Colon	Ussing	Gly-L-Phe	50	>2	[254]
Human	Ileum	Ussing	EDTA	10	7†	[146]
Human	Colon	Ussing	Mannitol	10	5†	[104]
Human	Colon	Ussing	FD4	10	7†	[104]
Human	Colon	Ussing	FD4	26	17†	[157]
Human	Colon	Ussing	EDTA	10	2†	[105]
Human	Colon	Ussing	HRP	10	2†	[105]

† Drop in TEER across mucosae treated with C_{10}

Table III: Absorption-promotion properties of C_{10} in animal models.

Species	Marker/Drug	Intestinal Region	C_{10} (mM)	Model	Enhancement Ratio	Ref
Mouse	Ampicillin	Rectum	—	Suppository	—	[255]
Mouse	Ceftizoxime	Rectum	—	Suppository	—	[255]
Rat	Insulin	Duodenum	50	*In situ* loop	1	[256]
Rat	LMWH	Duodenum	—	Instillation	24	[257]
Rat	Calcein	Duodenum	—	Instillation	19*	[180]
Rat	*O. japonicus* polysaccharide	Duodenum	—	Solid dosage delivery	5-20	[258]
Rat	Cefoxitin	Jejunum	50-100	Perfusion	>5	[113]
Rat	Cefotaxime	Jejunum	13	Single pass perfusion	3.1	[189]
Rat	Danshensu	Jejunum	25	Single pass perfusion	3.3	[246]
Rat	Salvianolic acid B	Jejunum	25	Single pass perfusion	1.1	[246]
Rat	Acamprosate	Jejunum	16	Perfusion	2	[245]
Rat	Mannitol	Jejunum	50	Single pass perfusion	9	[106]
Rat	Carboxyfluorescein	Jejunum	20	*In situ* loop	1.4	[139, 259]
Rat	Insulin	Jejunum	50	*In situ* loop	—	[256]
Rat	FD4	Jejunum	50-125	*In situ* loop	3-13	[260]
Rat	FD4	Jejunum	205	*In situ* loop	12.7	[67]
Rat	FD4	Jejunum	103	*In situ* loop	4.7	[261]
Rat	Cefmetazole	Jejunum	13	*In situ* loop	6	[137]
Rat	Phenol red	jejunum	20	*In situ* loop	1.2	[262]
Rat	phenolsulfonphtalein	Jejunum	100	*In situ* loop	12	[263]

Rat	Fosphomycin	Jejunum	50	*In* situ loop	6	[109]
Rat	FD4	Jejunum	250	Instillation	2	[127]
Rat	FD4	Jejunum	100	Instillation	33	[106]
Rat	Sulpiride	Jejunum	—	Solid dosage delivery	2.5	[112]
Rat	Sulpiride	Jejunum	—	Solid dosage delivery	>6	[119]
Rat	Carboxyfluorescein	Ileum	20	*In situ* loop	2.5	[139, 259]
Rat	Insulin	Ileum	50	*In situ* loop	1.7	[256]
Rat	FD4	Colon	100	Instillation	33	[106]
Rat	FD70	Colon	100	Instillation	<1	[106]
Rat	sCT	Colon	5	Instillation	—	[154]
Rat	Carboxyfluorescein	Colon	30	Instillation	5	[134]
Rat	Phenol red	Colon	—	Solid dosage delivery	4	[252]
Rat	FD4	Colon	20	Single pass perfusion	4.5	[101]
Rat	Cefmetazole	Colon	13	*In situ* loop	10	[108]
Rat	Phenol red	Colon	100	*In situ* loop	2.8	[264]
Rat	Glycyrrhizin	Colon	100	*In situ* loop	37	[264]
Rat	FD4	Colon	100	*In situ* loop	85	[264]
Rat	FD4	Colon	103	*In situ* loop	6.7	[261]
Rat	FD4	Colon	205	*In situ* loop	12.7	[67]
Rat	FD10	Colon	100	*In situ* loop	1587	[264]
Rat	FD40	Colon	100	*In situ* loop	193	[264]
Rat	Phenolsulfonphtalein	Colon	100	*In situ* loop	7.7	[263]
Rat	Rebamipide	Colon	100	*In situ* loop	18	[265]
Rat	Carboxyfluorescein	Colon	20	*In situ* loop	13	[139, 259]
Rat	Insulin	Colon	50	*In situ* loop	9	[256]

Rat	Cefmetazole	Colon	50	*In situ* loop	11.6	[188]
Rat	Fosphomycin	Colon	50	*In situ* loop	35	[109]
Rat	Phenol red	Colon	20	*In situ* loop	2.7	[266]
Rat	Antipyrine	Rectum	50	Recirculated perfusion	1.7	[206]
Rat	P-aminobenzoic acid	Rectum	—	*In situ* loop	—	[116]
Rat	Phenolsulfonphtalein	Rectum	—	*In situ* loop	—	[116]
Rat	Trypan blue	Rectum	—	*In situ* loop	—	[116]
Rat	FD4	Rectum	—	*In situ* loop	—	[116]
Rat	FD10	Rectum	—	*In situ* loop	—	[116]
Rat	hEGF	Rectum	100	*In situ* loop	>68	[26]
Rat	Decapeptide	Rectum	500	Instillation	5	[99]
Rat	Glycyrrhizin	Rectum	50	Instillation	2.7	[267]
Rat	Erythropoietin	Rectum	515	Instillation	>300	[111]
Rat	Insulin	Rectum	50	Instillation	24	[110]
Rat	Carboxyfluorescein	Rectum	100	Instillation	>20	[268]
Rat	Phenolsulfonphtalein	Rectum	—	Instillation	5	[92]
Rat	P-aminobenzoate	Rectum	—	Instillation	15	[92, 218]
Rat	Ampicillin	Rectum	—	Instillation	50	[92, 218]
Rat	Ampicillin	Rectum	160	Infusion	9.9	[269]
Rat	Ampicillin	Rectum	154	Infusion	26	[270]
Rat	Cefoxitin	Rectum	220	Infusion	>9	[271]
Rat	Cefoxitin	Rectum	—	Suppository	2.4	[159]
Rat	Acyclovir	Rectum	—	Suppository	2.2	[272]
Rat	Atenolol	Rectum	—	Suppository	1.4	[273]
Rat	5-fluorouracil	Rectum	—	Suppository	3.7	[128]
Rat	Ampicillin	Rectum	—	Suppository	6	[179]

Rat	Ampicillin	Rectum	—	Suppository	—	[255]
Rat	Ceftizoxime	Rectum	—	Suppository	—	[255]
Rat	Antipyrine	Rectum	50	Circulated perfusion	1.7	[179]
Rat	Cefotaxime	Oral	—	Oral	2.4	[189]
Rat	DMP 728	Oral	—	Oral	2.5	[125]
Rat	DMP 532	Oral	—	Oral	1.1	[274]
Rat	Acamprosate	Oral	—	Oral	2	[245]
Rat	Ardeparin	Oral	—	Oral	1.4	[244]
Rat	Insulin	Oral	—	Oral	3.6	[275]
Rat	Danshensu	Oral	—	Oral	1.6	[246]
Rat	Salvianolic acid B	Oral	—	Oral	1.4	[246]
Rat	Glycyrrhizin	Oral	200	Oral	63	[267]
Rabbit	Rebamipide	Rectum	—	Suppository	41	[276]
Rabbit	Eel calcitonin	Rectum	—	Suppository	1.6	[112]
Rabbit	Atenolol	Rectum	—	Suppository	1.5	[273]
Rabbit	Vancomycin	Rectum	—	Suppository	3.7	[277]
Rabbit	Ampicillin	Rectum	—	Suppository	—	[255]
Rabbit	Ceftizoxime	Rectum	—	Suppository	—	[255]
Rabbit	Norfloxacin	Oral	—	Oral	1.7	[278]
Dog	Acyline	Duodenum	—	Instillation	5.5-15.9	[227]
Dog	Zoledronic acid	Duodenum	—	Instillation	—	[230]
Dog	Alendronate	Duodenum	—	Intubation	3.3	[25]
Dog	LMWH	Duodenum	—	Intubation	—	[25]
Dog	Alendronate	Oral	—	Oral	2.3	[25]
Dog	Isis oligonucleotides	Oral	—	Oral	—	[194]
Dog	DMP 728	Oral	—	Oral	3.8	[274, 279]

Dog	DMP 728	Oral	—	Oral	1.4	[125]
Dog	Uracil	Rectum	—	Instillation	—	[280]
Dog	Ampicillin	Rectum	—	Suppository	—	[255]
Dog	Ceftizoxime	Rectum	—	Suppository	—	[255]
Pig	ISIS olionucleotides	Jejunum	—	Instillation	—	[115]
Cattle	Ampicillin	Rectum	—	Suppository	3.5	[281]

Data were pooled from literature that examined enhancement in a number of *in situ* and *in vivo* models. In some studies, the local concentration of C_{10} and enhancement ratios cannot be calculated although they do demonstrate improved delivery of marker/drug.

Table IV: Absorption-promotion properties of C_{10} in human trials.

Marker/Drug	C_{10} (mM)	Model	Enhancement Ratio	Ref
LMWH	—	Intrajejunal intubation	—	[25]
Ceftizoxime	—	Rectal suppository	—	[216]
Ceftizoxime	—	Rectal suppository	—	[219, 220]
Ampicillin	—	Rectal suppository	—	[219, 220]
Ampicillin	—	Rectal suppository	1.8	[22]
Ampicillin	—	Rectal suppository	—	[223]
Glycyrrhizin	—	Rectal suppository	—	[221]
Cefoxitin	—	Rectal suppository	2.4	[168]
Penicillin V*	18	Rectal perfusion	0.4	[215]
Antipyrine*	18	Rectal perfusion	0.9	[215]
Alendronate	—	Oral	—	[24]
LMWH	—	Oral	—	[24]
ISIS oligonucleotides	—	Oral	—	[26, 123]
Acyline	—	Oral	—	[228]
Zoledronic acid	—	Oral	—	[231]

*No improvement detected in these studies from same group

Table V: CMC reported for capric acid or C_{10} in different buffers and by different methods of measurement.

Buffer	CMC (mM)	Temperature	Ref
Saline	140	20	[271]
Saline (0.9% w/v)	25-30	25-37	unpublished data
HEPES with NaCl and KCl	45	Not specified	[183]
HEPES with NaCl, KCl and cholate (5mM)	5	Not specified	[183]
HBSS with 25mM HEPES without Ca^{++}	13	22	[96, 97]
HBSS without Ca^{++}or Mg^{++}	13	Room temperature	[142]
HBSS without Ca^{++}or Mg^{++}	51	Not specified	[100]
HBSS without $CaCl_2$	28.8	37	[160]
HBSS	28.1	Not specified	[93]
Borate buffer (50mM) pH 9	80-100	25	[166]
Water	95.5-106	25-50	[282]
Water	50	22-25	[163]

Table VI: Cytotoxicity of C_{10} in Caco-2 monolayers

C_{10} (mM)	Cytotoxicity	Ref
13	● Membrane damage measured by uptake of PI ● No effect on enterocyte structure in TEM	[96]
50	● Morphological deterioration in light microscopy	[99]
26	● Membrane damage measured by uptake of trypan blue integrity test and protein and DNA release assays ● No cytotoxicty in neutral red dye uptake assay	[100]
1-10	● Conc-dependenct cytotoxicity and membrane damage measured by MTT conversion and LDH release assays, respectively	[101]
13-16	● Impaired respiration measured by a drop in cellular dehydrogenase activity and ATP levels ● Mitochondrial swelling (TEM)	[97]
5-10	● Membrane damage measured by bilateral LDH release	[143]
10-20	● Cytotoxicity measured by MTT conversion assay ● Irreversible membrane damage measured by trypan blue dye exclusion assay	[185]
10	● No cytotoxicity or membrane damage measured by MTT conversion and LDH release assays, respectively	[94]
10	● Membrane damage measured by LDH release and uptake of PI	[182]
10	● Focal enterocyte perturbation in anisotonic conditions in TEM ● No damage or PI uptake in isotonic conditions	[142]
10-13	● Cytotoxicity measured by MTT conversion assay	[187]
5-10	● Cytotoxicity measured by MTT conversion assay	[242]
10	● Cytotoxicity and membrane damage measured by MTT conversion and LDH release assays, respectively	[98]
5	● No Cytotoxicity or membrane damage when co-administered with glycyrrhizin as measured by Neutral red dye uptake assay and LDH release assays, respectively	[154]
5-10	● Cell lysis measured by LDH and alkaline phosphatase assays	[183]
10	● Focal enterocyte damage in TEM	[93]
10	● No effect on enterocyte structure in TEM	[107]
13-50	● Concentration-dependent cytotoxicity measured by MTT assay	[244]
25-40	● Concentration dependent cytotoxicity measured in MTT assay	[246]

Table VII: Cytotoxicity of C_{10} in isolated intestinal mucosae

Tissue	*Ex vivo* Model	C_{10} (mM)	Cytotoxicity	Ref
Rat jejunum	Everted sac	13	● Membrane damage measured by an increase in total protein release but significantly lower than positive control ● Mild mucosal damage in light microscopy	[171]
Rat jejunum	Sac	13	● No effect on rat jejunal enterocytes in TEM	[189]
Rat colon and jejunum	BBMV	10-13	● Membrane perturbation measured by release of CF from BBMV ● Disorder in both the lipid and protein domains in bilayers measured by fluorescent polarization studies	[108] [137] [171]
Rat ileum	Ussing	10	● No effect on enterocyte structure in TEM	[103]
Rat colon	Ussing	10	● Mild morphological damage in light microscopy and TEM ● Membrane damage measured by LDH release although significantly lower than positive control ● Inhibition of electrogenic chloride secretion induced by the cholinomimmetic, carbachol	[104]
Rat colon	Ussing	0.1-1	● Inhibition of electrogenic chloride secretion induced by carbachol and forskolin	[283]
Rat colon	Isolated crypts	1	Intracellular acidification	[283]
Rat colon	Ussing	10-20	● No significant increase in LDH release	[136]
Rat colon	Ussing	26	● Modulation of electrogenic ion transport	[156]
Rat jejunum and colon	Ussing	20	● Membrane damage in the jejunum and colon measured by protein release	[135]
Human colon	Ussing	10	● No effect on enterocyte structure in TEM ● Swelling of the mitochondria and endoplasmic reticulum in TEM ● Drop in short circuit current	[105]
Human ileum	Ussing	10	● Mitochondrial swelling (TEM) ● No enterocyte membrane damage ● Impaired respiration measured by a drop in cellular ATP levels and ECP	[146]

Human colon	Ussing	10	● Significant morphological damage in light microscopy and TEM ● Membrane damage measured by LDH release although significantly lower than positive control ● Inhibition of electrogenic chloride secretion induced by the cholinomimmetic, carbachol	[104]

Table VIII: Toxicity of C_{10} in animal models

Species	C_{10} (mM)	Toxicity	Ref
Mouse	—	● Capric acid LD50 129mg/kg by i.v. route	[84]
Mouse	—	● No Damage in histology after 13 weeks oral administration	[193]
Rat	—	● Capric acid LD50 3730mg/kg by oral route	[85]
Rat	—	● No damage to gastric mucosa in histology upon 150 day repeat oral exposure (~5g/kg/day)	[86]
Rat	25	● No release of phospholipids or proteins into the intestinal lumen in an *in situ* jejunal loop	[109]
Rat	13	● Release of phospholipids but not protein into the intestinal lumen in an *in situ* colonic loop	[108]
Rat	20	● Release of phospholipids and proteins into the intestinal lumen in an *in situ* colonic loop	[266]
Rat	—	● Release of LDH and protein into the intestinal lumen in an *in situ* jejunal loop	[261]
Rat	13	● Release of LDH into the intestinal lumen, but significantly less than positive control in an in situ single pass jejunal perfusion	[189]
Rat	20	● Release of protein into the intestinal lumen, but not phospholipids in jejunal in situ loop	[262]
Rat	205	● Release of LDH in an in situ jejunal loop ● Increased histological damage score in a in situ jejunal loop	[67]
Rat	205	● Mucosal damage in histology including erosion of the membrane structure in an in situ jejunal loop ● Release of LDH into the intestinal lumen in an in situ jejunal loop	[190]
Rat	—	● Release of protein into the intestinal lumen following solid dosage delivery to the colon ● No damage in histology following solid dosage delivery to the colon	[252]
Rat	5	● No damage in histology in an in situ colonic instillation	[154]

Rat	—	● No damage in histology in an in situ rectal instillation	[99]
Rat	—	● No damage in histology following oral administration	[244]
Rat	100	● Mucosal damage in histology including coagulation necrosis in an in situ colonic loop	[265]
Rat	100	● Rectal contraction in an in situ colonic instillation	[268]
Rat	400	● Increased histological damage score following rectal delivery ● Rectal contraction following rectal delivery	[191]
Rat	50-100	● Mild mucosal damage in histology and TEM in an in situ colonic instillation	[106]
Rabbit	—	● Rectal damage in histology including mucosal erosion, flattening of epithelial cells and inflammatory cell infiltration following insertion of a rectal suppository	[276]
Rabbit	—	● Irritation on uncovered rabbit belly following topical administration ● Severe corneal injury in rabbits following topical administration	[85]
Dog	—	● No damage in histology after oral administration ● No unusual finding in clinical chemistry after oral administration	[193, 194]
Dog†	—	● Emesis only at high concentrations (0.9g/kg/day) after oral administration ● No unusual findings in ECG, haematology, plasma biochemistry or urinalysis following oral administration ● No damage in histology following oral administration	[24]
Pig	—	● No damage in histology in an in situ jejunal instillation ● No abnormalities in post mortem examination in an in situ jejunal instillation	[115]
Cattle	—	● No adverse reaction was observed after in insertion of a rectal suppository	[281]

†GIPET^{-TM} formulation containing C_{10}

Table IX: Toxicity of C_{10} in humans

Route	Toxicity	Ref
Intra-jejunal	● No reported toxicity	[24]
Topical	● Irritant to human skin	[284]
Rectal*	● Increased histological damage score ● Mucosal damage in TEM including sloughing of cells, discontinuities in the epithelium and flattening of microvilli	[22]
Rectal	● Slight increase in scores on defecating sensation, but not pain, itching and burning	[191]
Rectal	● Diarrhea in 3.6-8.6% of patients receiving suppositories of containing ampicillin or ceftizoxime with witepsol H-5 and C_{10}	[219, 220]
Rectal	● Severe irritation to the rectal mucosa at the highest concentration tested	[221]
Rectal	● No adverse effects reported	[215]
Rectal	● Little or no irritation in 5 day repeat administration ● Slight sensation of a foreign body and defecation but no pain or burning at the highest dose tested ● No irritation in children	[216]
Rectal	● Gastrointestinal disturbances reported in minority of patients including diarrhoea, perianal pain and redness	[223]
Oral†	● No reported toxicity	[24]
Oral	● No adverse effects in routine physical examination ● No adverse effects in routine clinical chemistry tests	[26]
Oral	● Mild gastrointestinal disturbance upon repeated high dose administration	[123, 124]
Oral†	● No adverse effects in clinical chemistry tests	[228]
Oral†	● No reported toxicity	[231]

* Reported toxicity not directly attributable to C_{10}
† GIPET^{-TM} formulation containing C_{10}

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