



Systemic delivery of peptides by the oral route: Formulation and medicinal chemistry approaches

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ABSTRACT

In its 33 years, ADDR has published regularly on the potential of oral delivery of biologics especially peptides and proteins. In the intervening period, analysis of the preclinical and clinical trial failures of many purported platform technologies has led to reflection on the true status of the field and reigning in of expectations. Oral formulations of semaglutide, octreotide, and salmon calcitonin have completed Phase III trials, with oral semaglutide being approved by the FDA in 2019. The progress made with oral peptide formulations based on traditional permeation enhancers is against a background of low and variable oral bioavailability values of ~1%, leading to a current perception that only potent peptides with a viable cost of synthesis can be realistically considered. Desirable features of candidates should include a large therapeutic index, some stability in the GI tract, a long elimination half-life, and a relatively low clearance rate. Administration in nanoparticle formats have largely disappointed, with few prototypes reaching clinical trials: insufficient particle loading, lack of controlled release, low epithelial particle uptake, and lack of scalable synthesis being the main reasons for discontinuation. Disruptive technologies based on engineered devices promise improvements, but scale-up and toxicology aspects are issues to address. In parallel, medicinal chemists are synthesizing stable hydrophobic macrocyclic candidate peptides of lower molecular weight and with potential for greater oral bioavailability than linear peptides, but perhaps without the same requirement for elaborate drug delivery systems. In summary, while there have been advances in understanding the limitations of peptides for oral delivery, low membrane permeability, metabolism, and high clearance rates continue to hamper progress.

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1. Introduction

Oral peptide delivery has been a recurrent theme area of ADDR for more than 30 years. Using a definition of a peptide being less than 50 amino acids, there are almost 70 peptides marketed and over 150 in current clinical development [1], with the vast majority administered by the injected route. Attractive features for parenterally-delivered peptides include high potency and specificity, low systemic toxicity, and some reduction in manufacturing costs compared to even 10 years ago [2]. In taking stock of progress in oral macromolecule delivery, a browse of the Inaugural ADDR Issue from 1987 revealed an article by the late Joe Robinson and colleagues [3], where the authors highlighted the problems of intestinal peptidase threats to peptide stability and the low epithelial permeability for large molecular weight (MW) biologics in the small intestine. They further predicted that future biologics could pose an immunogenic problem in the GI tract and advocated promoting absorption via the lymphatic system, neither of which came to be realized. In 1989, an ADDR article [4] suggested that large proteins from the diet could be absorbed intact by intestinal epithelia and Peyer's patches, and further suggested that stable lectins could be used to target liposomes to enterocytes and M cells to deliver macromolecules. The former proved to be largely incorrect, while the liposome-targeting approach turned out to be possible in theory, but so far it has not delivered sufficient material to either cell type for therapeutic application [5]. The topic was also a research area of the former Editor-In-Chief of ADDR, Vincent Lee, who reviewed the barrier properties of mucosal epithelia to peptides and proteins and assessed the potential and toxicity risks for the intestinal permeation enhancers (PEs) that had been identified by 1989 [6].

Between 1987 and 2010, apart from the marketed oral formulations of cyclosporine (CsA) and desmopressin, no other peptide had progressed beyond Phase II in an oral dosage form, and detail around target product profiles and formulation specifics was lacking. In the intervening period, our understanding of GI physiology advanced with respect to the role of mucus, the make-up and regulation of epithelial tight junctions (TJs), and the fate of molecules in the GI tract. This led to a myriad of oral peptide "platform" formulations, but despite much hype the majority did not progress to clinical development. The field has eventually arrived at a point where an oral formulation of the glucagon-like peptide 1 receptor agonist (GLP-1-RA), semaglutide (Novo Nordisk, Copenhagen, Denmark), underwent almost a dozen oral Phase III trials for type 2 diabetes (T2D) before approval by the FDA in 2019 [7]. An oral formulation of salmon calcitonin (sCT) completed a Phase III trial for osteoporosis in 2012 [8] and, following a complete response letter (CRL), the sponsors are seeking funding for additional Phase III trials for use in both osteoporosis and osteoarthritis. An oily suspension of octreotide completed a Phase III trial for acromegaly patients in 2015 [9] and, following a CRL, this formulation is undergoing further Phase III trials. All this would suggest progress, but enthusiasm is somewhat tempered because the oral bioavailability from each of these three formulations using established PEs was very low, at ~1%, while the efficacy of sCT and octreotide achieved from those particular oral formulations in humans was debatable.

In this Review, we examine the technologies that reached Phase III for oral peptides, ones in earlier clinical phases, selected preclinical molecular-based approaches aimed at temporarily opening epithelial TJs, as well as the re-emergence of lipid-based systems. We also review developments in passive and active nanoparticle design, where initial promising preclinical studies on insulin entrapped in polyalkylcyanoacrylate from 1988 [10] have not yet been built upon. Drug-device combination systems incorporating needles and patches offer an exciting disruptive approach to challenge traditional oral formulation; selected preclinical studies are reviewed. Finally, oral peptide formulators are commonly challenged by being provided with high MW, unstable molecules originally designed for parenteral delivery in order to obtain proof of concept outcomes. In addition, other advantages need to be demonstrated for an oral peptide if there is an established approved injectable counterpart. Consequently, the work of medicinal chemists in creating stable potent macrocyclic peptides suited to oral delivery may prove to be just as (or indeed more) helpful as formulation advances. Progress in oral macrocycles is comprehensively assessed in the second half of this review.

1.1. The physiology and formulation problem, a brief recap

The main challenges of developing oral biologic formulations, including those of peptides, for systemic delivery have been summarized [11,12], and also in Fig. 1. Peptides and proteins can typically survive both stomach acid and the degrading efforts of the stomach peptidases, renin and pepsin, by protecting them in methacrylate-based polymeric enteric-coated oral dosage forms. Once reaching the higher pH values the small intestine, dissolution of the enteric coating allows release of the payload, which would then be subjected to pancreatic serine proteases, especially trypsin, chymotrypsin, and elastase. The normal role of these enzymes in digestion is to clip proteins into di- and tri-peptides amenable for PepT1- and other carrier-mediated systems, and possibly for paracellular flux across the epithelium. Luminal mucus represents an under-estimated barrier for a number of oral delivery approaches, with recent studies providing greater understanding of the composition and properties of mucus (both loosely adherent and closely-associated with the epithelial surface) in different intestinal regions [13]. There is debate over the merits of mucoadhesive and muco-permeant peptide formulations, with arguments in favour of using hydrophilic neutral, zwitterionic, or anionic polymer-coated biologic-entrapped nanoparticles [14], or constructs incorporating mucolytics [15] to negotiate it. Older literature tended to be dominated by mucoadhesive chitosan and polycarboxyl-based constructs [16]. Many of these polymers were rendered ineffective by rapid mucus turnover and failed to access the epithelium in standard formulation approaches.

At the epithelium, hydrophilic macromolecules do not partition in lipid bilayers and are effectively excluded from entry. The paracellular route via TJs may be an alternative for some of the lower MW molecules of this group, but only if the TJs are transiently-opened using clinically-acceptable pharmacological approaches. There is debate over whether traditional Generation 1 TJ openers comprising excipients and surfactants including medium chain fatty acids (MCFAs), calcium chelators,

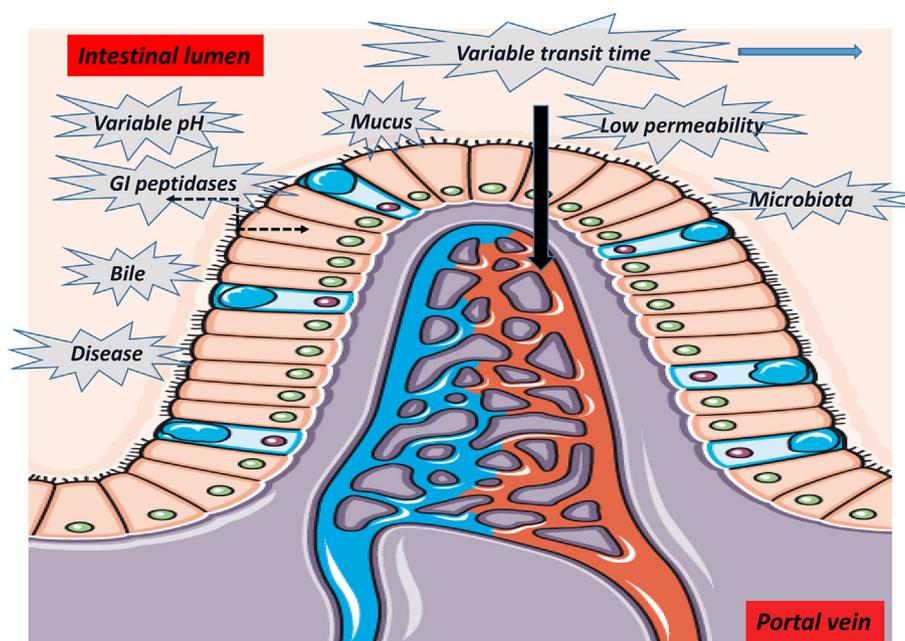


Fig. 1. Impediments for oral peptides. Inherent barriers include permeation of mucus, GI peptidase metabolism in the lumen and in epithelia of the stomach and small intestine, along with low epithelial permeability. Acid pH can destroy labile peptides, but this can be circumvented by enteric-coating tablets and capsules. Inter-subject physiological variables for include GI transit time, the influence of bile and the microbiome. Regional delivery of formulations using pH-dependent polymer coatings is notoriously variable between subjects. The impact of disease on permeability is likely to be substantial and has barely been explored. Modified from Servier Medical Art under a Creative Commons Attribution License.

and bile salts might be less efficacious and more toxic than more precise molecular approaches targeting specific proteins (e.g. claudins) of the TJs (i.e. Generation 2 agents) [17]. Some believe that Generation 2 PEs are less likely to result in long term toxicity due to their specificity and a more tightly-regulated effect on TJ openings than Generation 1 agents [18]. Though attractive, this argument has some weakness in that these (typically) peptide-based Generation 2 agents can be chemically unstable and slow to act hence none have yet reached clinical trials. Since they are new chemical entities (NCEs) with unknown toxicology, there is uncertainty with respect to the perceived elevated safety risk compared to Generation 1 agents. By comparison, some of the Generation 1 PEs have been designated as Generally-Regarded-As-Safe (GRAS), while others have food additive status, or are established excipients performing roles as chelators, wetting agents, and emulsifiers. In addition, the mechanistically-“dirtier” Generation 1 agents may offer additional efficacy by forming a mixed micelle population in which the peptide can associate, thereby creating a depot with potential for transcellular uptake [19], although this is more likely to be observed with hydrophobic actives. For these reasons, older PEs currently dominate the oral peptide formulations that are being evaluated in clinical trials [20].

Another possible entry route for peptides across the epithelium arises from synthesis of chemical conjugates that are recognized by carriers. For example, Garcia-Castillo et al. [21] conjugated GLP-1 to glycosphingolipids with ceramide domains containing small fatty acids and promoted epithelial cell uptake via the GM1 carrier that normally mediates cholera toxin uptake. The principle of using non-toxic chimeras of bacterial exotoxins to conjugate peptides for endocytosis following oral delivery has been researched in preclinical studies [22]. Alternatively, incretin peptides including GLP-2 can be made more lipophilic by conjugating to short- and medium chain fatty acids, thereby assisting membrane insertion and epithelial translocation in a passive process [23]. These conjugating approaches (including prodrugs) involve NCEs, so in order to compensate for the increased costs and risk compared to oral formulations of unmodified peptides, a competitive development strategy requires oral NCE formulations to produce better bioavailability data than the latter.

Much focus has been on the mucus and epithelial biological barriers to overcome, in addition to coping with the physiological variables of luminal pH, luminal, brush border and cytosolic peptidases, as well as formulation approaches to ensure sufficient epithelial contact time. Recently, Mantaj et al. [24] demonstrated that the mucosal basement membrane comprising collagen and laminin could also impede translocation of 100 nm nanoparticles and fluorescein isothiocyanate (FITC)-dextran 4000 (FD-4) across Caco-2 monolayers grown on an artificial substrate mimicking the basal membrane. While this study highlights the difficulties in predicting in vivo outcomes from in vitro studies lacking essential components, it also challenges assumptions on systemic delivery being guaranteed once the enterocyte epithelial apical membrane is overcome, at least by nanoparticles. Still, a recent study from Merck researchers examined fluxes of a set of cyclic peptides (designated as BCS Class III) in the presence and absence of the excipient PE, Labrasol® (Gattefosse, St. Priest, France) and generated good correlations between Caco-2 monolayers and rat in situ duodenal instillations [25], emphasising the continuing importance of these screening tools in cell membrane permeability assessment of oral peptides, even if they have limitations in assessing actual oral formulations.

2. Oral peptide formulations that are undergoing or have completed Phase III

Pivotal Phase III clinical trials in the past 5 years for three oral peptides in a range of PE-based formulations reveal a snapshot of the current status, allowing detailed pharmacokinetics (PK) and pharmacodynamics (PD) to be analyzed. Benchmarks are now available for other peptide formulations in earlier Phases. These Phase III trials also set targets for disruptive device-based technologies to compete against, bearing in mind that PK and PD requirements will be specific to the peptide candidate. They give pertinent information about the leading PE-based delivery systems from Enteris Biopharma/Tarsa Therapeutics (NJ, USA) and also by Novartis (Basel, Switzerland)/Nordic Biosciences (Herlev, Denmark) for sCT, by Novo-Nordisk for semaglutide, and by Chiasma (Jerusalem, Israel) for octreotide.

2.1. Oral salmon calcitonin

Efforts to orally deliver sCT (MW 3432 Da) to treat post-menopausal osteoporosis (OP) and osteoarthritis (OA) have contributed greatly to the oral peptide field even though an oral sCT formulation has yet to be approved by the FDA. Injectable and nasal sCT formulations have been second-line OP therapies for over 30 years. A recombinant sCT oral formulation has undergone a rather tortuous path via Unigene (NJ, USA), Enteris Biopharma, and other companies before being licensed by Osteon Therapeutics (NJ, USA) in 2019. TBRIA™ is an oral tablet coated with a pH-dependent polymer designed to dissolve at luminal pH values (> 5.5) at and beyond the duodenum. Removal of the outer coating exposes a polymeric sub-coat, whereupon sCT is released from citric acid-based vesicles. The overall strategy was termed Peptelligence™ (Enteris Biopharma). Citric acid protects sCT against peptidases by maintaining a local acidic pH around the sCT. Its effects as a PE are minimal in dilute solutions [26], so the hypothesis was that just enough intact sCT could traverse the small intestinal epithelium in equivalent quantities to the nasal comparator product [27]. Although lauroyl carnitine chloride (LCC) was used as a PE in Enteris/Unigene formulations with other peptides and in early iterations for sCT [28], it was apparently not present in the sCT formulation tested in the 2012 ORACAL Phase III trial (NCT00959764) [8], but there is still some confusion in the literature over this.

In the ORACAL trial, TBRIA™ (0.2 mg or 1200 IU sCT/day) was tested against nasal sCT (200 IU) (Miacalcin®, Novartis, NJ, USA) and placebo using evening dosing in 585 post-menopausal women over 48 weeks, with the change in lumbar spine bone mineral density (BMD) selected as the primary end-point. TBRIA™ improved the BMD by 1.5% versus 0.8% (nasal) and 0.5% (placebo). While a statistical difference was observed between oral TBRIA™ and placebo, the lack of effect of nasal sCT was problematic, and in addition, BMD changes at other sites were not different between TBRIA™ and placebo. Side-effects of TBRIA™ were present in 80% of subjects, but most were mild-to-moderate. This formulation was not approved by the FDA, but there are now efforts underway to fund a new Phase III trial where presumably a nasal placebo will also be examined. In 2014, Binkley et al. [29] followed up the ORACAL study with one in post-menopausal, osteopenic women and, using the same formulation, they confirmed slight changes in lumbar spine BMD over 24 months, as well as a reduction in the bone reabsorption biomarker, C-terminal telopeptide of type 1 collagen (CTX-1) (NCT01292187). PK data was not published from either of these trials, but it is reasonable to assume a maximum oral bioavailability value of <1%, given that nasal sCT was the comparator in the ORACAL trial. For highly potent peptides like sCT, protection against stomach acid and intestinal peptidases may therefore suffice for commercially-acceptable oral bioavailability if the PD outcome is achieved and the cost of peptide production can be borne.

With a different oral sCT formulation approach from Peptelligence™, Novartis and Nordic Biosciences carried out a Phase III study (NCT00525798) with oral sCT where OP was also the target [30]. This formulation, SMC021, consisted of 0.8 mg sCT matched with Emisphere's (NJ, USA) Eligen®-based PE, 5-CNAC (8-(N-2-hydroxy-5-chloro-benzoyl)-amino-caprylic acid), along with vitamin D and calcium. It appears that no enteric-coating was required for these tablets, the same as for another more important Eligen® carrier, salcaprozate sodium (SNAC), when it in turn was formulated with other therapeutics. Bone fractures, spinal BMD, and biomarkers were measured over 36 months in response to SMC021. While lumbar spine BMD was increased to a similar level as in the ORACAL study, the primary endpoint of preventing new fractures was not reached in NCT00525798. PK data was limited, but it suggested that sCT plasma levels were at the limit of detection. This PE-based formulation was therefore discontinued for OP. However, Nordic Biosciences, also tested the same formulation in two further independent Phase III trials targeted at knee OA [31], NCT00486434 (CSMC021C2301) and NCT00704847 (CSMC021C2302).

Over 24 months, the oral sCT was administered in tablets twice daily in 0.8 mg doses, with 200 mg 5-CNAC administered in 50 mL water to OA patients, but neither the required target end-points of joint space narrowing nor the pain scores were positively-affected versus placebo in either trial. The authors concluded that because there was a known relationship between plasma levels of sCT and CTx-1 as determined from Phase I and II trials, the reasons for failure was insufficient sCT delivery from the particular oral formulations used in Phase the III trials, reflecting a failure of this Eligen® carrier formulated with sCT. Thus, exposure to sCT in the NCT00704847 trial was surprisingly less than in earlier studies with the same formulation. Karsdal et al. [32] subsequently analyzed some of the variables in relation to the oral 5-CNAC-sCT formulation in Phase III trials and noted positive effects of exploiting circadian rhythms and of dosing ahead of food, as well as using a 50 mL volume of water. A useful outcome amid disappointment was the good safety data on 5-CNAC with sCT in the three Phase III trials; this was relevant for the later trials of SNAC with semaglutide. A final consideration with respect to sCT is that its efficacy as a second-line OP treatment even by any route is regarded as rather low [33]. The relatively weak BMD data from the marketed nasal sCT formulation is consistent with this point and suggests that future oral delivery efforts to treat OP and OA may result in more benefit if disease-modifying agents are used instead of this peptide.

2.2. Oral octreotide

Chiasma's oily suspension technology was designated as "Transient Permeation Enhancement" (TPE™) technology. Octreotide (MW 1020 Da) is a cyclic octapeptide, with a pendant threonine derivative, that pharmacologically mimics somatostatin. Its cyclic structure makes it partially resistant to cleavage by small intestinal exopeptidases, thereby offering some increased stability over linear peptides [34]. The rationale was to move patients from painful injections of long-acting somatostatin analogues with low gauge needles to daily oral octreotide capsules in order to promote patient acceptability. Tuvia et al. [35] outlined the composition of an oral formulation in which the moderately efficacious medium chain fatty acid PE, sodium caprylate (C₈), was mixed with octreotide in an aqueous buffer, followed by lyophilisation and suspension in oil-based excipients, including polysorbate-80 and other surfactants. The oily suspension was loaded into enteric-coated hard gelatin capsules for oral dosing. TPE™ is regarded as a TJ-opening technology, arising from the altered TJ protein expression in intestinal epithelial tissue and from rat intestinal instillation studies showing induction of MW-dependent flux of fluorescent dextran (FD) molecules [35]. If this is the case, an argument might be made for including a better TJ-opener than C₈, as well as other excipients that also act on TJs. In addition, the combined roles of the incorporated surfactants and excipients are consistent with membrane perturbation in parallel; in any event the technology is not simply an ad-mixture of peptide with C₈. An exclusive temporary MW-dependent action on TJs is an attractive hypothesis in terms of countering the argument that increased permeability of intestinal pathogens or lipopolysaccharide (LPS) fragments might be a safety risk for chronic administration.

A Phase I study in healthy subjects from 2012 revealed equivalent bioavailability to 0.1 mg octreotide by the sub-cutaneous (S.C.) route required 20 mg by the oral route, a relative oral bioavailability of 0.5% [36], the same order as the oral sCT formulations described above. Melmed et al. [9] have described a first Phase III study of oral octreotide (Mycapssa™, formerly Octreolin™) in 150 patients with acromegaly (NCT01412424). The oral formulations of 20–80 mg day⁻¹ were able to control levels of the biomarkers, Insulin Growth Factor-1 (IGF-1), and Growth Hormone, over 13 months in most pre-selected fasted acromegaly patients known to respond to S.C.-administered octreotide. Dose-related proportional increases in plasma levels were achieved from the twice-a-day oral octreotide capsule. Adverse events appeared

to be consistent with the somatostatin class and the associated GI related side-effects resolved in most subjects. On the other hand, approximately half of the subjects enrolled in the extension period withdrew due to a combination of lack of plasma IGF-1 control or for side-effects. Ultimately, oral octreotide was not approved by the FDA arising from that first Phase III trial. Another Phase III trial (EudraCT Number: 2015-002854-11, MPOWERED™) was initiated for EMA submission and is due to report in 2020. A second Phase III trial with placebo controls (CHIASMA OPTIMAL, NCT03252353) under a Special Protocol Assessment agreement with the FDA was reported by the company to have met all initial endpoints in 2019 and FDA submission of Mycappsa™ was re-filed in 2020.

2.3. Oral semaglutide

Semaglutide (MW 4113 Da, Ozempic®, Novo Nordisk) was approved as a once-weekly S.C.-injected GLP-1-RA at a dose range of 0.1 mg – 1.0 for T2D in 2017 [37]. Semaglutide plasma stability arises primarily from di-acid C₁₈ acylation via a spacer at Lys-26, which confers affinity for albumin, as well as resistance to dipeptidyl peptidase-IV (DPP-IV). This is due to substitution of Ala-8 with alpha-aminoisobutyric acid and replacement of Lys-34 by Arg [38]. The once-a-day oral tablet version of semaglutide, Rebelsus®, was approved in 2019 [39]. Its high potency, stability, and long half-life ($t_{1/2}$) made it the most interesting peptide yet considered for oral delivery. The commercialisation factors that support financial viability for an oral daily dose of 7 mg or 14 mg are based on several assumptions. These included an absolute oral bioavailability of 0.4–1.0% [40], as well as an equivalent weekly tablet price to the once-weekly S.C. injection for T2D patients. The costing model will likely cater for a projected broader patient population of obese patients for which it will also be targeted in future, if approved for that indication. Factors relating to the cost-effectiveness of the oral version have been modelled by an academic group [41] and also by Novo-Nordisk [42]. In the latter study, the authors calculate that the net cost of achieving glycated hemoglobin A1c (HbA1c) and weight loss targets in T2D patients should be less for oral semaglutide than the injectable GLP-1-RA, liraglutide, the oral DPP-IV inhibitor, sitagliptin, and the oral sodium-glucose co-transporter-2 (SGLT2) inhibitor, empagliflozin.

For oral delivery, the optimal concentration in the tablet was 14 mg semaglutide paired with 300 mg of the Eligen™ carrier, SNAC, which had a 20-year history of achieving single digit oral bioavailability values with a range of payloads including vitamin B₁₂ [43] and unfractionated heparin [44]. Several factors may have influenced the decision to opt for SNAC over the competing PE including C₁₀. SNAC had an excellent safety profile supporting GRAS status [45], prior approval under medical food regulations for Eligen-B₁₂™ [46], and could be incorporated in simple non-enteric coated tablets. The majority of the ten Peptide InnOvation for Early diabEtes tReatment (PIONEER) Phase III trials of oral semaglutide across ~9000 T2D patients have been published since 2018 and were summarized recently [47]. The capacity of oral semaglutide to lower HbA_{1c} to a similar degree as Ozempic® has been demonstrated, along with capacity to induce weight loss, and with no requirements for dose adjustment in renal and liver-compromised patients. In addition, it demonstrated superior efficacy in respect of the HbA_{1c} biomarker over oral DPP-4 and SGLT-2 inhibitors, and there were no drug interactions with patients concomitantly on proton-pump inhibitors, loop diuretics, warfarin, metformin, digoxin, statins, or estrogen-based contraceptives [47]. A meta-analysis against marketed injectable GLP-1-RAs concluded that the 14 mg daily oral dose of semaglutide was second only to once-weekly S. C.-administered 1 mg semaglutide in lowering HbA_{1c} levels and achieving body weight loss over a 6-month period [48]. It is unclear if oral semaglutide has a cardiovascular benefit compared to that demonstrated for patients on injectable GLP-1-RAs; such data may emerge upon completion of a large cardiovascular assessment trial [NCT03914326]. It is also not

apparent that it can alleviate chronic kidney disease, a benefit of injectable GLP-1-RA molecules. The niche for oral semaglutide in the treatment paradigm is for T2D patients not well-controlled on current oral anti-diabetic small molecule therapies, for patients with needle phobia, and for patients preferring to opt for GLP-1-RA therapy earlier than they would have with just injectable options. On the other hand, in ingesting the semaglutide tablet, patients must wait 30 min before eating to avoid interference of absorption by food. This might prove a barrier to take-up, given the long-term nature of the therapy: whether patients will prefer this regimen over a once-a week S.C. injection of Ozempic® remains an open question, but experience indicates that patient compliance with oral formulations is almost always greater than for injections and that they typically get used to chronic dosing regimens. Nevertheless, as global sales of Ozempic® in 2019 were \$1.65 billion, there is potential for Rybelsus® to be an even greater blockbuster.

PK parameters from oral semaglutide tablets in normal and T2D patients have been established from NCT01037582 and NCT01686945 [49]. The $t_{1/2}$ values in plasma were 152–165 h and 165–184 h for oral semaglutide versus Ozempic® respectively. Maintaining the high $t_{1/2}$ in the oral version should compensate for large individual variation in oral bioavailability. The oral bioavailability value for semaglutide tablets in dogs was $\sim 1.2\% \pm 0.25$ with a 10 mg dose [59]; human values stated on the Package Insert are slightly less [40], perhaps reflecting species differences in stomach dilution, residence time, and pH values in the fasted state. A novel gastric epithelial absorption mechanism of action (MoA) was proposed for the semaglutide-SNAC tablet [59]. In ligated dogs, where small intestinal absorption was precluded, gastric permeability accounted exclusively for semaglutide absorption [59]. SNAC provided pH-elevating buffering very close to the tablet in the stomach offering protection against pepsin. The tablet dissolved over 60 min, and the released peptide was presented in a pepsin-resistant monomeric absorbable format, which in turn traversed the gastric epithelium, presumably aided by SNAC's PE actions on epithelia. If the buffering to high pH was a bulk effect in the stomach, then drug interactions would have been expected with co-administered omeprazole, weak acids and bases, which was not the case. These data supported a long-held hypothesis that payload and PE need to be co-released in high concentrations close to the epithelium and that unformulated diluted mixtures would be sub-optimal (reviewed in [60]). Using antibodies to semaglutide [59], the authors found co-location with parietal cells and this supported a transcellular permeability process for semaglutide (Fig. 2).

Using a gastric cell line grown as a monolayer on filter inserts, the authors also concluded from in vitro studies that permeability increases were only detectable with semaglutide and not liraglutide, and also that SNAC could not be substituted by another Eligen™ carrier. This inferred a unique interaction between semaglutide and SNAC and suggested that this gastric MoA could not necessarily be extrapolated to SNAC with other payloads, or to the Eligen® carriers in general. This discovery of a gastric MoA was in the context of prior work with SNAC with other payloads, which had assumed small intestinal absorption of a non-covalently bound lipophilic soluble complex [60]. Studies using biophysical methods should further decipher the nature of the interactions between SNAC and semaglutide. Noting the structure of SNAC as a salicylamide - C₈ derivative, its surfactant nature, as well as its capacity to complex non-specifically with other macromolecules, claims for a specific interaction between SNAC and semaglutide should be further investigated at the level of structural biology. Finally, it would be interesting to see if there is absorption of semaglutide from enteric-coated SNAC tablets, as this would reveal whether the gastric mechanism was obligatory, or if there is still a small intestinal contribution. In the light of demonstration of semaglutide gastric absorption in ligated dogs, perhaps the absorption regions of vitamin B₁₂- and heparin-SNAC from tablets could also be evaluated further?

An important aspect of the clinical development of oral semaglutide was toxicity assessment in hundreds of control subjects and T2D

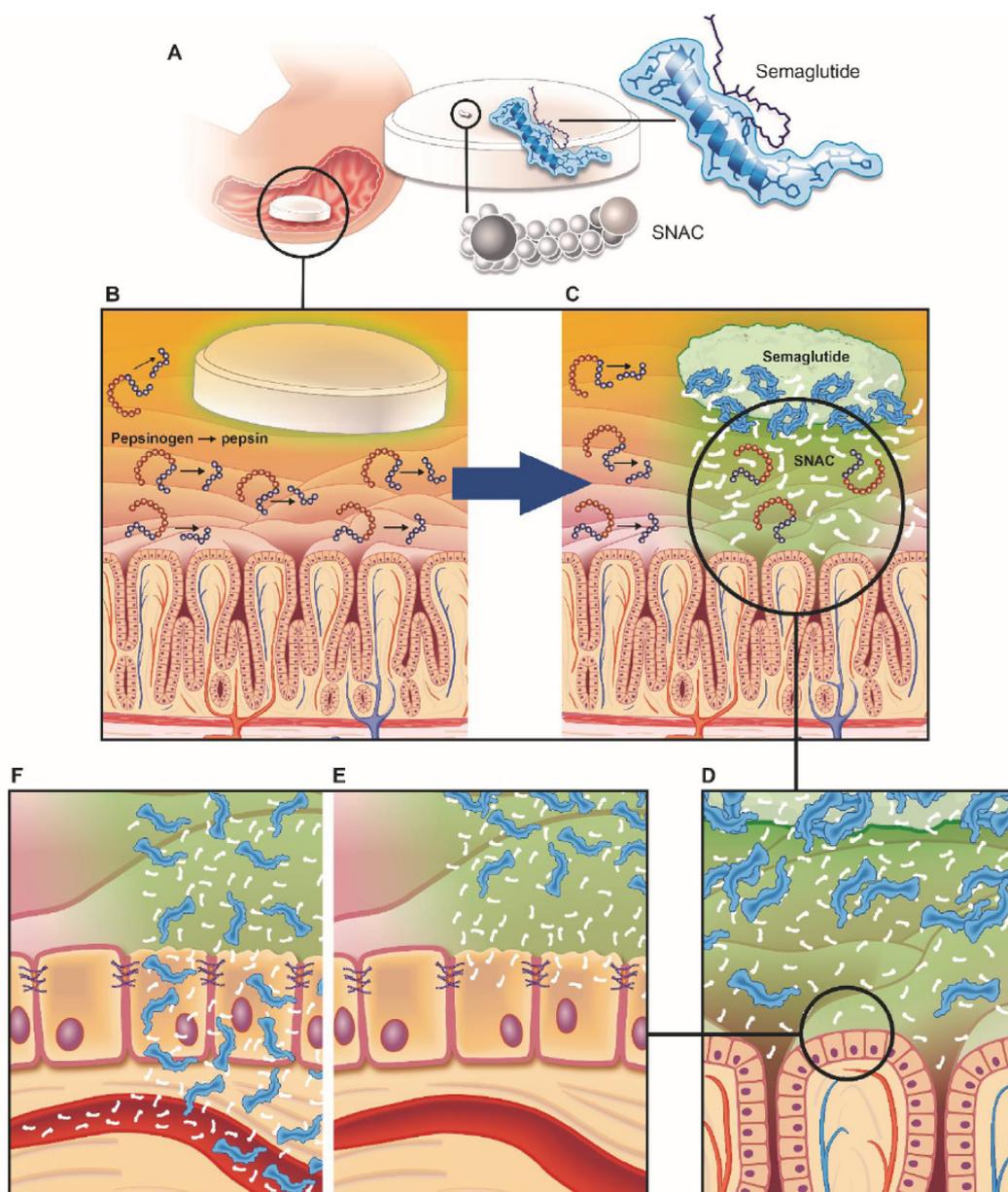


Fig. 2. Proposed mechanism of action of SNAC in inducing absorption of semaglutide following oral administration [59]. (A) Semaglutide tablet is co-formulated with SNAC, which is then absorbed from the stomach. (B) Pepsin is normally produced from pepsinogen at stomach pH. (C) As the tablet erodes in the stomach over 60 min, SNAC neutralizes the pH, thereby preventing pepsin activation. Semaglutide is released from the tablet as multimers, which are protected from pepsin and acidic stomach pH in the immediate region around the tablet. SNAC and semaglutide solubility increases. (D) SNAC causes monomers of peptide to be formed. (E) SNAC inserts in the plasma membrane of the gastric epithelium and perturbs it via fluidization, but without directly opening TJs. (F) Semaglutide fluxes across the epithelium by a transcellular route. Image reproduced from [59] under a License from the American Association of Advancement for Sciences.

patients. From the perspective of whether administering 14 mg semaglutide and 300 mg SNAC a day could be problematic, the PIONEER studies assessed cohorts of normal subjects, T2D patients, and patients with moderate-to-severe renal impairment over 26 and 52 weeks, with some studies lasting 78 weeks. The main adverse events were nausea and GI-related symptoms, leading to withdrawal rates of ~10% across trials [47–49,54,58]. These were mild-to-moderate and were offset by ramping the dose escalation of oral semaglutide. Moreover, the GI-related side-effects appear to be a class effect of GLP-1-RA and have not been ascribed to SNAC. Nonetheless, the Rybelsus® label recommends against use for patients with pancreatitis, thyroid tumors, or multiple endocrine neoplasia, although it would be surprising to see its use in patients with a history of stomach or duodenal ulcers or Crohn's disease. Overall, the approval of oral semaglutide seems so

far to have addressed assertions that PEs in such formulations might be problematic due to the possibility that they would permit unintended absorption of bystander pathogens or LPS fragments [61], or that they might induce autoimmune disease [62]. The data emerging has so far raised no concerns that could specifically be ascribed to the 300 mg dose of daily SNAC. Eligen-B₁₂TM containing 100 mg SNAC (a 3-fold lower dose than in Rybelsus®) was approved as a medical food for daily administration over 6 years ago and was recently made available as an over-the-counter product. Only post-marketing surveillance of chronic daily regimens, however, will reveal whether PEs in oral peptide formulations might cause concerning intestinal problems. For Rybelsus®, even though it has achieved exceptional PD outcomes, there are cost-saving incentives to further reduce both the doses of semaglutide and SNAC [63]. Table 1 summarizes the completed Phase

III studies using oral formulations of each of these three peptides in the last decade.

3. Traditional permeation enhancers in clinical and preclinical development

In focusing on SNAC, 5-CNAC, and C₈ (as part of an oily suspension), the agents that have completed Phase III trials for oral peptides, this does not allow a conclusion that they are superior to PEs in other formulations currently being examined in preclinical and clinical phases. One of the downsides of using SNAC is its rapid absorption. Together with low potency as a PE, it is therefore difficult to maintain a threshold concentration for long enough at the intestinal wall and is the reason why a 300 mg concentration of SNAC has to be used in semaglutide tablets. Gradual release of SNAC over 60 min in the stomach seems to delay its absorption so that it has more time to act, but it will likely be absorbed as soon as it is released. Many factors have to be considered in PE selection for a translatable oral product: payload and PE availability, Good Manufacturing Practice (GMP) quality, and cost, PE compatibility with the payload, potency and efficacy of both the PE and the payload in an oral dosage form, scale-up potential, and an extensive toxicology package. The commercial risk factors are especially high for a new PE with no history in humans and this tends to skew selection for development towards conservative excipient options. Here, we discuss a selection of other PEs in preclinical research and clinical trials.

3.1. C₁₀, acyl carnitines, EDTA, and bile salts

C₁₀ (1% w/w) was first demonstrated to increase paracellular flux in rat colonic epithelia in 1988 [64], later confirmed in Caco-2 monolayers as an effect on TJs at concentrations >10 mM [65]. This anionic surfactant initially appears to fluidize the plasma membrane of enterocytes, triggering phospholipase C to activate an intracellular cascade that leads to calcium elevation, followed by interactions between calmodulin and myosin light chain kinase (MLCK). Mitochondrial ATP is also reduced. These intracellular mechanisms induced by C₁₀ (reviewed in [66]) merge to temporarily open the TJs, permitting paracellular flux arising from removal of tricellulin and altered expression of claudin-5 [67], the latter also evident for C₁₂ [68]. More recently, interaction of C₁₀ and a co-administered peptide with bile salts and mixed micelles in the small intestine at concentrations above C₁₀'s critical micellar concentration (CMC) suggests a complex vesicular mechanism of phospholipid interaction in vivo [69]. Although C₁₀ has never been formally designated as GRAS-listed, it has a long history of use in humans, is present in milk at low mM concentrations, and also in butters and oils in high mM concentrations. It is also allowed as a food additive by the European Food Safety Authority with no current requirement for a maximum acceptable daily intake value [70]. It is possible that C₁₀ could also achieve GRAS status, although no Pharma company has to our knowledge sought to achieve GRAS status by performing acute and chronic safety testing as was performed for SNAC. Enteric-coated tablets of C₁₀ with a range of peptides and BCS Class III small molecules reached

Table 1
Completed Phase III trials for oral peptides in the last decade (selected)^a.

Peptide	Technology	Description	Comments	Ref
sCT (TBRIA™)	Peptelligence™ (Enteris)	Citric acid protection in enteric tablet	ORACAL trial: Small reduction in spinal bone mineral density similar to nasal delivery in an OA study	NCT00959764, [8]
sCT	Eligen®, (Emisphere, Novartis, Nordic Biosciences)	5-CNAC as PE in tablet (SMC021)	Spinal BMD was increased, but primary endpoint of preventing new fractures was not reached in an OP study.	NCT00525798
sCT	Eligen®, (Emisphere, Novartis, Nordic Biosciences)	5-CNAC as PE in tablet (SMC021)	CSMC021C2301 study for knee OA: no benefit, as no effect on joint space narrowing.	NCT00486434, [31,32]
sCT	Eligen®, (Emisphere, Novartis, Nordic Biosciences)	5-CNAC as PE in tablet (SMC021)	CSMC021C2302 study for knee OA: 4% reduction in WOMAC score was not significant.	NCT00704847, [31,32]
Octreotide	TPE™ (Chiasma)	Oily suspension with C ₈ as PE	20–80 mg day ⁻¹ oral octreotide controlled plasma IGF-1 and GH levels in acromegaly patients over 13 months in pre-selected patients that responded to s.c. octreotide.	NCT01412424, [9]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC (300 mg) as PE in tablet with semaglutide	PIONEER 1: achieved HbA1c reduction (3, 7 and 14 mg); weight loss (14 mg) over 26 weeks in T2D patients.	NCT02906930, [50]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC with 14 mg semaglutide	PIONEER 2: at 52 weeks, HbA1c and body weight were reduced versus the oral SGLT2 inhibitor, empagliflozin (25 mg) in T2D patients.	NCT02863328, [51]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC with 7 and 14 mg semaglutide	PIONEER 3: greater reductions in HbA1c over 26 weeks with both doses compared to oral DPP-IV inhibitor, sitagliptin (100 mg), in T2D patients not controlled with other oral therapies.	NCT02607865, [52]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC with 14 mg semaglutide (maintenance dose).	PIONEER 4: non-inferior to liraglutide (1.2 mg daily maintenance dose; s.c.) in decreasing HbA1c, and superior in decreasing body weight at week 26. Both had similar side-effects.	NCT02863419, [53]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC as PE in tablet with 14 mg semaglutide	PIONEER 5: Oral semaglutide was efficacious in T2D patients with renal impairment. Mild-to-moderate nausea seen occasionally.	NCT02827708, [54]
Semaglutide	Eligen®, (Emisphere, Novo-Nordisk)	SNAC as PE in tablet with 14 mg semaglutide	PIONEER 6: No major cardiovascular events in post hoc analysis of individual patients after 19 months on oral semaglutide	NCT02692716, [55,56]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC as PE in tablet with 3, 7, 14 mg semaglutide	PIONEER 7: a higher percentage of T2D patients achieved HbA1c of <7% with oral semaglutide than with sitagliptin (100 mg) at 52 weeks using flexible dosing.	NCT02849080, [57]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC as PE in tablet with 3, 7, 14 mg semaglutide	PIONEER 8-Insulin add-on: Oral semaglutide was superior to placebo in reducing HbA1c and body weight when added to insulin with or without metformin in T2D patients. 11–23% of patients on semaglutide had nausea.	NCT03021187, [58]
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC as PE in tablet with 3, 7, 14 mg semaglutide	PIONEER 9: Compared oral semaglutide with s.c. liraglutide (0.9 mg daily) in Japanese T2D patients. Completed; not yet published.	NCT03018028
Semaglutide	Eligen®, (Emisphere, Novo Nordisk)	SNAC as PE in tablet with 3, 7, 14 mg semaglutide	PIONEER 10: Compared oral semaglutide with weekly dulaglutide (0.75 mg, s.c.) as an adjunct to current oral therapy in Japanese T2D patients. Completed; not yet published.	NCT03015220

Abbreviations: BMD: bone mineral density; WOMAC: Western Ontario and McMaster Universities Arthritis Index; 5-CNAC: (8-(N-2-hydroxy-5-chloro-benzoyl)-amino-caprylic acid); SNAC: salcaprozate sodium; OA: osteoarthritis; OP: osteoporosis; T2D: Type 2 diabetes. IGF-1: Insulin Growth Factor-1; GH: Growth Hormone. A detailed summary of the primary and secondary end points achieved in each of the first 8 PIONEER trials is given in [39].

^a Sourced from either www.clinicaltrials.gov or peer-reviewed literature.

clinical trials, initially as Elan Pharma's (Athlone, Ireland) PROMDAS™ system, and subsequently by Merion Pharmaceuticals (Dublin, Ireland) as GIPET™, summarized in [71]. The most salient features of these studies were relatively low single digit oral bioavailability values with very large coefficients of variation (CVs), but acceptable toxicity profiles.

In parallel, Ionsys (San Diego, CA, USA) advanced an oral antisense molecule into Phase I using a C₁₀-based tablet, achieving somewhat higher oral bioavailability values than peptides, but with similarly large CVs [72]. Following licensing from Merion, an extensive 8-week Phase II trial was published in 2019 by Novo Nordisk using once-a-day C₁₀ matrix tablets incorporating a basal insulin (designated I338) with a long t_{1/2} and increased stability against intestinal enzymes [73], a peptide with some structural advantages, similar to the approach taken with oral semaglutide and SNAC. Glycaemic control from the oral formulation was equivalent to an S.C.-injected insulin analogue, but there was more individual variation with the former. Notably, the oral bioavailability of I338 was 1–2% and the formulation was abandoned, likely over cost and PK variability reasons. Most side-effects were mild and resolvable, with 12% of subjects reporting diarrhea, consistent with other trials with C₁₀-containing tablets. Similar to SNAC, a major issue for C₁₀ in clinical trials with several macromolecules was the high dose required, due to its low potency and likely sub-optimal formulation in GIPET™. Tablets typically contained >500 mg C₁₀ and several trials involved taking multiple tablets per dose. On the other hand, some concerns over potential PE toxicity were alleviated, at least in short-term trials, as there was no evidence of infection from GI absorption of LPS fragments or pathogens. This was in keeping with rat data [74], where it was demonstrated that C₁₀-induced intestinal permeability increases (although associated with mild membrane perturbation) were local and transient, and required contemporaneous exposure of C₁₀ and payload in high concentrations to enable increased absorption. Currently, C₁₀ is included as a PE in an insulin prodrug tablet (Insulin tregopil) from Biocon (Bangalore, India) that completed a Phase I trial for T2D [75]. That study confirmed that the C₁₀ in the PEGylated alkylated insulin tregopil tablet had little effect on the PK of co-administered metformin tablets administered 20 min later, more evidence that C₁₀ only works as a PE on a payload presented in close association and that it did not pose interaction problems even when subjects were taking multiple oral drugs. When PK was examined from tablets of insulin tregopil in dogs, the estimated relative oral bioavailability was 0.82% [76], the same order as that seen for oral I338 in humans. For an apparent gold-standard PE that emerged from *in vitro* intestinal epithelial bioassays, C₁₀ has ultimately proved to be a lot less effective in the dynamic environment of the human GI tract *in vivo*. Yet, its clinical performance is still on a par with SNAC, despite also being hampered by its own rapid and complete absorption in the upper small intestine.

The amphoteric surfactant long-chain acyl carnitine salts, palmitoyl carnitine chloride (PCC) and LCC, induce paracellular permeation-enhancement for poorly-permeable solutes in isolated rat colonic mucosae in Ussing chambers, as discovered by Merck scientists in 1993 [77]. Using porcine mucosal explants, Danielsen and Hansen recently showed that LCC permeabilized enterocytes, fused microvilli, and blocked apical membrane trafficking in boosting flux of the normally impermeable small molecule dye, Lucifer Yellow [78]. As with other surfactant PEs, the mechanistic journey of the acyl carnitines has evolved from what was thought to be a specific TJ-opening effect to include interactions with phospholipids and lipid rafts in the plasma membrane. With a good preclinical safety profile, LCC, became the PE of choice in the Peptelligence™ enteric-coated oral delivery system of Enteris Biopharma (NJ, USA) [28]. Oral formulations of leuprolide (endometriosis) and difelikefalin (opioid for pain control) are in Phase II using Peptelligence™. A Phase I trial with tobramycin (Tobrate™) tablets using this technology (presumably incorporating LCC) was recently published in which efficacy was demonstrated following tablet ingestion [79].

Ethylenediaminetetraacetate sodium (EDTA) and the bile salts, sodium glycocholate (NaGC), sodium taurocholate (NaTC), sodium deoxycholate (NaDC), are well established PEs for macromolecules in oral studies described over several decades [80]. In a pertinent example, EDTA, omega-3 fatty acids, and selected bile salts were incorporated into oral peptide enteric-coated formulations along with peptidase inhibitors, including aprotinin and soya-bean trypsin inhibitor by Oramed (Jerusalem, Israel) [81], and designated as the Protein Oral Delivery (POD™) technology. Phase II outcomes for the POD™ technology have been published from a trial for Type 1 diabetics (T1D) using their ORMD-0801-Type 1 insulin formulation consisting of 8 mg of rapid-acting human insulin administered thrice daily (NCT00867594) [82]. It lowered blood glucose and was well-tolerated in 15 patients. According to Oramed, the technology is being leveraged for an oral GLP-1-RA (ORMD-0901) and for insulin (ORMD 0801-Type II), both for T2D. The MoA of EDTA and bile salts is multi-modal: calcium-chelating, TJ openings, and membrane perturbation, but with additional mild detergent surfactant actions for the latter.

3.2. Alkyl maltosides, Labrasol™, and sucrose laurate esters

Alkyl maltoside non-ionic surfactants were originally developed as the basis of the Intravail™ technology for improving nasal delivery by Aegis Therapeutics (San Diego, CA) [83]. The lead PEs are n-dodecyl-β-d-maltopyranoside (DDM, 12-carbon alkyl chain length) and n-tetradecyl-β-d-maltopyranoside (TDM, 14-carbon). Due to its non-toxic features, TDM has been leveraged from its original use in nasal delivery for oral delivery of macromolecules, with oral gavage studies of octreotide in mice showing improved bioavailability, albeit just 4% relative to S.C. injection [84]. Membrane perturbation was the predominant effect of TDM in enhancing bioavailability of sCT in a rat colonic loop model [85]. Studies examining the MoA in Caco-2 monolayers using modified versions of fasted- and fed-state simulated intestinal fluids revealed that above the CMC values of alkyl maltosides, monomer concentrations fell as they interacted with bile salt/lecithin mixed micelles, thereby retarding flux of FD-4 *in vitro* and *in vivo* [19]. This emphasizes the relevance of using bio-relevant buffers across bioassays in attempting to predict translational outcomes. Interaction with luminal constituents to reduce free concentrations of alkyl maltosides to sub-therapeutic levels could explain in part why these PEs and many others have yet to demonstrate efficacy in clinical trials for oral peptides.

Labrasol™ (Gattefosse, St Priest, France) is a self-emulsifying non-ionic surfactant excipient comprising mono-, di- and triglycerides, as well as mono (C₈ and C₁₀)- and di- fatty acid esters of polyethylene glycol (PEG)-8, and free PEG-8. It is best known as a solubilising agent and emulsifier in lipid-based formulations of BCS Class II small molecules and was a component of a recently-approved oral formulation of the androgen receptor inhibitor, enzalutamide, for prostate cancer treatment [86]. In 2002, it was initially discovered that Labrasol™ could enable delivery of insulin from intestinal instillations in rats [87]. This was followed up in more detail recently when McCartney et al. [88] revealed that an ad-mixture could deliver insulin from instilled jejunal and colonic loops of rats with similar efficacy as C₁₀. Moreover, they showed that multiple components in Labrasol™ contributed to the effects of membrane perturbation and TJ openings. Additionally, *in vivo* efficacy in the loop model was not due to actions of lipases that might liberate free C₈ and C₁₀ from medium chain glycerides and macrogl glycerides. A previous *in vitro* study using sets of gastric and duodenal lipases had suggested that the compounds present in Labrasol™ were hydrolysed by such lipases to form MCFAs and concluded that it was simply a prodrug [89], a conclusion that may require revisiting. Labrasol™ was used in a recent Pharma screen in rats for improving oral bioavailability of BCS Class III cyclic peptides [25]. Its efficacy as a PE has become widely recognized, and in addition, its ease of formulation in oral emulsions [90] is compatible with gelatin capsules. It is also attractive in part due to its excipient status and extensive safety

package, as described in monographs on Caprylocaproyl- Polyoxyl-8 glycerides and Macrogol-8 glycerides in the United States Pharmacopoeia – National Formulary (USP-NF) and the European Pharmacopoeia (pH.Eur.), respectively.

The renewed interest in oil-in-water (o/w) emulsions (Section 5) as technologies that can be used for oral peptides has led to other sources of lipid-based PEs to be examined that are compatible with such systems. Sucrose laurate esters fall into this category as they have a hydrophilic-lipophilic balance (HLB) of 16, similar values as the emulsifying excipients, Kolliphor® HS 15 and Cremophor® RH 40 (BASF, Ludwigshafen, Germany). The food grade surfactant, sucrose laurate, is in the FDA database of food additives and it has a high acceptable daily intake level in food products [91]. The attraction for sucrose laurate as a PE arises not only because it is amenable to emulsion formation, but also because C₁₂ is the most efficacious MCFA PE *in vitro*; however its formulation potential may be curtailed in conventional oral formulations by its low CMC [92]. Sucrose laurate was initially evaluated for rectal administration of insulin in rats [93]. An extensive study confirmed its efficacy for insulin delivery with characteristics consistent with an MCFA effect, including a major effect on TJ opening [94]. Its efficacy at promoting insulin absorption from rat jejunal and colonic instillations was equivalent to C₁₀ and Labrasol™.

4. Molecular approaches to epithelial tight junction opening: pre-clinical research

Strategies described in the previous Sections have the common theme of agents (or mixtures of agents) whose PE properties were identified through *in vitro* and/or *in vivo* screens. Despite the enthusiasm associated with their identification, there are still many issues that hamper their translation to oral peptide products. One major issue is that finding these agents through empirical methods does not provide a defined MoA and this information can take years to decipher or may never be fully elucidated. While this does not preclude clinical development in oral peptide products (e.g. SNAC in Rybelsus®), it could slow the process.

Firstly, extensive and multiple safety studies are likely to be required since it is not possible to predict potential toxicological outcomes without a defined MoA. Furthermore, potential toxicity-related pathways relevant to an agent without a known MoA could be missed due to species differences. For example, a DNA-sensing pathway present in humans is not found in mice [95]. In some cases, elements of a MoA have now been identified, but without a tractable method to test these proposed mechanisms, the critical MoA actions are still unclear, with implications for long-term toxicology. Moreover, recent studies have highlighted the fact that specific pathways relevant for potential toxicological actions may not be present in pre-clinical test species. There are caveats: while toxicity can be predicted from MoA studies, this is not necessarily the case for many drugs.

Secondly, translating from pre-clinical studies to clinical trials can be especially problematic. Initial studies in rodents typically involve extensive optimization of these PEs in a specific formulation; this process of optimization is typically repeated as the agents are tested in subsequent pre-clinical models (e.g. dog, pig, non-human primate). Due to significant physiological differences between each of these species and humans, an 'optimized' formulation that provides reasonable improvements in oral bioavailability in these pre-clinical models is often less effective than anticipated in clinical trials. Due to the high cost of clinical trials where further optimization opportunity is constrained, the ultimate performance of these PEs will therefore likely be less than the initial promise.

Some of the most promising PEs are those that appear to moderately open TJs to transiently enhance paracellular solute transport. An alternative to the historical, empirical approach to identify PEs that are active on TJ structures is the idea of designing agents that can modulate established cellular processes. The rationale for this idea is based upon

the fact that TJ structures are dynamic and all intestinal epithelial cells are constantly turning over through mechanisms of senescence, growth, and repair. Enterocyte TJs are some of the most dynamic in the body with the entire intestinal epithelium turning over every 5–7 days [96], requiring complete deconstruction and reconstruction of TJs. Further, TJs open and close rapidly in order to allow the transmigration of innate immune cells such as neutrophils [97]. Finally, intestinal TJs, also respond to nutritional elements that can result in particularly rapid changes in TJ function. The molecular mechanism of this rapid nutrient-response involves the uptake of essential amino acids and glucose at the apical plasma membrane at rates that are above the Michaelis constant (K_m) for these sodium ion (Na^+) co-transporters [98]. In order to maintain a low intracellular Na^+ level, enterocytes export Na^+ in exchange for calcium ions (Ca^{2+}), which leads to the Ca^{2+} /calmodulin-mediated activation of MLCK, which in turn leads to phosphorylation of scaffolding associated with TJ structures. Phosphorylated myosin light chain (pMLC) modifies the TJ to not only modestly increase its paracellular permeability to act as a second avenue for nutrient uptake, but also induces increased expression of claudin-2 in TJ structures. Claudin-2 is known for its positive charge perm-selective properties, which minimize uptake of negatively-charged exotoxins during periods of increased paracellular permeability [99]. TJs modified in this way are rapidly returned to the resting state of reduced paracellular permeability as soon as the apical nutrient levels drop below the K_m of Na^+ co-transporters for these essential nutrients.

A way to exploit this nutrient-driven enhancement of paracellular permeability has been described that involves the counter-balance enzyme in the process described above. Once the high levels of essential nutrients have dropped below the K_m of the Na^+ co-transporters, MLC phosphatase (MLCP) dephosphorylates the pMLC to reduce the paracellular permeability to basal levels. MLCP is a multimeric protein complex where the PP1 phosphatase is regulated by either MYPT1 or CPI-17 proteins [100]. Co-crystal structures were used to identify short peptides to emulate the interfacial contacts between PP1 and MYPT1 or CPI-17, which were modified to make them membrane permeable and then synthesized using all D-amino acids in a retro-inverso format to increase their stability in the intestine. Two membrane permeable inhibitors of MLC phosphatase (PIP) peptides, PIP-250 and PIP-640, were shown *in vitro* and *in vivo* to alter intestinal TJ barrier properties in a manner that was similar to the nutrient-driven enhancement of paracellular permeability mechanism outlined above. These two PIP peptides enhanced the oral bioavailability of insulin in rats [101], and also the enterocyte uptake of calcitonin and exenatide in a charge-dependent manner [102]. Fig. 3 shows confocal images of Caco-2 cell monolayers following exposure at their apical (luminal) surface to either a biotinylated form of PIP-640 or to control peptides (PIP-641, PIP-642), which were rendered inactive by single amino acid replacements [103]. Intracellular labelling of these biotinylated PIP peptides with fluorescent streptavidin (green) shows co-localization of PIP-640 with occludin (red), demonstrating its specific actions at MLCP localized to TJ structures. The lack of intracellular localization of PIP-641 or PIP-642 at TJ structures shows the precise structure/function understanding of this approach to prevent MLC de-phosphorylation as a MoA to enhance peptide flux. As anticipated from using an endogenous mechanism of enhancing paracellular permeability, no inflammatory or cytotoxic actions have been observed with these PIP peptides [103]. Nonetheless, even with the MoA worked out for these peptide-based PEs, physiological impediments could reduce their performance in a dosage form in the GI tract *in vivo*.

The idea of using endogenous mechanisms that are known to modulate TJ function has also examined pathogen-related intestinal epithelial changes. A variety of bacteria can infiltrate enterocytes, leading to dramatic changes in paracellular permeability, although these are typically associated with significant cytotoxicity. For example, the human pathogenic bacterium *Clostridium perfringens* secretes an enterotoxin that targets claudins through its C-terminal receptor-binding domain

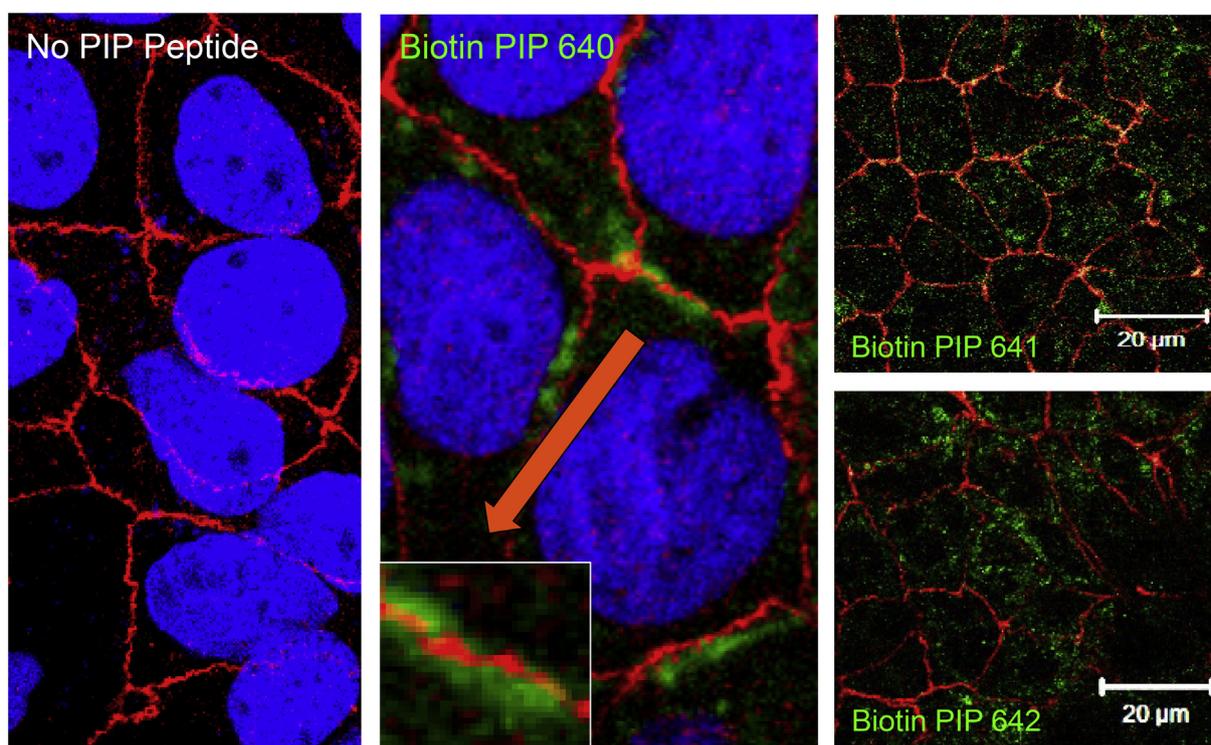


Fig. 3. Intracellular localization of the permeable inhibitor of myosin light chain phosphatase (PIP) peptide 640, evolved from molecular approaches. Apical application of biotin-labelled PIP 640 or two non-active sequences (PIP 641, PIP 642) from this peptide family were localized using streptavidin-Alexa 488 *in vitro*. TJ elements were defined by immunofluorescence imaging of occludin. Nuclei were labelled with DAPI. Original Figure obtained with permission of Elsevier from [103] and modified.

(C-CPE), causing dissociation of claudins at TJs that results in epithelial barrier breakdown [104]. The potential use for oral drug delivery through its actions on claudin-4 has been described [105]. While this approach would likely have potential toxicity issues, information garnered from the mechanism(s) used by such toxins could lead to new approaches to enhance oral peptide delivery. One additional drawback to this approach is a kinetic one: several hours are required for such enterotoxins to act, with still more hours needed for epithelial recovery. This may hinder application in dynamic conditions. To get around the toxicity issues of toxins and the fact that derived PE molecules tend to be unstable peptides, Watari et al. [106] recently screened a library of claudin-4 binders to see if other candidates could compete with C-CPE. From it, a cyclic antibiotic, thiostrepton, emerged and had capacity to enhance absorption of FD4 in a rat loop gut model. Finally, toxins are also being used by academic groups in nanoparticle constructs to deliver insulin orally. One example was to decorate the surface of an insulin-entrapped pluronic nanoparticle with zonula occludens toxin (ZOT) peptide [107], with proof-of-principle being demonstrated in diabetic rats.

5. The re-emergence of lipid-based systems for oral delivery of biologics

Self-emulsifying drug delivery systems (SEDDS) are isotropic mixtures of oil, surfactant/co-surfactant and solvent/co-solvent that spontaneously emulsify when diluted in aqueous fluids [108]. They have a successful track record in oral formulation of both lipophilic small molecules and also for CsA, an atypical hydrophobic peptide. Their renewed potential for oral peptides has emerged from a set of recent studies demonstrating that peptides could dissolve in the oil phase using the principle of hydrophobic ion pairing (HIP) through which peptide lipophilicity could be increased [109]. Loading values for peptides in SEDDS via HIP have achieved up to 10% for 2–3 peptides, e.g. [110], which is typically much higher than most nanoparticle constructs. The principle

of HIP involves matching peptides to surfactants through electrostatic attraction between the opposite charges on amino acids and ionizable surfactants. This is a type of salt formation, although unlike the formation of soluble drugs salts to improve dissolution, the hydrophobic moiety of the amphiphilic counter-ion reduces aqueous solubility and increases partitioning in non-aqueous vehicles. The net increase in hydrophobicity of the non-covalent complexes generated by HIP is also thought to promote transcellular permeation across gut epithelia. Mahmood and Bernkop-Schnürch have extensively reviewed the various combinations of hydrophilic macromolecules and surfactants that have been matched as HIP for incorporation in SEDDS, along with the rodent studies demonstrating efficacy [111]. For our purposes, we have modified their tabulated work in [111] to abstract the HIP combinations with surfactants for the following peptides: exenatide, insulin, leuprolide, and lanreotide (Table 2).

In vivo studies of SEDDS with HIP of peptides are beginning to be published and some of the initial data is encouraging. For example, exenatide was paired with sodium docusate in precise molar ratios and incorporated into a SEDDS [109]. The log *P* of exenatide was increased, along with the capacity to increase mucus permeation. Oral bioavailability relative to S.C. delivery was 14% in normal healthy rats upon oral gavage. The same group also combined octreotide with deoxycholate and formed a SEDDS, which translated to 18% higher oral bioavailability over controls in pigs [112].

Other groups have also generated HIPs with high complexation, high log *P* values between peptides (desmopressin and leuprolide) and docusate using a different method: pre-emulsion and hot high pressure homogenization allowed formation of nanostructured lipid carriers with a particle size of <200 nm and a high encapsulation efficiency [113]. In this way, HIP lipid-based systems can be combined with nanotechnology. Another method also uses HIP as the starting point for oral peptide formulation via rapid nanoprecipitation, whereby highly-loaded peptides are located in the hydrophobic core of the carrier [114]. *In vivo* data is awaited from both formulation approaches. Finally,

Table 2
HIP examples of peptides formulated in SEDDS.

Peptide	Surfactant	SEDDS	Loading (% w/w)	Log P
Desmopressin	Sodium docusate	5% Transcutol HP, 20% Peceol, 10% Capryol 90, 35% Labrasol ALF, 30% Tween 20	10.7	0.3
Exenatide	Sodium docusate	35% Cremophor EL, 25% Labrafil 1944, 30% Capmul-PG 8 and 10% Propylene Glycol	1.0	2.1
Insulin	Sodium docusate	30% Tetraglycol, 30% Peceol, 40% Labrasol	10.7	2.0
Lanreotide	Sodium deoxycholate	25% Capmul MCM, 30% Kolliphor EL, 45% Miglyol 840	6.4	2.6
Leuprolide	Sodium docusate	5% Transcutol HP, 20% Peceol, 10% Capryol 90, 35% Labrasol ALF, 30% Tween 20	10.7	2.8
Leuprolide	Sodium oleate	30% Cremophor EL, 30% Capmul MCM, 10% Propylene Glycol, 30% Captex 355	0.4	–

Modified with permission from table 1 in [111] where source references for examples are cited.

we note that excipients in some of the SEDDS that are being used for HIP formulations also act as efficacious PEs, a good example being Labrasol®. It will be interesting to see if HIP in SEDDS formulations can prove to be a real platform for translation for oral peptides. Maintaining stability in the GI tract for these emulsion-based systems is one of the major obstacles.

6. Nanoparticles and oral peptide delivery

The early years of ADDR contained articles from leaders in the field that were unreservedly enthusiastic about the potential of nanoparticles to solve many oral drug delivery problems, including those of peptides e.g. [115,116]. The rationale was obvious, but unfortunately at that time the mechanistic understanding was not biologically-sound: the hypothesis was that peptide-entrapped nanoparticles released in the upper GI from an enteric capsule could provide protection from peptidases and be efficiently absorbed intact across the mucosal epithelium. However, these predictions did not translate. Assumptions were made predicting very high epithelial uptake of nanoparticles in vivo based predominantly on Caco-2 and M-like cell-based studies using particle compositions based on polystyrene, poly (lactide) co-glycolide (PLG), poly (ethylene glycol) (PEG), and liposome constituents. To our knowledge, correlation of particle uptake with in vivo intestinal uptake from these in vitro bioassays has not been shown and inferences for in vivo have proved to be overly-optimistic. Another assumption was that nanoparticles would not prematurely release payload in the lumen of the intestine, which proved a very difficult parameter to control. A lack of in vivo uptake of many different types of nanoparticles also brought into focus the impact of luminal mucus in the GI tract in vivo. For example, the mucus barrier in respect of particle diameter, hydrophobic compositions, as well as cationic surface charge was initially underestimated. Unfortunately for the majority of nanoparticle constructs in the literature, peptide loading was invariably low and there was little focus on fabrication methods using biomaterials that could be scaled for manufacturing. The generation of nanoparticles with ligands targeting enterocytes or M cells also seemed especially attractive [117], until the complexity of reproducible synthesis proved problematic, along with addressing the variable GI physiology within and across species. These factors have so far mitigated against obtaining reproducible in vivo pharmacology for oral peptide nanoparticles.

6.1. Conclusions from the EU FP7 oral peptide nanoparticle project, TRANS-INT

TRANS-INT (2012–2017) was a major EU consortium investigating nanoparticle concepts for oral peptide delivery [118]. Nineteen partners researched non-ligand targeted (passive) constructs across a range of diverse structures made using established biomaterials: nanocapsules, polymeric nanoparticles, and nanocomplexes. One of us (DB) was the Deputy Coordinator and another, (RJM), was a scientific advisor. Human insulins and GLP-1 analogues provided by Sanofi (Paris, France) were used as model peptides due to the simple readout of blood glucose reduction, along with validated ELISA and LC-MS analytical measurements. An ADDR Issue on oral peptide delivery using nanoparticles was put together by the consortium in 2016 [119]. In the

context of this Review, it is timely to reflect on the approach, effort, and conclusions of TRANS-INT.

In TRANS-INT, a set of common criteria limits (particle size, loading, release in intestinal buffers, resistance to peptidases, and cytotoxicity) were set for a nanoparticle formulation to qualify for further investigation. Also important was stability in storage and lyophilisation, as this permitted inter-lab transfer for assessments. A unique aspect was to have lab reference centres to compare formulations under the headings of cytotoxicity, Caco-2 assays, particle uptake and fluxes in rat and human intestinal mucosae in Ussing chambers, in vivo non-diabetic rat intestinal instillations, in vivo biodistribution in rats, and (for lead candidate nanoparticles) testing in a porcine model of T2D. A number of collaborative papers emerged, and prominent examples are cited in Table 3. While many of the publications from TRANS-INT offered a positive interpretation on in vivo rodent data, there was no insulin or GLP-1 analogue entrapped in a nanoparticle construct that stood out as being sufficient for further investment for scale up and translation to clinical trials. Moreover, the majority of the insulin-entrapped nanoparticles delivered less peptide to the systemic circulation than was achieved with ad-mixtures of peptides with PEs. Common difficulties were that several prototypes could not be lyophilised for inter-lab transfer; some had different characterisation upon reconstitution following lyophilisation, while others performed well in an in vivo bioassay in one lab, but not in another.

There were other problems concerning the TRANS-INT nanoparticle prototypes. These included low peptide loadings (with some exceptions) and variable release in simulated small intestinal fluid (SIF), noting that no tested prototype stayed fully intact in that buffer, which in turn reduced the possibility of particle endocytosis being a pathway to efficacy. Evidence of particle uptake for some prototypes was presented in Caco-2 cells, but rarely for human intestinal tissue mucosae, although it was technically challenging to provide accurate comparative quantitative data using nanoparticles entrapped with fluorescent probes. The relevance of particle uptake data in Caco-2 cells in a static 2-D configuration is questionable given their lack of mucous cover, but perhaps this can be improved with 3-D and spheroid cultures in microfluidic designs. A major difficulty was that chemically-conjugated fluorophores that were used to track particles materially impacted particle characteristics and behaviour. When particle uptake was assessed in vivo by fluorescent microscopy using rat jejunal instillations, the majority adhered to mucus, with just occasional pockets of epithelial uptake observed for some prototypes [126]. This was confirmed for selected examples where In Vivo Imaging Systems (IVIS) imaging revealed that the signal was primarily located in the stomach and small intestine following oral gavage to rats, e.g. [123]. One publication to date from TRANS-INT provided data on batch-to-batch variability in nanoparticle synthesis and characterisation [125]. This data will be useful if a prototype is ever to be advanced to a large animal study and translated to humans.

On the other hand, most of the prototypes were non-cytotoxic in Caco-2 cells according to a battery of assays. They did not release peptide in simulated gastric fluid, offered protection against pancreatin, and there was no evidence of intestinal histological damage in instillation studies, reflecting the decision to focus on established excipients and polymers. When PEs including the cell penetrating peptide (CPP), poly-arginine, were included in one nanoparticle construct, insulin

Table 3

Selected oral insulin nanoparticle papers from the TRANS-INT consortium.

Nanoparticle construct	Loading (w/w)	Key data	Ref.
Insulin-entrapped protamine nanocapsules with a bile salt and Miglyol 812® in core	< 1%	Blood glucose reduction in diabetic rats following instillation	[120]
Insulin complexed with amphiphilic octa-arginine derivative, with an envelope of PEG-poly (glutamic acid, PGA), abbreviated as ENCPs	18–41%	SPECT-CT in rats following oral administration showed most in GI tract, some in bladder, suggestion of interaction with GI epithelium; however lyophilized nanocarriers given in mini-capsules to normal rats had no effect on blood glucose versus insulin control.	[121]
Modified ENCPs: insulin complexed with C ₁₂ -modified octa-arginine, but with increased PEG surface density	16–29%	low mucin interaction, high Caco-2 uptake, but no in vivo data in this study	[122]
Insulin in an oily core of deoxycholate, oleic acid, and Span-80, surrounded by a poly-arginine/poloxamer 188 coat	Not cited; (AE was ~80%)	IVIS imaging from labelled carriers in mice showed GI signal over 24 h. Intra-duodenal instillation to normal rats caused glucose reduction over 2 h. This was prolonged when deoxycholate was added.	[123]
Insulin complexed with an amphiphilic cationic cyclodextrin, with a PEGylated phospholipid dextran sulphate as excipients	10%	Reduction in blood glucose following jejunal administration to normal rats. Relative bioavailability of 5.5% versus s.c. administration.	[124]
Insulin in a core comprising L-arginine and zinc in ratios, overlaid with a silica shell	59%	Reduction in blood glucose following jejunal administration of insulin particles to normal rats.	[125]

delivery was enabled to some extent in a rat instillation model [123]. The idea of co-formulation of PEs in nanoparticles as a generalised concept has led to subsequent papers. In one example [125], a core nanoparticle was formed between insulin, the enhancer L-arginine and zinc, which was then coated with silica and instilled to rat jejunal loops where insulin was delivered. Efficacy was enabled by L-arginine increasing epithelial uptake of the released insulin at the epithelium. This strategy is a response to the realisation that, despite occasional dramatic images of uptake of untargeted nanoparticles by rodent small intestinal segments [127], the majority do not get that far and, therefore, permeability assistance for the released payload must be provided. Finally, it was noted by the Consortium that most of the insulin nanoparticle papers from the 1980s and 90s provided PD data from induced diabetic animal models, none of which led to translation. One possibility is that diabetic models may have inherent bias for insulin due to hypersensitivity compared to normal models.

Even though TRANS-INT examined over 10s of prototypes across 5 different classes of constructs, an argument could be made that it may have missed promising prototypes outside the expertise of the selected team. The EU's ALEXANDER FP7 project [128] comprised 14 academic and industrial partners and ran from 2012 to 2016; its goal was to research muco-permeating nanoparticles across multiple epithelial barriers. One example of a muco-permeating particle was a construct made from chitosan and chondroitin [129], but oral peptides were not the focus of this consortium and its main objective was to provide better mucus models for bioassays and understanding of particle-mucus interaction(s) [130]. Similarly, the COMPAQ consortium of 23 partners from the EU's Innovative Medicine Initiative ran from 2011 to 2015. Its aim was to use chemistry and delivery technologies to increase delivery of macromolecules across barriers including the GI tract. Its publication outputs have been detailed [131]. While nanofibres, PLG particles, and other modalities were described by this Consortium for oral peptide delivery, there were no animal studies reported. Unfortunately, there is no evidence therefore that any breakthroughs on oral peptide nanoparticles were made by EU consortia in the last decade. Perhaps untargeted nanoparticles will never provide enough oral peptide delivery to achieve target product profiles, or else even if they can, they simply do not out-perform less complex and cheaper systems.

6.2. Targeted nanoparticles: still lost in translation?

The concept of a targeted particle using surface-decorated ligands for oral peptides was initially advanced with the vitamin B₁₂ receptor as a potential target for insulin-entrapped dextran nanoparticles conjugated on their surface with B₁₂ [132]. The principle behind using a targeted particle is based on maximizing intestinal epithelial uptake beyond that of passive constructs and avoiding peptide release either in

the intestinal lumen or at the epithelium. This construct achieved plasma glucose reduction in rats, but it was not advanced further. Since then, other types of nanoparticle compositions have been used to try and further the concept in diabetic rat models. Examples include calcium phosphate insulin-entrapped nanoparticles coated with B₁₂ grafted to chitosan-alginate polyelectrolyte complex [133], trimethylated chitosan nanoparticles also with B₁₂ on the surface [134], and B₁₂-modified alginate nanoparticles [135]. Issues for B₁₂-targeted peptide entrapped nanoparticles relate to insufficient peptide loading, premature release in the GI tract, and low capacity uptake by the B₁₂ transporter. In addition, the targeted nanoparticle must be able to present to the receptor on enterocytes when there might be competing vitamin B₁₂ as well as other molecules from bile, cell debris, and mucus in the intestinal lumen milieu in vivo. Kelly et al, have demonstrated that polystyrene nanoparticles decorated with transferrin do not orient or present correctly at the receptor in the presence of a corona induced by plasma proteins [136]. Whether there is a corona effect on nanoparticles generated by proteins in the GI lumen is unknown, but it seems highly plausible and, if present, this could mitigate against efficient targeting with a nanoparticle conjugated with B₁₂ (or another targeting ligand) on the surface.

Further research is required in deciphering the intracellular fate in the epithelium following particle uptake via the B₁₂ pathway. Epithelial cell trafficking of B₁₂ seems to diverge from clathrin-dependent uptake in its soluble form to caveolae-dependent uptake when conjugated to polystyrene nanoparticles [137]. The assumption that a nanoparticle can easily access the circulation from epithelial cells once lysosomes are circumvented has also been challenged lately [24], where it was demonstrated that a basement membrane coating of the substrate on filter inserts could impede nanoparticle transport, in contrast to movement of soluble macromolecules. Whether there is unimpeded movement of nanoparticles across endothelia of the hepatic portal vein has also to be determined. From a manufacturing perspective [138], complex targeted nanoparticles aimed at receptors on small intestinal enterocytes are difficult to scale up given the challenge of reproducibly ensuring that there is sufficient ligand conjugated to the surface and that it is in the correct orientation for recognition. In our literature analysis of various iterations of the B₁₂-targeted insulin nanoparticle over 20 years, in vivo pharmacology has largely been limited to oral gavage of nanoparticles to rodents or diabetic rodent models, but to date, to our knowledge, there has been no translation of a targeted peptide-entrapped nanoparticle in a coated tablet or capsule to a large animal model.

Han et al. [139] have provided a wide-ranging analysis of potential targets in the GI tract that can mediate peptide transport via conjugates or targeted nanoparticles. These targets include bile acid transporters, lectin receptors, PepT1, CD44, and monocarboxylate transporters. Recently Kim et al. [140] conjugated the bile acid, glycocholic acid, to

polystyrene nanoparticles and observed increased epithelial uptake in rats following oral gavage. The authors ascribed it to uptake via the ileal apical sodium-dependent bile acid transporter (ASBT). Their hypothesis was that systemic delivery was achieved using chylomicron delivery into the lymphatics. It seems there may be significant competition for access to this transporter from endogenous sources. Another target that has been probed in detail with targeted particles containing insulin is the neonatal Fc receptor (FcRn) that mediates IgG transport along the small intestine. In 2013, Pridgeon et al. synthesized an insulin-loaded nanoparticle using a biocompatible PLG-PEG copolymer and functionalized it with conjugated Fc on the surface [141]. The targeted nanoparticle was efficacious in wild-type mice (at a low 1.1 IU kg⁻¹ dose), but not in FcRn knock-out mice, thereby demonstrating that receptor expression mediated both particle uptake and plasma glucose-lowering. Elegant imaging studies of fluorescently-labelled targeted particles revealed signal in the small intestinal epithelium following oral gavage to mice, supporting an uptake mechanism for the FcRn-targeted particle. While this important study laid down key principles for a targeted oral peptide particle concept using well-known particle biomaterials and a high capacity, well-expressed target on the epithelium, it is not known if it can become a potential platform for oral peptides via achieving data in large animals, and also whether such data compares favorably with simpler enhancer- and device-based technologies.

Others are also pursuing the FcRn target with a different type of nanoparticle construct: highly concentrated insulin entrapped porous silicon nanoparticles surface coated with albumin, and parceled in pH-sensitive HPMC particles for protection [142]. The principle here was to hijack the FcRn receptor for translocation of the construct using albumin as a ligand. The same group broadened the concept by recently entrapping GLP-1 into porous silicon nanoparticles functionalized with Fc [143]. Undecylenic acid, a PE [144], was also incorporated into the modified porous silicon nanoparticles. The *in vitro* data on Caco-2 cell uptake was promising for both examples, but this is not uncommon for targeted concepts.

In 2012, a new potential targeting approach to attach nanoparticles to intestinal goblet cells rather than normal enterocytes was discovered using *in vivo* phage display [145]. CSK (CSKSSDYQC) is a targeting oligopeptide that was combined a polymeric coating of trimethyl chitosan chloride (TMC) to generate insulin-entrapped nanoparticles for targeting goblet cells. The construct induced leading to hypoglycemia in rodents upon oral delivery. The goblet cell-targeting ligand would need to be considerably more efficient than those targeting receptors and carriers expressed on enterocytes, given that goblet cells are a minority cell population. The MoA of the CSK-coated nanoparticle on goblet cells seems to be a combination of altering internalization routes along with TJ openings [146], but its access to goblet cells was (somewhat ironically) impeded by mucus. Still, the concept has been advanced further with non-peptide drugs as payloads including gemcitabine, where oral bioavailability in rodents was improved to 60% with a CSK-TMC conjugates [147]. A recent attempt to address the mucus-association problem for CSK-entrapped nanoparticles was to make the nanoparticle charge-neutral by using a block *co*-polymer made from cationic dextran and PLG. Exenatide was used as the payload and the construct induced a 9.2% relative bioavailability in diabetic rats following oral gavage [148]. Whether targeting goblet cells using a ligand on a nanoparticle generates better data than an untargeted construct is open to question. For example, by combining exenatide with zinc chloride in association with a PEG-PLG *co*-polymer reacted with the dual CPP and PE (low MW protamine), similar relative oral bioavailability (8.4%) was achieved by the same team in the same rat model with the same peptide [151].

Multifunctional polymeric nanoparticles for oral peptides have therefore been synthesized with highly elegant designs incorporating PEs (e.g. protamine), surface ligands to target receptors (e.g. CSK), mucolytics (e.g. papain and bromelain), and pH-dependent coatings (e.g. HPMC). Still, Chater et al. have expressed safety concerns over the use of mucolytics

in nanoparticle systems for oral delivery, arguing that the mucus layer must retain its capacity to protect with adequate flow and re-annealing characteristics, which might be lost if the layer is removed [152]. In recent years, a paradox has been outlined in respect of formulation charge interactions with mucus and the apical membrane of GI epithelia. Bernkop-Schnurch has summarized the problem [153]: poly-cationic polymeric materials (e.g. chitosan and cell penetrating peptides (CPPs)) on the nanoparticle surface have a particularly strong electrostatic affinity to anionic mucus glycoproteins, which should restrict mucus permeation, but these same positively-charged structures are desirable to electrostatically interact with anionic glycoproteins (e.g. glycocalyx) and lipid rafts in the plasma membrane to facilitate uptake and cell penetration. This problem might explain why attempts to decorate nanoparticles with linear poly-arginine-based cationic CPP have so far led to relatively modest efficacy for oral peptides in rat models [123]. To address the cationic charge dilemma, nanoparticle systems with capacity to change their surface charge depending on pH, redox, or enzymatic changes have been synthesized, so that cationic charges can present at the epithelium having bypassed mucus. This approach, though elegant, adds an additional layer of complexity to an already-sophisticated nanoparticle drug delivery system. While prodrug approaches have shown that it is possible to rely on chemical and enzymatic processes in humans, it is not yet clear if inter and intra subject variability in dynamic mucus secretions will permit a consistent response.

A good example of such a “flip-flop” mechanism was demonstrated with a SNEDDs formulation, where the zeta potential became more cationic as phosphate groups were gradually removed by the actions of alkaline phosphatase at the brush border membrane [154]. Similarly, another construct was built around zeta potential changing polyphosphate nanoparticles [155]. Another approach to solve the charge dilemma was to synthesize a self-assembled double coated nanoparticle where the outer layer was comprised of N-(2-hydroxypropyl) methacrylamide (pHPMA), which could muco-permeate according to studies in mucus-producing intestinal epithelial co-cultures [156]. The authors showed that the outer layer gradually dissolved as the particle permeated mucus to reveal an inner layer of the cationic CPP, penetratin. These concepts have emerged as our understanding of both the barrier and protective role of mucus *in vivo* has become better understood. Finally, the CPP field itself has expanded to include numerous improved motifs based on structures beyond arginine [157]; perhaps these more stable peptide structures can overcome the sequential double barrier with advantageous features? A recent example was when a stable cyclic arginine-rich CPP was used to coat a liraglutide-entrapped PLA nanoparticle [158], where radiolabeled liraglutide was detected in rat plasma following oral administration. When the first ADDR articles were published on oral peptide delivery in 1987, a widely-held view was that promoting mucoadhesion using sticky polymers such as chitosan and polycarbophil would be important in solving the problem, but knowledge of mucus as a formidable barrier to nanoparticle transport, mucus composition, and mucus turn-over time has vastly increased since then. While these polymers are still relevant in oral formulation, their more recent role is thought more in terms of acting as peptidase inhibitors, PEs, and as bioadhesives for devices. Stealth coatings of nanoparticles with polymers including hydrophilic neutral PEGs and poly (sialic) acid have also become more prominent in the design of targeted nanoparticles. Table 4 summarizes some of the most interesting targeting approaches for oral peptide nanoparticles.

Three interesting concepts concerning untargeted inert nanoparticle constructs have recently emerged. First, it was recently demonstrated that a commercial silica nanoparticle available for research could activate integrin receptors on the apical membrane of intestinal epithelia to elicit insulin delivery via TJ openings in mice when they were co-administered [159]. This receptor-based mechanism applied only to anionic nanoparticles and, moreover, it was the first time that unloaded nanoparticles had been advocated as PEs *per se*; it challenges current thinking about nanoparticle GI mechanisms. At the level of controlling

Table 4
Selected targeted nanoparticle prototypes for oral peptide delivery (preclinical).

Enterocyte target	Nanoparticle construct	Key data	Ref.
Vitamin-B ₁₂ -Intrinsic Factor receptor	B ₁₂ -conjugated succinic acid-modified cross-linked dextran nanoparticles containing insulin	Plasma glucose reduction, 29% oral bioavailability relative to s.c. in diabetic rats at dose of 20 IU kg ⁻¹	[132]
Neonatal Fc receptor, FcRn	Poly(lactic acid)- <i>b</i> -poly(ethylene glycol) (PLA-PEG) block copolymer nanoparticle grafted with Fc of IgG	Absorption efficiency of 13.7% per hour in mice at 1.1 IU kg ⁻¹	[141]
Bile acid transporter, ASBT (in part)	Glycocholic acid conjugated fluorescent carboxylate polystyrene nanoparticles	Oral bioavailability of 47% at for fluorophore at a dose of 20 mg kg ⁻¹ in rats	[140]
CSK peptide transporters on goblet cells	Block copolymer of CSKSSDYQC (ligand)-cationic dextran-PLG loaded with exenatide designed to mucus-permeate	Relative bioavailability of 9.2% in rats after oral administration at 100 µg kg ⁻¹ dose	[147]
Biotin (Vitamin B ₇ receptor)	Biotin-conjugated DSPE in liposome membranes comprising soybean phosphatidylcholine, and cholesterol, and insulin	Hypoglycemia in diabetic rats and relative bioavailability of 8% in rats after oral administration at 20 IU kg ⁻¹ dose	[149]
Transferrin receptor	PEG-PLG nanoparticles with transferrin-modified exenatide-zinc	Relative bioavailability of 6.5% in rats after oral administration at 100 µg kg ⁻¹ dose	[150]

TJ openings via intracellular MLC phosphorylation mechanisms, Artursson and Lundquist noted that these nanoparticles seem to activate similar pathways as the cell-permeable PIP peptide TJ openers, as well as with C₁₀ [160].

Secondly, Xu et al. [161] discovered a mechanism in which micelle-loaded lipid nanocapsules entrapping exenatide could also activate entero-endocrine L cells to secrete GLP-1, thereby creating a synergistic effect between the delivery of exenatide and the promotion of GLP-1 secretion. This was the first time that the capacity of a nanoparticle to promote endogenous beneficial physiology had been considered. Components in the formulation that seem to help trigger GLP-1 release included lipid-based excipients, Labrafac® WL 1349, Span® 80, and Kolliphor® HS 15. Thirdly, there are exciting attempts to solve the fundamental issue of how to improve peptide loading in nanoparticle prototypes: inverse flash nanoprecipitation is a scalable self-assembly process whereby peptides can be loaded at levels 5–15-fold higher than most current attempts [162]. The peptide is loaded into a hydrophilic core surrounded by a PLA shell with a PEGylated surface using a process enabled by solubilisation using amphiphilic copolymer stabilizers. While currently being optimized for injectable peptides, there is potential for adapting it to oral delivery. This entrapment process does not rely on HIP or electrostatic attraction.

Despite papers from academic groups showing proof-of-principle of targeted and untargeted nanoparticle constructs in rat models, it is still not clear that the systems offer advantages over conventional simpler PE-based formulations made with GRAS additives or excipients, which is the comparator used by the Pharma industry. Consequently, a perusal of clinical trials of oral peptide nanoparticles shows only three constructs that have reached either Phase I or Phase II [20]. The first is from Oshadi (Rehovot, Israel), which is in Phase II with a nanoparticle comprising a silica core overlaid with peptides, polysaccharides, and oils. Peptide cargoes in the most advanced Oshadi formulation are a combination of insulin, pro-insulin, and C-peptide (termed Oshadi-icp) for T1D where plasma glucose lowering has been demonstrated along with a good safety profile (NCT01973920) [163]. The second was a bioadhesive calcium phosphate insulin particle from NOD Pharma's subsidiary, Shanghai Bialaxy (Shanghai, China) [164]. The third was from Diasome (Cleveland, USA), who reported a Phase I study of their oral insulin-loaded liposome with a hepatocyte-targeting motif (HDV) aimed at a liver-specific galactoside in 2014 [165]. By 2019 however, publication of a Phase II study with this targeting technology using the S.C. route suggested that the focus of Diasome no longer on the oral route [166]. While there may be other clinical trial activity for oral peptide nanoparticles, it is impossible to gauge this accurately from company press releases, although our sense is that it is at quite a low level.

7. Disruptive technologies: medical device approaches

At the outset of ADDR in 1987, the solutions anticipated for oral peptides were in traditional oral pharmaceutical formulations. Borne of

frustration with the incremental benefits in oral bioavailability afforded by use of PEs for niche peptides in capsules and tablets, bioengineers have led efforts to create drug-device combination products over the past 10 years. The ambition is to design devices that lead to substantial increases in oral bioavailability for oral peptides. Approaches embraced the adaptation of technologies being developed for macromolecules across the skin (e.g. patches, microneedles, and iontophoresis), while others are entirely new concepts designed for oral delivery (e.g. microcontainers). Learnings were also leveraged from the creative gastroretentive formulation designs developed by veterinary pharmaceutical engineers for intra-ruminal delivery of antibiotics and anthelmintics to cattle in the 1970s. Understanding the interaction(s) of such devices with the intestinal mucosa in the context of human GI physiology is the key to progressing these technologies [167]. For invasive needle-based device approaches, the hypothesis is based on completely by-passing the epithelium to achieve significant bioavailability, but this is offset by increased toxicological risk due to mucosal perforation that could become more extensive due to muscular peristalsis and repeated damage that would come with chronic administration. This may potentially be alleviated by designing microneedles that melt upon affixing to the GI epithelium. Some patches and micro-container designs reflect the principle of unidirectional co-release and co-localisation of peptide and PEs in high concentration gradients at the epithelial wall; this could never be achieved in a traditional tablets or capsules where dilution and spreading of released payload in the GI lumen is the norm.

7.1. SOMA, LUMI, the Robotic Pill, patches, and micro-containers

In 2019 researchers from MIT and Novo-Nordisk created an oral “self-orienting millimeter-scale applicator (SOMA)” system to deliver insulin across the stomach wall [168]. The principle of the system was that the device can correctly “right itself” at the gastric epithelium and, upon fluid ingress, actuates spring-loaded peptide-filled milliposts, which traverse the epithelium but not the underlying smooth muscle layers. The milliposts were made by compressing 0.3 mg of powdered insulin with poly(ethylene oxide); these dissolved in 60 min. In porcine studies, the authors demonstrated insulin delivery following oral administration. Histology of the porcine stomach was normal and the authors showed that the device could orient correctly in pigs from several geometric starting points. This was the first study to show that gastric delivery to the systemic circulation can be achieved for peptides via physical disruption, a parallel discovery to the oral semaglutide/SNAC tablet, which also exploited the gastric site by chemical means [59]. Attractive features of the SOMA platform potential include the loading of powdered insulin, while issues to be addressed include a limited maximum loading capacity of 700 µg, extensive toxicology, more control over the triggering of the actuation, manufacturing scale-up requirements, and whether it can be further adapted to house injectable liquids.

Members of the SOMA team also created a capsule-based injection designed for delivery of molecules across the small intestine via

microneedles, termed the “Luminal Unfolding Microneedle Injector (LUMI)” [169]. These capsules, with 9×30 mm dimensions, are composed of previously approved, osmotic-controlled release systems that used a pH-dependent methacrylate coating designed to dissolve at a pH of >5.5 . Upon capsule dissolution a spring is actuated, which leads to the release of LUMIs; each LUMI is a 1 mm long patch that contains 32 drug-entrapped, dissolvable microneedles. Microcomputer tomography studies with needles comprising barium sulfate demonstrated depth of injection and lack of perforation in ex vivo human and porcine tissue. Proof-of-principle was demonstrated for the LUMI system in pigs when a patch was loaded with 0.6 mg insulin and achieved relative oral bioavailability of 10%. This data, if confirmed, seems to be more impressive than that seen with PE-based oral formulations in large animals. Moreover, the authors could account for the non-biodegradable components of the device in feces. Pathology of the actuation sites in the small intestine appeared normal, but effects upon chronic administration would need to be further examined.

A simpler needle-based small intestinal capsule delivery system for peptides has been advanced to a Phase I trial using placebo devices by Rani Therapeutics (San Jose, CA). Termed the “Robotic Pill”, the first description of the RaniPill™ technology appeared in 2019 in a paper by Hashim et al. [170]. This technology is also based on insertion of microneedles in the small intestinal epithelium. Again, a pH-dependent coating is used on a hydroxypropyl methyl cellulose (HPMC) capsule of dimensions 28.0×11.0 mm. When the coating dissolves in the upper GI tract, fluid enters the capsule and actuates a self-inflating balloon system, which in turn activates a “micro-syringe”:

a dissolvable sucrose-based microneedle system, which pierces the epithelium. In the Hashim study [170], proof-of-principle to an extent was achieved in pigs with insulin-loaded devices, which were manually inserted and oriented in the jejunum, presumably to have a higher chance of success than with oral delivery at this point in development. According to the company, a total of 10 peptides, proteins, and antibodies have been testing in the RaniPill™ in large animal models with oral bioavailability asserted to be on a par with S.C. injection, but neither these data nor the Phase I study have yet been published. It seems that the main differences between LUMI and RaniPill™ are the actuation method following fluid ingress (spring versus balloon) and vector for the needles (patch versus micro syringe), as the principles of enteric-coated capsules and dissolvable microneedles are similar for both. Another Phase I study, registered at [clinicalTrials.gov](https://clinicaltrials.gov) (NCT03798912), is enrolling 46 human subjects for assessment of octreotide in the RaniPill™.

Patch systems for oral peptide delivery have been described by a number of academic groups. A prototype comprises large surface area mucoadhesive patches made from Carbopol/Eudragit® E PO, pectin, and sodium carboxy methyl cellulose (CMC) [171]. These patches were loaded with insulin, dimethyl palmitoyl ammonio propanesulfonate (PPS) as the PE, citric acid as the peptidase inhibitor. An impermeable ethyl cellulose backing was used to ensure unidirectional insulin release upon membrane attachment, with the systems being loaded in size 9 enteric-coated and non-coated capsules. Following oral delivery, a PD effect of insulin was demonstrated for both iterations in non-diabetic rats [171]. Borrowing from the transdermal field and building

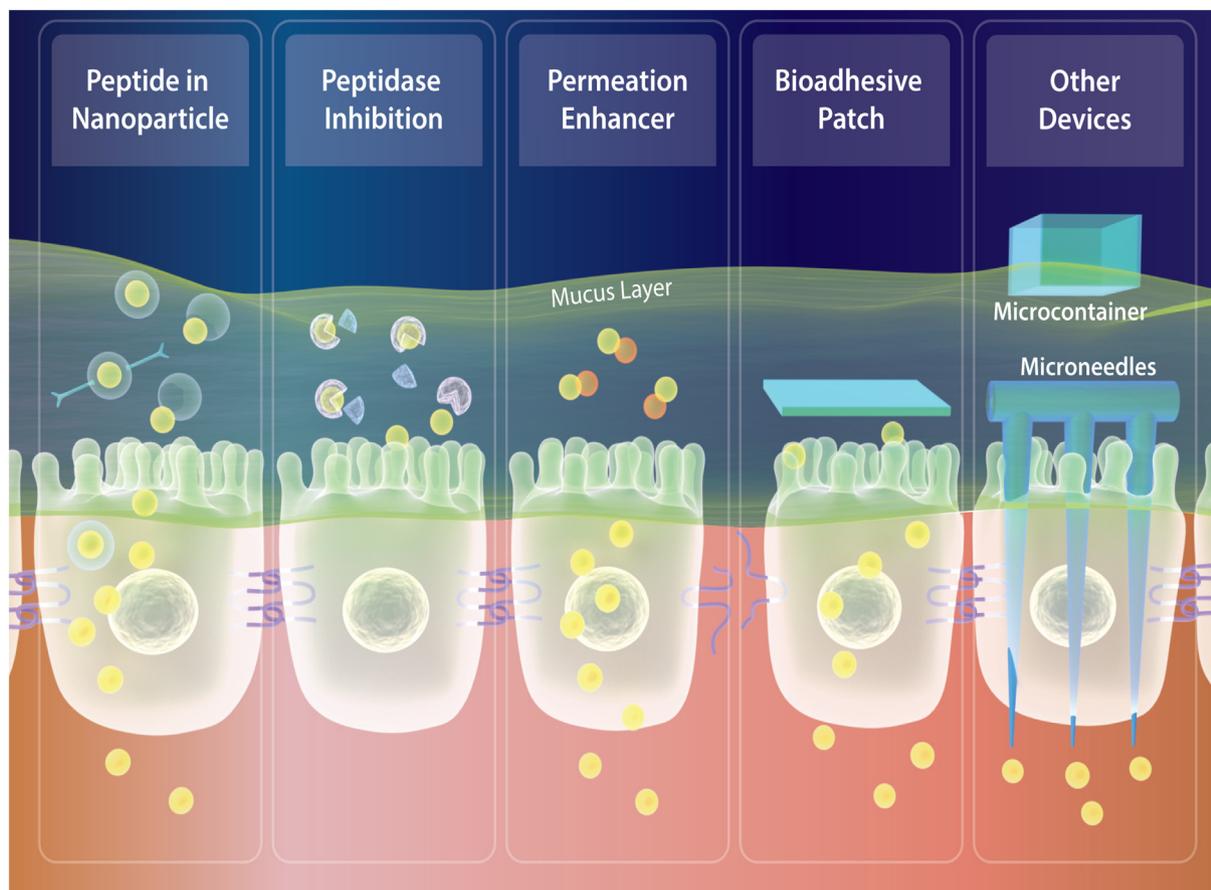


Fig. 4. Strategies for improving oral peptide delivery across intestinal epithelia. From the left: entrapment in nanoparticles; one nanoparticle model predicts peptide release close to the epithelium, while another predicts quantitative epithelial particle uptake if the particle is targeted to a receptor. Peptidase inhibitors can be included in tablets and capsules, but unlike pH modifiers, their long-term safety is questionable. Permeation enhancers (PEs) such as SNAC can bind weakly via non-covalent linkage to peptides and cause a detergent-like effect on epithelia. Some PEs like C_{10} also open tight junctions transiently. Device technologies include mucoadhesive patches, micro-containers, and biodegradable microneedle constructs. Adherent mucus is present above the epithelium and must also be negotiated. Inspired from a design [183].

upon a proof-of-concept discovered almost 20 years ago in using iontophoresis to increase intestinal flux [172], the Mitragotri group recently designed an oral iontophoretic patch for insulin delivery and efficacy was demonstrated with electrically-activated insulin-containing patches placed in rat loops [173].

In keeping with the strategy of designing a device with high concentrations of peptide and an associated PE, Jorgensen et al. synthesized micro-containers containing insulin and C₁₀ which also release in a unidirectional fashion in vitro [174]. Typically, the micro-containers are made from poly- ϵ -caprolactone, have a 0.3 mm diameter and are coated with Eudragit®-S100, which in turn can be loaded into a larger delivery system. To date, a micro-container manufacturing process has successfully been designed for poorly-soluble small molecules [175], but there might also be potential for peptides. In an in vitro Caco-2 study [174], the authors showed that the highest permeability across Caco-2 epithelial monolayers was achieved when insulin and C₁₀ were co-released from the micro-container as close as possible to the monolayer. Yet, no insulin absorption was detected when they attempted to reproduce the effect in vivo by gavaging rats with Eudragit®-L100-coated micro-containers comprising insulin, the enhancer, sodium dodecyl sulfate (SDS), and the peptidase inhibitor, soya-bean trypsin inhibitor (SBTI) [176]. The authors concluded that failure resulted from the lack of retention and correct orientation of the micro-containers in mucus. It seems that achieving micro-container proximity and attachment to the epithelium, and

unidirectional release is going to be a key challenge for patch and microneedle systems. Perhaps revisiting the potential of expanding hydrogels with meshes forming from pH-dependent polymers [177] may have potential in hybrid-device systems? Such technology may address the problems of ensuring access and adhesion of patches and microneedles to the intestinal epithelium. With respect to PE-loaded patches, from the sub-optimal results seen in a study of hexarelin absorption in a rat single pass perfusion, Dahlgren et al. [178] suggested that this outcome may have been due to spatial separation of hexarelin from C₁₀ during the perfusion, data consistent with studies in humans for tablets of insulin with the same PE [73]. To improve on its performance as a PE, C₁₀ could present better as a highly-concentrated reservoir suspension at pH 7.4. Micro-containers may eventually address this challenge for C₁₀ if enough of it (or preferably a more potent PE) can be loaded as a suspension. Clearly, there is much work to be done to understand the optimal release kinetics for PEs in bulk fluid and in the dynamic micro-environment where unidirectional release is required.

Another disruptive technology applicable for oral protein delivery uses ink-jet printing to make layer-by-layer enteric devices for insulin delivery [179]. In this approach, the authors designed a scalable process for fabricating a highly loaded planar micro-device with enteric polymers used as capping. Other device modalities that are delivering large molecules and RNA into colonic epithelia thereby overcoming the initial, permeability barrier include ultrasound [180,181]. A hand-

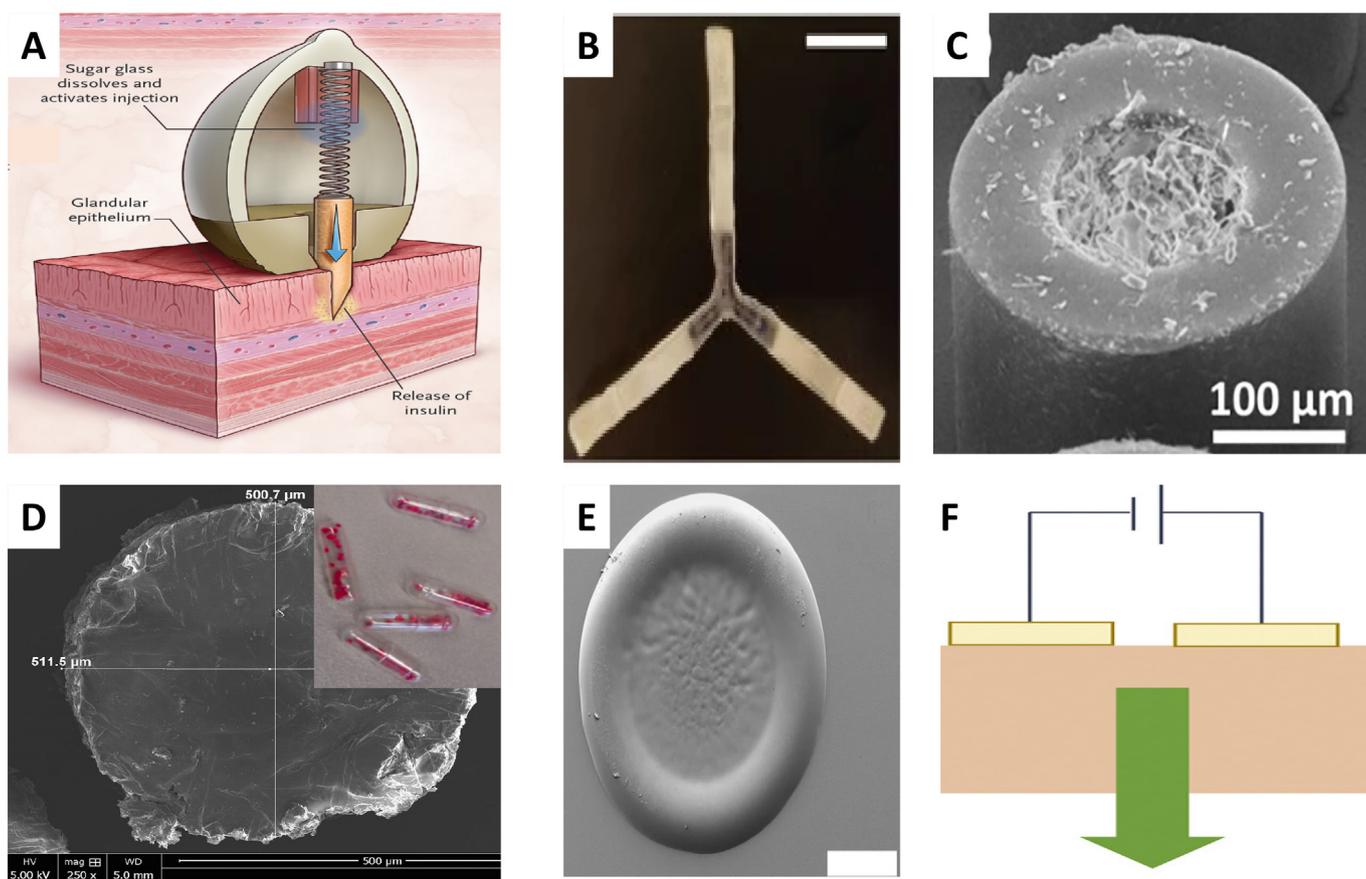


Fig. 5. 69 Examples of devices being researched for oral peptides. (A). A self-orienting millimeter-scale applicator (SOMA) lands on the gastric mucosa under the force of gravity and orients against the tissue [168]. A spring-loaded release mechanism fires and the mucosa is penetrated by a drug-containing millipost. Image re-used from [184] with publisher permission. (B) Overhead (top) image of an unfolded luminal unfolding microneedle injector (LUMI) [169]; peptide is entrapped in dissolvable microneedles on the three unfolded arms; bar = 1 cm. Image courtesy of MIT News [185]. (C) Representative scanning electron microscopy (SEM) image of a micro-container filled with C₁₀; image re-used from [174] with publisher permission. (D) SEM image of Eudragit®-L100-coated micropatches (bar = 500 μm); rhodamine dye-loaded coated muco-adhesive patches placed inside size 9 capsules (inset). Images re-used with publisher permission from [171]. (E). SEM image of micro-device synthesis using ink-jet printed silicon wafers, layer-by-layer additions, and baking at 22 °C. bar = 100 μm; reproduced from [179] with publisher permission. (F). Principle of enhanced oral insulin delivery from an iontophoretic mucoadhesive patch with anode and cathode poles; re-used from [173] with publisher permission.

held device has also been used to deliver a steroid into the buccal epithelium using ultrasound in dogs [182]. Such approaches are set to move into the next phase of advanced safety and proof-of-principle studies with peptides in large animal models, and ultimately clinical trials. Aside from physical methods to overcome the epithelial barrier, PEs (including nanoparticles) are likely to play components of micro-containers, micro-devices, and patch systems. It is possible that current sub-optimal features of stand-alone PE oral dosage forms in terms of localization of high concentrations of payload and PE might be compensated for in hybrid devices. Microdevices that promote unidirectional release of PE and payload into the adherent mucus gel (a low water microenvironment relative to bulk intestinal fluid) may however, present solubility and dissolution problems. Fig. 4 summarizes the full range of approaches being considered for oral peptide delivery, encompassing PEs, peptidase inhibitors, nanoparticles, and device types.

Fig. 5 and Table 5 summarize specific device approaches for delivering oral peptides to the systemic circulation.

8. Macrocytic peptides: towards oral drugs

Peptides tend to be polar, water soluble, polymers of amino acids that are zwitterions due to positive and negative charged ends. The ends are recognized by degrading hydrolytic enzymes (exopeptidases), such as aminopeptidases, carboxypeptidases, and dipeptidases, which use water to split off amino acids from the N- and C-terminus. Endopeptidases such as pepsin, trypsin, chymotrypsin, and elastase cut within polypeptides by recognizing short peptide sequences [186]. Such proteolytic enzymes ('proteinases,' 'peptidases,' or 'proteases') are important in the small intestine for protein digestion and in blood and cells for pruning peptides to activate or deactivate hormones and signaling proteins. The ends of polypeptides and proteins can be protected from truncation by joining them together to form cyclic peptides. Nature uses cyclisation as a post-translational modification in part to make polypeptides resistant to proteolytic degradation and in part to control functions [187,188].

Cyclisation can also occur through joining amino acid side-chains to one another (e.g. disulfide bonds), or to the N- or C- terminus, or to the amide backbone (e.g. N-alkylation) [189]. These cyclisation processes can protect peptide sequences from recognition by proteases, often by promoting folding of the peptide backbone into turn, helix, or sheet structures that are not recognized by proteases [190]. These structures are stabilized by intramolecular hydrogen bonds, ionic contacts, and side-chain packing [191]. Such control over peptide folding also enables cyclisation to 'pre-organise' the structure of a peptide for favorable binding to a target protein, often conferring higher affinity over linear analogues [192]. Folding through cyclisation helps to bury some peptide polarity in the interior of the macrocycle making its exterior surface less polar, more hydrophobic, and consequently more permeable through lipid membranes [193]. These and other advantages have led to approval of ~40 macrocyclic peptide drugs (Table 6), with many other macrocyclic peptides in clinical trials or in development [194,195]. Nevertheless, like almost all linear peptides, most cyclic peptides are not particularly membrane permeable; only a few are absorbed from the intestine, and even fewer have sufficient oral bioavailability to be effective by the oral route [193]. Here, we summarize examples of orally-administered cyclic peptides, as distinct from other macrocycles [196], and point to some observations on these and model cyclic peptides that may help lead to improvements in cyclic peptides as oral drugs.

8.1. Current cyclic peptide drugs: injectable versus oral delivery

Cyclic peptide drugs have been approved for clinical use in a number of disease settings [195,197,198] (Table 6), with bacterial and fungal infections, diabetes, and cancer being most common [193,194,197–199]. Most cyclic peptide drugs are delivered by injection, with very few

used orally due to the issues raised in this review (Fig. 6). Since almost all such drugs have low-to-negligible oral bioavailability in humans and animals, they tend to be used orally to target local conditions of the GI tract for which they do not need to be absorbed, otherwise they must rely upon very high potencies in order to deliver only trace amounts to the circulation. Thus, oral cyclic peptide antibiotics (Fig. 6) are given mainly to treat GI infections, including difficult-to-treat infections caused by *Clostridium difficile* and *Staphylococcus aureus*, and its methicillin-resistant strains [194], while others like linacotide and plicanotide (Fig. 6) are also used to locally treat Irritable Bowel Syndrome with constipation (IBS-C) and other conditions restricted to the GI tract [200–202].

An exception is CsA, which is the only currently marketed macrocyclic peptide drug in Table 6. Its appreciable oral bioavailability of 20–40% depends on lipid-based formulations for efficacy by the oral delivery route [203]. It is sold in several dosage forms (oral capsule, oral solution, eye drops, and injectable) including a micro-emulsified oral formulation, Sandimmun Neoral® [204]. A typical oral dose is 2.5–5 mg kg⁻¹ day⁻¹, with a single 200 mg oral dose to humans resulting in a C_{max} of 500–1000 ng mL⁻¹, a T_{max} of ~1.5–3 h, an AUC of 4–5 µg.h.mL, and a t_{1/2} of 3.5–5 h. It is approved for clinical use as an injectable- and orally-administered immunosuppressant that has efficacy in preventing graft-versus-host rejection during bone marrow and organ transplants, as well as in treating severe rheumatoid arthritis, psoriasis, and other inflammatory and autoimmune conditions [204]. There are ongoing efforts to improve CsA' solubility, permeability, and metabolic stability as these factors still limit oral bioavailability. Strategies to enhance its absorption include biodegradable polymeric nanoparticles, liposomal formulations, and emulsion-based beads [205].

The unusually high oral bioavailability of CsA has been the source of inspiration for developing other orally- absorbed macrocycles, prompting studies of its chemical structure, function and mechanism of action. CsA is a non-ribosomally synthesized cyclic peptide of 11 amino acids, including one D-alanine and seven N-methyl amino acids, and it is metabolized by cytochrome P450 3A to a range of 15–30 less bioactive metabolites. CsA has good oral exposure, despite being much larger (MW 1202 Da) than conventional orally bioavailable small molecule drugs (MW < 500). It violates Lipinski's parameters [206] and is not very water soluble (0.03 mg mL⁻¹, 25 °C). Clues to its desirable properties were sought from X-ray crystallography and NMR spectroscopic studies, which revealed it to be a conformationally-flexible macrocycle, adopting one major conformation in solvents like acetone and chloroform due to four stabilizing intramolecular backbone amide NH—OC hydrogen bonds, but different conformations in polar solvents compete for these hydrogen bonds and destabilize the structure [207]. When bound to its cytosolic protein receptor cyclophilin, CsA turns inside-out to adopt yet a different conformation without transannular hydrogen bonds [208,209]. In summary, the oral bioavailability of CsA has been attributed to seven N-methylated amides that reduce hydrogen bond donors, four intramolecular hydrogen bonds (three transannular) that bury polar groups in the interior of the cycle, and eleven hydrophobic amino acids that shield some of the polar backbone from water. These combined features promote CsA's passive diffusion across lipid membranes.

These properties contrast with most current cyclic peptide drugs (Table 6, Fig. 6), which have highly polar surfaces, more than five hydrogen bond donors, many rotatable bonds, few intramolecular hydrogen bonds, or some positively-charged amino acids. These features tend not to favour oral absorption [193]. For example, desmopressin acetate (DDAVP) is a potent antidiuretic hormone analogue, with deamination of Cys1 and D-Arg8 leading to enhanced metabolic stability and antidiuretic activity. However, it has fourteen atoms bearing hydrogen bond donors, three exocyclic amides, a tyrosine hydroxyl, a positively-charged arginine sidechain, and three pendant amino acids. These features contribute to high polarity and flexibility for DDAVP. It is effectively absorbed when administered intranasally, but has very low oral

Table 5
Device approaches for oral peptide delivery (preclinical).

Strategy	Device design	Key data	Ref.
Self-Orienting Millimeter-scale Applicator (SOMA)	Device comprising core of stainless steel and poly-caprolactone (PCL) overlain with a spring attached to an insulin-loaded millipost, which is actuated by fluid: designed for attachment at the gastric epithelium.	Intra-gastric administration of SOMA with 0.3 mg insulin to pigs delivered insulin and caused hypoglycemia. Histology of stomach normal after week and devices recovered in feces.	[168]
Luminal Unfolding Microneedle Injector (LUMI)	Enteric-coated capsule designed for pH-dependent release in the small intestine. Device has three spring-loaded degradable arms to propel a patch with drug-loaded dissolvable microneedles designed for the small intestine.	Intra-jejunal porcine delivery of 0.6 mg insulin in two devices: 4% lowering of blood glucose and estimated 10% systemic uptake.	[169]
RaniPill™	Enteric-coated HPMC capsule whereby fluid enters and actuates a self-inflating balloon in the upper GI, which in turn activates a dissolvable sucrose-based microneedle system.	Glucose-clamp study in swine where device containing 0.69 mg human insulin was auto-injected manually in jejunum. 98% relative bioavailability to s.c. injection of insulin.	[170]
Uni-directional mucoadhesive patches with a PE and a peptidase inhibitor	Enteric-coated capsules with 15 mg citric acid (peptidase inhibitor). Patches made from Eudragit® E PO, pectin, and SCMC, with PPS enhancer (0.2 mg), and an ethyl cellulose backing	L100 coated insulin-PPS patches with free CA in capsule (50U kg ⁻¹ insulin, ~0.2 mg PPS and ~15 mg CA. Mini-capsules gavaged to rats at an insulin dose of 50U kg ⁻¹ . Plasma glucose reduction by 66% in 6 h.	[171]
Iontophoretic patch	Same design as [171] but without PE and with electrodes attached.	Rat jejunal manual instillation study with patch linked to external battery. 50 IU kg ⁻¹ insulin. Plasma glucose reduction by 63% in 3 h. 100% relative bioavailability to s.c. injection of insulin.	[173]
Micro-containers with PE and a peptidase inhibitor	Eudragit® L 100-coated micro-containers filled with insulin: PE: SBTI powder (6:2:2, w/w/w), using either SDS or C ₁₀ was the PE. 3 mm diameter devices were made from PCL.	Oral gavage to rats failed to reduce blood glucose at an insulin dose of 104 IU kg ⁻¹ . Failure attributed to mucus impediment and orientation issues.	[176]
Ink-jet printed layer-by-layer enteric devices	Silanized wafer surface on which polymer solution is dispersed in picolitres, solvent is evaporated to form device; peptide is entrapped; device is capped with Eudragit® S 100.	100 ng insulin is deposited per device and it is stable. Multiple devices may be incorporated in a capsule.	[179]
Hand-held ultrasound device	Printed device has a 5 mm diameter aluminum half-wave horn that generates 40 kHz. Device includes a chamber filled with 1 mL therapeutic.	Proof-of-principle with isolated porcine intestine and uptake of FD3 to FD500. Budesonide was delivered to hamster-cheek pouches in vivo.	[182]

bioavailability of 0.08–0.16% in humans [210]. The commercial success of desmopressin therefore depends entirely on its high potency. Ramoplanin is a glycolipopeptide antibiotic with an even larger and much more polar than CsA; its exocyclic amino acids, sugars, and a positively charged ornithine render it poorly absorbed after oral administration [211]. Vancomycin and teicoplanin are hydrophilic fused tricyclic macrocycles with exocyclic sugars, phenolic hydroxyl groups and short $t_{1/2}$ values, which only permit intravenous administration and as a last resort antibiotic in hospitalized patients [212].

An FDA-approved oral formulation of vancomycin for treating pseudomembranous colon inflammation also does not require oral absorption [213]. Linaclotide (14 residues) and plecanatide (15 residues) are cyclic peptide drugs with multiple disulfide bonds (Fig. 6) that target guanylate cyclase C on the apical side of the epithelium. They are

delivered orally to treat IBS-C [201,202], having negligible oral bioavailability (Rat: 10 mg kg⁻¹ p.o., AUC_{0–6h} ~ 20 ng.h.mL, and oral bioavailability ~0.1%) [201]. Romidepsin is a small hydrophobic cyclic depsi-pentapeptide prodrug that inhibits zinc-containing histone deacetylase enzymes and is FDA-approved for treating T cell lymphomas. It has D-valine, D-cysteine and (3S, 4E)-3-hydroxy-7-mercapto-4-heptenoic acid residues and was discovered in the bacterium *Chromobacterium violaceum* [214]. It is Lipinski rule-of-5 compliant (MW 541, H-bond donors 4; H-bond acceptors 10, miLogP 1.6, rotatable bonds 2; total polar surface area 143 Å²) and is orally absorbed (Rat: 10 mg kg⁻¹, oral bioavailability = 16%) [215]. Yet it is still only administered by i.v. infusion to humans. Dactinomycin (actinomycin D) has two cyclic depsi-pentapeptides, each with an L-proline and two N-methyl amino acids, bridged by a phenoxazinone linker. It suppresses transcription and is

Table 6
Current cyclic peptide drugs.

Peptide	Use	Delivery	Peptide	Use	Delivery
Anidulafungin	Antifungal	i.v.	Micafungin	Antifungal	i.v.
Atosiban	Premature birth	i.v.	Octreotide	Acromegaly	i.v., i.m. s.c.
Bacitracin A	Antibiotic	topical, i.m.	Oritavancin	Antibiotic	i.v.
Bremelanotide	Sexual Dysfunction	s.c.	Oxytocin	Labour	i.v.
Capreomycin	Antibiotic	i.v.	Pasireotide	Cushing disease	s.c.
Carbetocin	Postpartum bleeding	i.v., i.m.	Pentetreotide	Diagnostic	i.v.
Caspofungin	Antifungal	i.v.	Plecanatide	Chronic idiopathic constipation	p.o.
Colistin	Antibiotic	topical	Polymixin B	Antibiotic	topical, i.m, i.v, intrathecal
Cyclosporine	Immunology	i.v., p.o.	Ramoplanin	Antibiotic	p.o.
Dactinomycin	Cancer	i.v.	Romidepsin	Cancer	i.v.
Dalbavancin	Antibiotic	i.v.	Somatostatin	Cancer	i.v.
Daptomycin	Antibiotic	i.v.	Teicoplanin	Antibiotic	i.v., p.o.
Depreotide	Diagnostic	i.v.	Telavancin	Antibiotic	i.v.
Desmopressin	Diabetes Insipidus	i.n., p.o., s.c.	Terlipressin	Blood pressure	i.v.
Edotreotide	Cancer	i.v.	Vancomycin	Antibiotic	i.v., i.p., p.o.
Eptifibatide	Anti-platelet	i.v.	Vapreotide	Variceal Bleeding	i.v.
Linaclotide	IBS-C	p.o.	Vasopressin	Diabetes Insipidus	i.v.
Lutetium Lu 177 dotatate	Cancer	i.v.	Ziconotide	Pain	Intrathecal
Lypressin	Diabetes insipidus	i.v.			

Abbreviations: i.v. = intravenous, i.m. = intramuscular, s.c. = subcutaneous, i.n. = intra-nasal, i.p. = intraperitoneal, p.o. = per oral. IBS-C: Irritable Bowel Syndrome (Constipated).

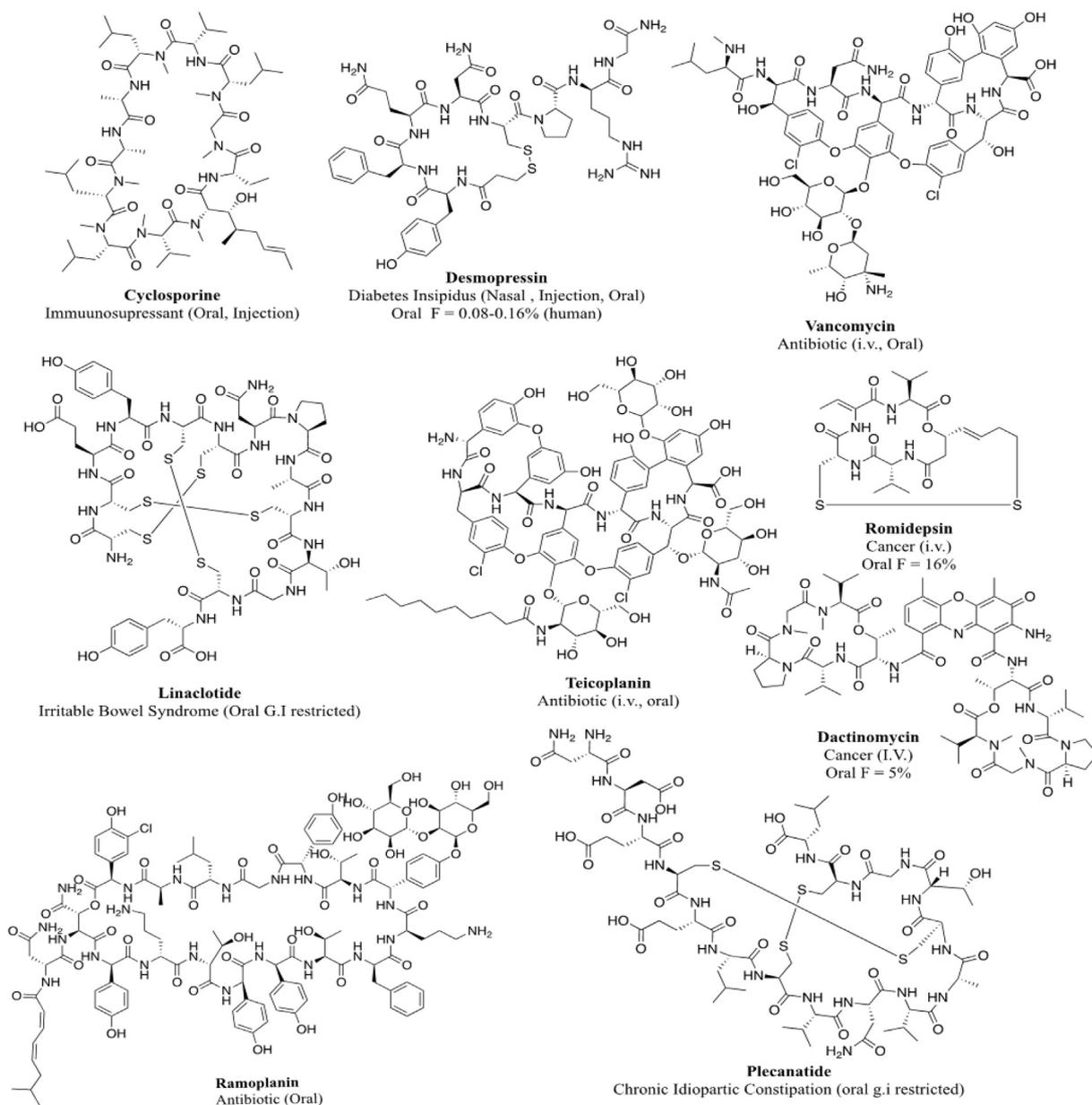


Fig. 6. Structures of current cyclic peptide drugs with oral activity.

Table 7

Experimental cyclic peptide drugs in clinical development.

Peptide	Use	Delivery ¹	Peptide	Use	Delivery ¹
Alisporivir	Antiviral	i.v, p.o	PL3994	Asthma	s.c.
ALRN-6924	Cancer	i.v.	Plitidepsin	Cancer	i.v.
AZP531	Prader-Willi Syndrome	s.c.	PMX-53	Arthritis	p.o.
Balixafortide	Cancer	i.v.	PM02734	Cancer	i.v
BMS-986189	Cancer	i.v.	POL6014	Cystic Fibrosis	Inhaled
BQ123	Cardiovascular	s.c.	PTG-200	Crohn's disease	p.o.
BT1718	Cancer	i.v.	PTG-943	Ulcerative Colitis	p.o.
Cilengitide	Cancer	i.v.	SCY-635	Antiviral	i.v, p.o.
Emodepside	Anthelmintic	p.o.	Somatropin	Acromegaly	s.c.
Kahalalide F	Cancer	i.v.	Setmelanotide	Obesity	s.c.
LFF571	Antibiotic	p.o.	Surotomycin	Antibiotic	p.o.
Murepavadin (POL7080)	Antibiotic	i.v.	Valspodar	Cancer	i.v, p.o.
Nepadutant	Gastrointestinal, Asthma	p.o.	Voclosporin	Immuno-suppressant	i.v, p.o.
NIM811	Antiviral	i.v, p.o.	Zilucoplan	Myasthenia gravis	i.v. s.c
NVB302	Antibiotic	i.v.			

Abbreviations as in Table 6.

highly cytotoxic to tumors [216,217], with moderate oral bioavailability of 5% [217].

8.2. Experimental cyclic peptide drugs with potential for clinical trials

Selected cyclic peptides that have progressed to clinical trials or are in development are shown in Table 7 [194,195,218]. Most have only low oral bioavailability due to high polarity, flexibility, or charged amines/carboxylates that all promote solvation by water. Among those shown to have oral exposure are alisporivir, valsopodar, voclosporin, NIM811, SCY-635, emodepside, LFF571, nepadutant, PMX53, surtomycin and kahalalide F (Fig. 7). Alisporivir, valsopodar, voclosporin, NIM811 and SCY-635 all maintain the 11-residue core structure of CsA, but with modifications at positions 1, 3, and 4. Modifications of CsA at residues 3 and 4 reduce immunosuppressive properties while increasing antiviral activity [219]. Alisporivir is administered orally (Rat: oral bioavailability = 46%) [220] and has good passive permeability and rapid absorption in humans [221], but Phase II trials for Hepatitis C (HCV) infections were discontinued in 2019. Valsopodar is an orally

bioavailable (42% in rat) cremophor® EL formulated inhibitor of P-glycoprotein, but lacks the immunosuppressive activity of CsA. In animal models valsopodar prevents cancer cell resistance to chemotherapeutics, however Phase III trials were not successful [222]. Voclosporin is a potent calcineurin inhibitor with immunosuppressant activity and clinical data across multiple indications. It inhibits expression of IL-2 and T-cell immune responses for preventing organ rejection in transplant recipients and recently completed a 52-week Phase III trial in patients to assess remissions for lupus nephritis. It is modestly absorbed after oral administration (rat 10 mg kg⁻¹: oral bioavailability = 7.8%) [223]. NIM811 is an orally-active cyclophilin inhibitor, with higher affinity for this target than CsA, but its complex with cyclophilin does not bind calcineurin and therefore NIM811 lacks immunosuppressive activity [224]. SCY-635 is rapidly absorbed and is orally bioavailable (Rat: 5 mg kg⁻¹ p.o., F = 23%; Monkey: 1.4 mg kg⁻¹ p.o.; oral bioavailability = 18%) and prevents the interaction with HCV NS5A protein with cyclophilin A, thereby blocking viral replication [225].

Emodepside is a cyclic octapeptide with four (depsi) ester bonds. It has been approved for treating nematode infections in animals, and

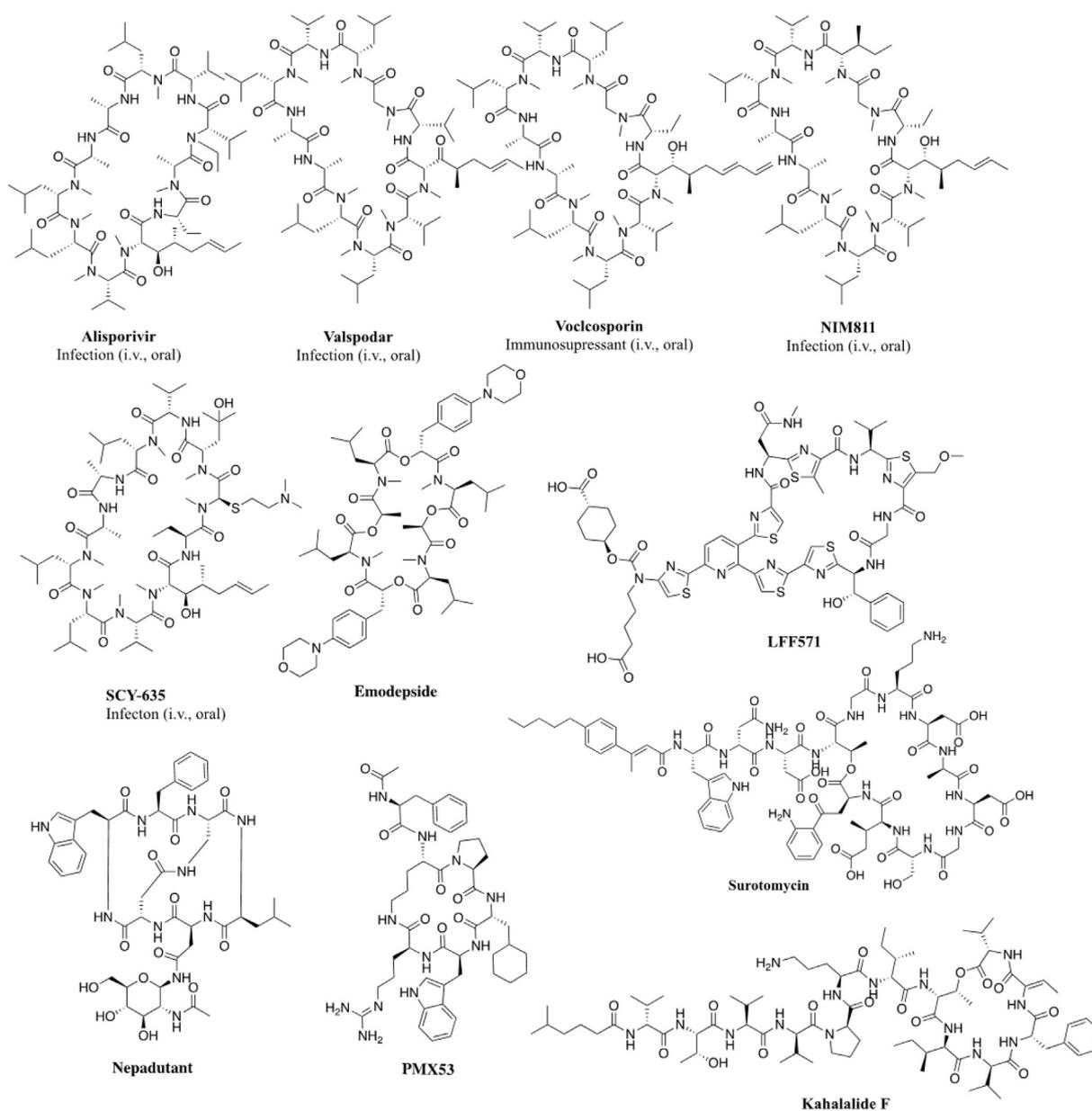


Fig. 7. Structures of cyclic peptide experimental drugs with oral activity.

has undergone Phase I human trials [226]. Since all four amides are *N*-methylated, there are no hydrogen bond donors and no transannular hydrogen bonds, resulting in the carbonyl groups being directed above and below the plane of the macrocycle. These create hydrophobic patches that confer good oral bioavailability (47–54%) [227]. LFF571 is a GI-restricted oral antibiotic, developed by Novartis from the natural product GE2270 A to treat *Clostridium difficile*, and it has completed a Phase II human trial [228]. Nepadutant is a glycosylated bicyclic hexapeptide tachykinin NK2 receptor antagonist, cyclized through both head-to-tail and side chain-to-side chain bonds, with an asparagine side chain attached to an amino-hexose that imparts amphiphilic character. It has some oral bioavailability (Mice 38 mg kg⁻¹ p.o. in castor oil: oral bioavailability = 5%) [229]. A pediatric Phase I trial located identified nepadutant in urine after 24 h, suggesting oral absorption. The Menarini group investigated its use in treating GI disorders and asthma [230]. PMX-53 (discovered as 3D53) is a designed anti-inflammatory hexapeptide that mimics the C-terminal turn of human complement protein C5a and antagonizes its binding to its GPCR (C5aR1) [231]. It has an exocyclic phenylalanine and five endocyclic amino acids (ornithine, proline, D-cyclohexylalanine, tryptophan, arginine). Despite a positively charged arginine limiting absorption, its long residence time on the receptor ($t_{1/2}$ = 15–20 h) [232] overrides high clearance and a low oral bioavailability of 1–2%, permitting efficacy to be detected in >20 rodent models of inflammatory disease and also in Phase II trials [233]. Several derivatives of PMX-53 are in development [234]. A crystal structure shows how it can bind to its receptor using 3 pi-cation interactions, extensive H-bonds with its backbone, and a hydrophobic face formed by tryptophan, dCha, proline, and phenylalanine side-chains [235]. Surotomycin is another GI-restricted oral macrocycle antibiotic, but development of this molecule was discontinued due to lack of superiority over vancomycin [236]. Kahalalide F, isolated from mollusks or green algae, is cytotoxic to cancer cells, contains a cyclic depsi-hexapeptide, and has completed a Phase I study; its high LD₅₀ (mouse: 300 mg kg⁻¹ p.o.) suggests very low oral bioavailability [237,238]. PTG-200 (an IL-23R antagonist) and PTG-943 (an $\alpha_4\beta_7$ integrin antagonist) are being tested in clinical trials sponsored by Protagonist (CA, USA) as orally-active GI-restricted drugs for Crohn's disease and ulcerative colitis, respectively [239].

8.3. Model cyclic peptides: enhancing oral absorption

Model cyclic penta-, hexa-, hepta-, octa- and deca-peptides containing hydrophobic amino acids (compounds **1–57**, Tables 8 and 9, Fig. 8) have provided interesting insights into oral bioavailability in rodents. The simple cyclic penta-L-leucine (**1**) obeys the Rule-of-5, but still has low oral bioavailability (4%) in rats (Table 8) [240]. Ring expansion to generate cyclic hexaL-leucine (**2**) violated the Rule-of-5 (MW = 679, 6 hydrogen bond donors, 12 hydrogen bond acceptors), but led to higher oral bioavailability (17.5%). The hydrophobic leucine side-chains seem to sufficiently shield the polar peptide backbone from water to confer some degree of oral exposure, without the need for *N*-methylation [240]. Interrupting the shielding by introducing one D-leucine (**3**) limits oral bioavailability (8.5%). Interestingly, the mirror image enantiomer of **2**, cyclic hexaD-leucine **4**, had less oral bioavailability and increased clearance despite having identical Lipinski Rule-of-5 parameters [241], highlighting the difficulty of applying empirical rules derived from small organic drugs to predict oral bioavailability for cyclic peptides [241].

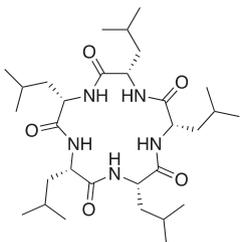
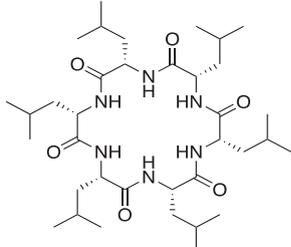
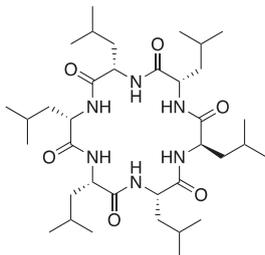
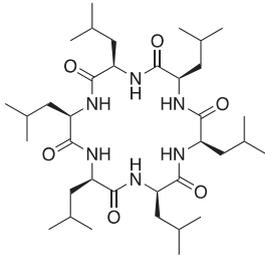
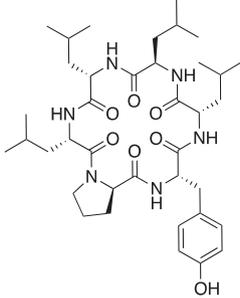
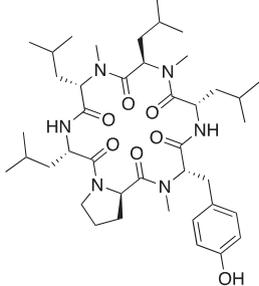
Related leucine-rich cyclic hexapeptides (**5–23**, Table 8), with hydrogen bond donors removed through amide *N*-methylation can show even higher oral bioavailability in rodents, the result of increased hydrophobicity and transannular hydrogen bonds cooperatively shielding the polar amides. Artificial membrane studies measuring permeability for stereoisomers of cyclo-[Leu-Leu-Leu-Leu-Pro-Tyr] found that cyclo-[Leu-D-Leu-Leu-Leu-D-Pro-Tyr] (**5**) had a similar passive diffusion rate to CsA under the same conditions [242]. The authors speculated that

water-to-lipid membrane-to-water conformational flexibility for (**5**) drives passive permeability that might confer oral bioavailability, by analogy to CsA [243], but (**5**) had minimal oral bioavailability (Mouse 5 mg kg⁻¹: oral bioavailability = 2%) [244]. *N*-methylation of amides not involved in hydrogen bonds gave six analogues of cyclo-[Leu-Leu-Leu-Leu-Pro-Tyr], two of which had high MDCK cell monolayer permeability comparable to propranolol; one compound (**6**) was metabolically stable in rat and human liver microsomes and orally bioavailable (Rat 10 mg kg⁻¹: oral bioavailability = 28%) [245]. The result for this model compound (**6**) sparked excitement in using it as a scaffold for further investigations. Structural studies by NMR and CD spectroscopy and molecular dynamics revealed that this compound did not change conformation in solvents of different polarities, instead remaining in a rigid conformation with two transannular hydrogen bonds [244,246]. Thus, the small size and low logP may allow enough aqueous solubility for it to stay in solution, but also to permeate lipid membranes. Some additional polarity could be tolerated to maintain both permeability and oral bioavailability. Permeability was maintained upon incorporating polar side chains into (**6**), via a serine (**7**) or threonine (**8**) analogue, but only the latter maintained oral bioavailability (2% vs 24%). Incorporating an aspartate (**9**) or lysine (**10**) greatly increased microsome stability, but reduced permeability and led to negligible oral exposure [247].

Another approach to predicting permeable compounds for PK studies was based on temperature shift coefficients in NMR spectra of diastereomers of (**4**), where (**11**) showed apparently greater oral bioavailability (33%) than (**6**), but with higher clearance, longer $t_{1/2}$, as well as lower AUC and C_{max} [248]. Enlarging the macrocycle, incorporating more polarity, and inserting gamma amino acids allowed introduction of two hydroxyl groups. These changes led to (**12**) with good cell permeability, low microsome clearance, and an oral bioavailability of 21%, equating to similar PK properties as (**6**) [249]. The effect of flexibility on oral bioavailability was explored by comparing addition of a rigid D-proline in (**13**) versus a less rigid D-leucine in (**14**), the conformationally-rigid compound having greater membrane permeability, metabolic stability and oral bioavailability [246]. This finding is consistent with less polar surface being exposed to solvent and a reduced entropy penalty for transition between polar and nonpolar environments. Novartis researchers then changed the tyrosine to phenylalanine (**15**) or aminobutyric acid (**16**) or alanine (**17**), with a new *N*-methylation pattern. Only (**15**) maintained MDCK cell monolayer permeability, while (**16**) and (**17**) showed reduced permeability. Oral bioavailability was in the opposite order: 7% (**15**), 39% (**16**), 23% (**17**), each having low AUC and a short $t_{1/2}$ [244]. Replacing tyrosine with different 2- pyridinylalanines (**18–23**) gave more impressive oral bioavailability values, with 2-pyridylalanine (**23**) being spectacularly high at 85%. NMR studies revealed that the pyridine nitrogen can form a hydrogen bond to the amide NH of the same residue, and the 2-pyridyl group increased aqueous solubility without hampering permeability in MDCK cells [250]. Another paper reported PK properties for (**23**), a mirror image of (**6**), and found greatly reduced oral bioavailability despite both compounds displaying equal permeability in the parallel artificial membrane permeability assay (PAMPA) [241]. This was attributed to greater clearance of the mirror image peptide, highlighting the challenge of predicting oral bioavailability based on physicochemical properties that are identical in enantiomers [241].

Sanguinamide A (**24**) (Table 8) is a head-to-tail cyclic heptapeptide isolated from the sea slug, *Hemigrapsus sanguineus*. NMR and modelling experiments predicted properties conducive to oral bioavailability, such as a contiguous patch of hydrophobic amino acids, only four amide NHs with two involved in transannular hydrogen bonds that shield amide polarity, as well as rigidifying heterocycles (two thiazoles, proline) that replace amides and rigidify the cycle structure. PK analysis revealed oral bioavailability of 7% [251], which was substantially improved to 51% in a structure made through an analogue design (**25**) guided solely by four key NMR observations (three-dimensional structures, solvent

Table 8
Model cyclic penta-, hexa- and hepta- peptides with oral bioavailability.¹

Peptide	P _{app} ^a	Cl _b ^(int)	Cl ^c	T _{1/2} ^d	AUC ^e	C _{max} ^f	F% ^g	Delivery	Ref
1 	1.7 MDCK	–	13	0.5	442	187	4	10 mg kg ⁻¹ in Olive Oil (Rat)	[240]
2 	10.2 MDCK	–	4.7	1.1	6289	1900	17.5	10 mg kg ⁻¹ in Olive Oil (Rat)	[240]
3 	11.9 MDCK	–	24	1	642	174	8.5	10 mg kg ⁻¹ in Olive Oil (Rat)	[240]
4 	1.6 PAMPA	90	42	0.4	291	109	7.3	10 mg kg ⁻¹ in Olive Oil (Rat)	[241]
5 	2.0 MDCK	95	82	0.2	6	3	2	5 mg kg ⁻¹ in Water (99%), Tween-80 (0.5%) and MC (0.5%). (Mouse)	[242–244]
6 	4.9 MDCK	30	5	2.8	11	852	28	10 mg kg ⁻¹ in 10% SEDDS: 90% (Rat)	[245]

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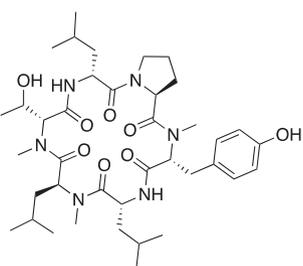
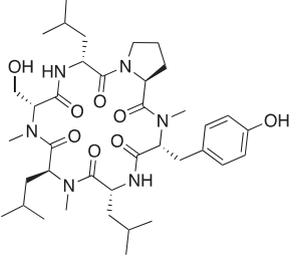
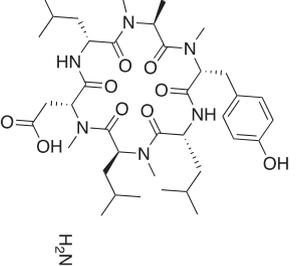
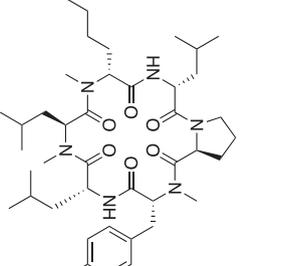
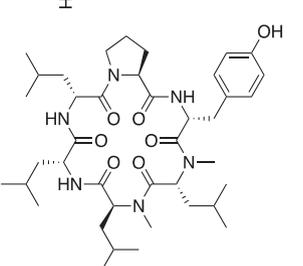
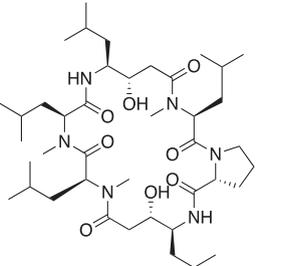
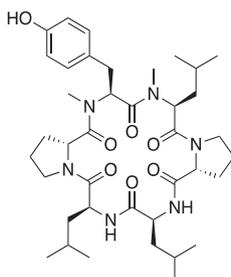
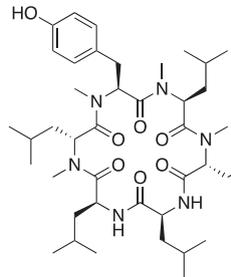
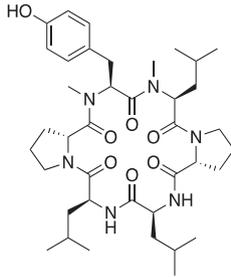
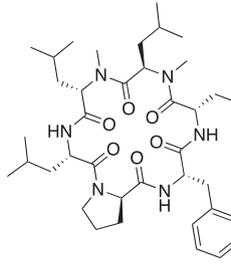
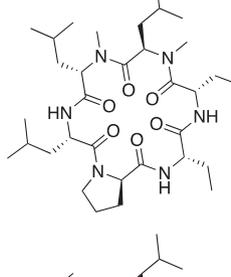
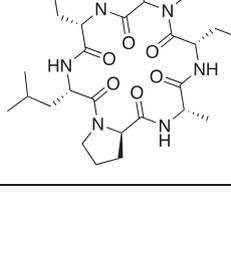
Peptide	P_{app}^a	$Cl_{b(int)}^b$	Cl^c	$T_{1/2}^d$	AUC^e	C_{max}^f	$F\%^g$	Delivery	Ref
7 	4.7 MDCK	44	64	1	105	201	24	10 mg kg ⁻¹ in 10% SEDDS: 90% water (Rat)	[247]
8 	1.6 MDCK	96	60	N.A.	42	N.A.	2	10 mg kg ⁻¹ in 10% SEDDS: 90% water (Rat)	[247]
9 	0.4 MDCK	<9	N.A.	N.A.	19	N.A.	0.5	10 mg kg ⁻¹ in 10% SEDDS: 90% water (Rat)	[247]
10 	0.3 MDCK	<9	N.A.	N.A.	18	N.A.	0.1	10 mg kg ⁻¹ in 10% SEDDS: 90% water (Rat)	[247]
11 	20 CACO-2	-	55	0.5	1003	117	33	10 mg kg ⁻¹ in Olive Oil (Rat)	[248]
12 	7.7 MDCK	30	10	1.6	1760	324	21	10 mg kg ⁻¹ in 10% propylene glycol, 5% Tween 80, 85% 20 mM phosphate buffer (Rat)	[249]

Table 8 (continued)

Peptide	P_{app}^a	Cl _b ^(int)	Cl ^c	T _{1/2} ^d	AUC ^e	C _{max} ^f	F% ^g	Delivery	Ref
13 	6.8 PAMPA	7	11	1	4320	878	30	10 mg kg ⁻¹ in Olive Oil (Rat)	[246]
14 	0.1 PAMPA	19	10	121	2918	768	18	10 mg kg ⁻¹ in Olive Oil (Rat)	[246]
14 	6.8 PAMPA	7	11	1	4320	878	30	10 mg kg ⁻¹ in Olive Oil (Rat)	[246]
15 	12 MDCK	488	2	5.5	896	77	7	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Mouse)	[244]
16 	4.0 MDCK	324	49	0.8	214	183	39	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Mouse)	[244]
17 	2.7 MDCK	654	105	0.4	59	59	23	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Mouse)	[244]

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Table 8 (continued)

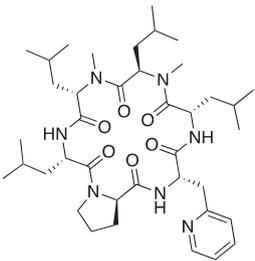
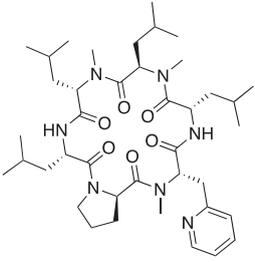
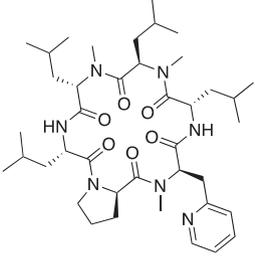
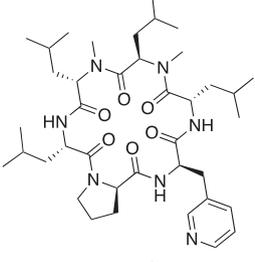
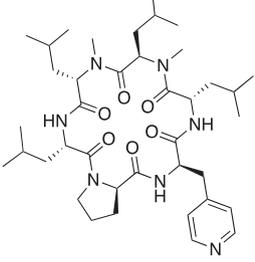
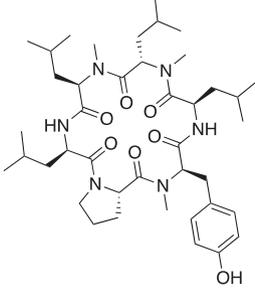
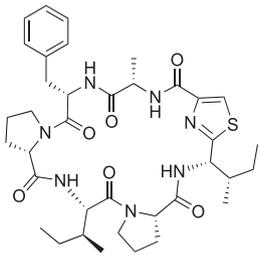
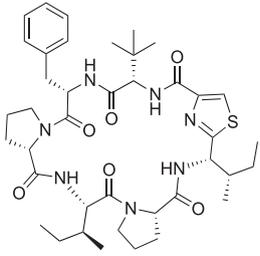
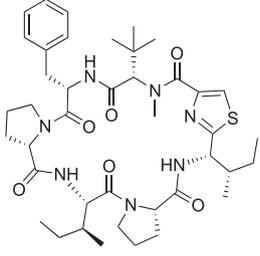
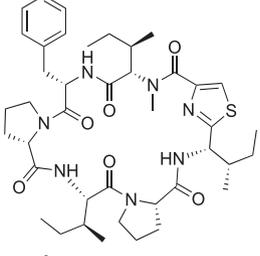
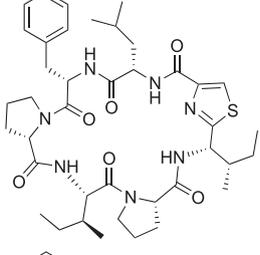
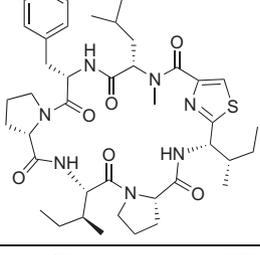
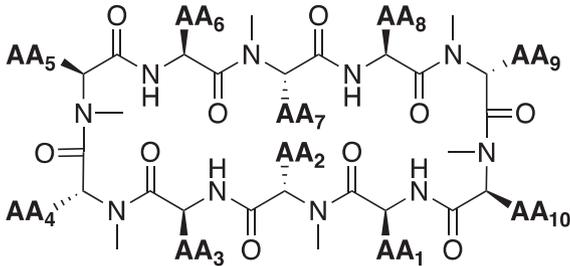
Peptide	P_{app}^a	$Cl_{b(int)}^c$	Cl^c	$T_{1/2}^d$	AUC^e	C_{max}^f	$F\%^g$	Delivery	Ref
18 	15 MDCK	–	53	0.8	434	9	5	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Rat)	[250]
19 	13 MDCK	–	5	1.6	4594	554	88	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Rat)	[250]
20 	13 MDCK	–	85	1.3	280	12	8	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Rat)	[250]
21 	8.9 MDCK	–	14	1.0	1628	282	41	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Rat)	[250]
22 	5.2 MDCK	–	76	0.6	311	22	13	10 mg kg ⁻¹ of water (99%), Tween80 (0.5%) and MC (0.5%). (Rat)	[250]
23 	1.0 PAMPA	100	23	121	4114	191	8	10 mg kg ⁻¹ in Olive Oil (Rat)	[250]

Table 8 (continued)

Peptide	P_{app}^a	Cl_{int}^b	Cl^c	$T_{1/2}^d$	AUC^e	C_{max}^f	$F\%^g$	Delivery	Ref
24	1.3 Caco-2	–	70	23	92	14	7	10 mg kg ⁻¹ in Olive Oil (Rat)	[251]
									
25	8 Caco-2	20	23	97	3372	726	51	10 mg kg ⁻¹ in Olive Oil (Rat)	[252]
									
26	14 Caco-2	60	13	65	2647	352	21	10 mg kg ⁻¹ in Olive Oil (Rat)	[252]
									
27	21 Caco-2	120	98	26	9	2	<1	10 mg kg ⁻¹ in Olive Oil (Rat)	[254]
									
28	11 Caco-2	120	85	85	192	60	10	10 mg kg ⁻¹ in Olive Oil (Rat)	[254]
									
29	19 Caco-2	400	105	110	69	105	4	10 mg kg ⁻¹ in Olive Oil (Rat)	[254]
									

¹ Footnotes: ^a P_{app} , apparent permeability coefficient ($\times 10^{-6} \text{ cm s}^{-1}$). ^b Cl_{int} , microsomal stability ($\mu\text{g}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}$). ^cPlasma clearance ($\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$). ^d $t_{1/2}$ (h). ^eArea under curve ($\text{ng}\cdot\text{h}^{-1}\cdot\text{mL}^{-1}$). ^fMaximum plasma concentration ($\text{ng}\cdot\text{mL}^{-1}$). ^gTotal oral bioavailability, F%. MDCK = Madin-Darby Canine Kidney Cell monolayers, PAMPA = parallel artificial membrane permeability assay, Caco-2 cell monolayers. MC = methyl cellulose. N.A. = not available.

Table 9
Model decapeptides with oral bioavailability¹ [255,256].



	AA1 + 8	AA2 + 7	AA3 + 6	AA4 + 9	AA5 + 10	CL ^a	AUC(i.v/p.o) ^b	F% ^c
30	L	L	L	a	A	66	256/69	27
31	L	A	L	a	A	64	277/28	10
32	L	L	L	G	P	121	144/4	3
33	A	A	A	G	P	56	377/5	1
34	L	A	L	f	P	5	4532/767	18
35	L	A	L	p	F	7	2206/1006	46
36	L	A	L	p	A	30	579/912	130*
37	L	A	L	p	V	43	379/40	11
38	L	F	L	p	A	4	3673/608	17
39	L	A	L	f	A	5	5773/487	10
40	L	A	L	l	F	1	12,368/491	4
41	L	A	L	p	F + X ^d	5	3219/1317	40
42	L	A	L	p	F + T	16	988/776	73
43	L	G	L	p	F	10	1490/322	22
44	L	L	G	p	F	21	723/214	29
45	L	A + L	L	p	F	7	2470/788	32
46	L	A + D	L	p	F	12	1192/32	2
47	L	A + K	L	p	F	8	1700/8	0.5
48	L	A + T	L	p	F	3	4354/728	15

¹ Footnotes: ^aPlasma clearance rate (mL.min⁻¹.kg⁻¹). ^bArea under curve, AUC (nM.h⁻¹.mL⁻¹). ^cOral bioavailability (F) %. ^dX = 3-pyridylalanine.

* Value exceeds 100% but has no SD reported [255]. Oral formulation for all compounds: 58% Cremophor RH40, 17% Labrafil M2125 CS, 8% propylene glycol, and 17% ethanol (w/w) [253,254].

exposed polar surfaces, amide H–D exchange rates, and temperature-dependent chemical shifts). A compact branched (tert-butyl glycine) side-chain was able to shield both polar atoms and intramolecular hydrogen bonds. *N*-methylation was detrimental (**26**) to oral bioavailability due to increased flexibility that exposed one H-bond to solvent. A combination of rigidity [246,252], stronger hydrogen bonds, and solvent shielding by branched side chains, enhanced oral bioavailability (F = 51%) [252]. Analogues of sanguinamide A were also studied to increase cell permeability (**27–29**) [253], but lower oral bioavailability was observed due to higher clearance [254]. Compounds **27–29** had greater rat liver microsome intrinsic clearance than **25–26**, possibly indicating that first-pass metabolism was limiting oral bioavailability. This study highlighted that compounds designed only on the basis of increasing membrane permeability may not in fact have the highest oral bioavailability. Compound (**29**) has a flexible conformation, changing from aqueous to non-aqueous conditions, supporting greater aqueous solubility but lower oral bioavailability due to metabolic instability, possibly because of easier access to P450 active sites [254]. The studies on Sanguinamide A identified important factors that can be incorporated into future design of orally-bioavailable peptides: hydrophobic patching, shielding of polarity and H-bonds, and rigidity versus flexibility in relation to metabolic stability and water solubility.

Novartis has reported oral bioavailability for a library of 18 cyclic decapeptides in mice (compounds **30–48**, Table 9). [255,256]. The study investigated whether parameters that improved PK of smaller cyclic peptides (Table 8), such as intramolecular hydrogen bonding and *N*-methylation of solvent-exposed amide NHs, could impart oral bioavailability to larger macrocycles. *N*-methylation and stereochemistry (L-versus D- amino acid) were varied along with side chain modifications, with NMR spectra revealing extensive and varied transannular hydrogen bonding patterns. Some compounds (**35**, **36**, **41**, **42**) had excellent oral bioavailability and low clearance. This was the first example of

designed compounds of similar MW to CsA having comparable or better oral bioavailability. Type II' β-turns observed in (**6**) were intended to be incorporated at opposite ends of a cyclic decapeptide to induce 4 transannular hydrogen bonds. To reduce polar surface area further, all amides not involved in hydrogen bonding were *N*-methylated. Macrocycles that maximized the number of transannular hydrogen bonds were more rigid and displayed greater cell permeability. Higher β sheet propensity, obtained by rigidifying the macrocycle, was found to be favorable for both permeability and oral bioavailability. Introducing polar and charged amino acids was detrimental to oral bioavailability, however inserting a pyridinylalanine or threonine led to excellent PK profiles, high oral bioavailability, and improved water solubility. A single polar modification at the β turn was well tolerated. In vitro permeability in MDCK monolayers did not, however, correlate with oral bioavailability; molecular dynamic studies suggested that increased membrane permeability correlated with a higher population of intramolecular transannular hydrogen bonded conformations in water [257]. This finding showed that designing peptides that shield polarity even in aqueous solutions could be advantageous for cell permeability, but it also presents a challenge for retaining water solubility.

While oral bioavailability has been achieved through design in model hydrophobic cyclic peptides like those above, so far there have been relatively few reports of applying the lessons learned to bioactive peptides. An example of improving PK properties of a natural product cyclic peptide is the anti-tuberculosis compound, griselimycin (**49**) [258]. Griselimycin was isolated from *Streptomyces* bacteria and has antibiotic activity [259]. In 2015 it was repurposed as an anti-tubercular agent (MIC = 1 μg mL⁻¹) with a novel mode of action against DnaN (DNA polymerase sliding clamp) and good PK properties (Mouse oral bioavailability 48%) [258]. It is cyclized through a side chain to the C-terminus bond, has eight amino acids in the cycle with one (depsipeptide) ester bond, and its PK and potency were improved by

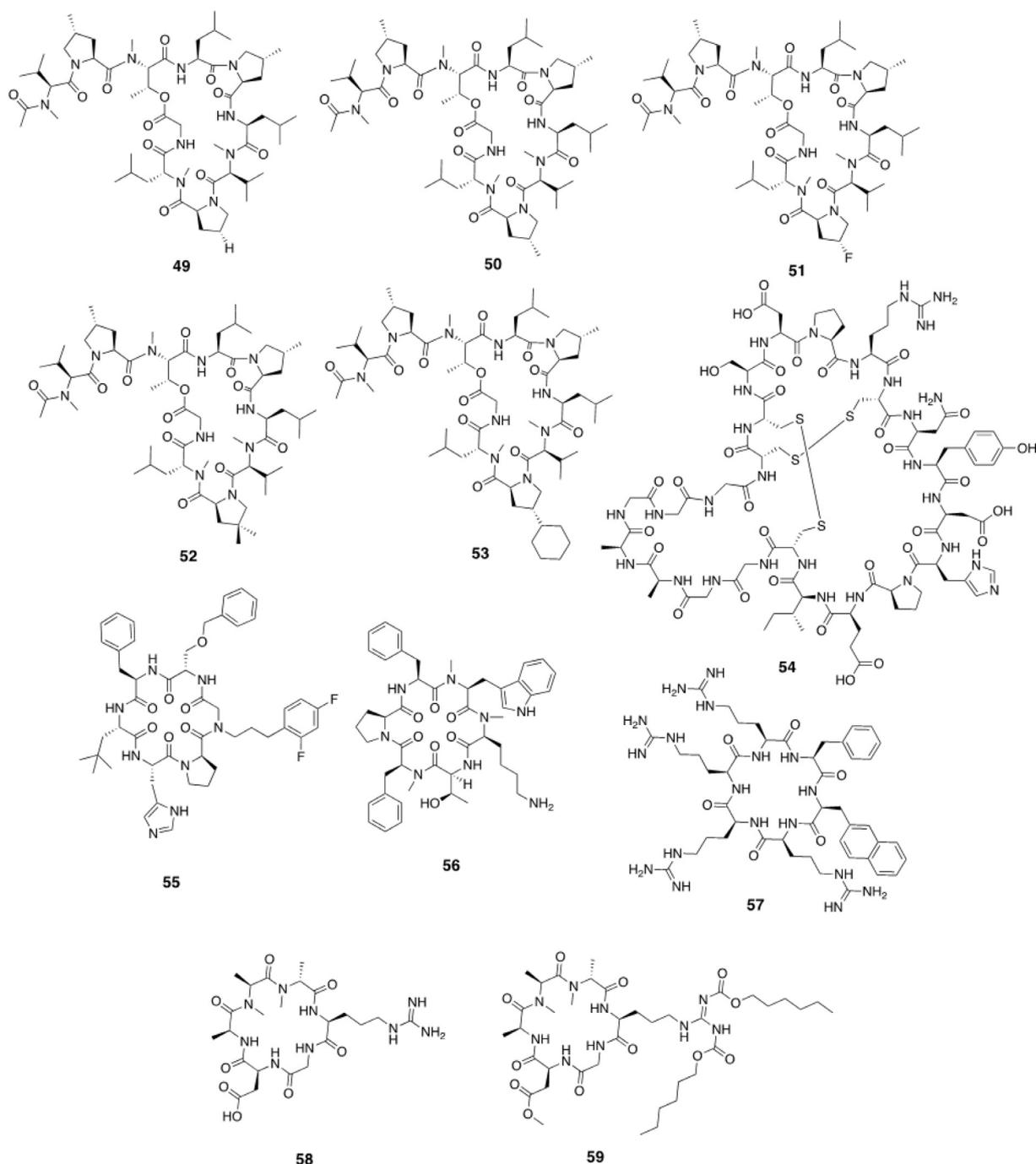


Fig. 8. Biologically active, cell penetrating and prodrug cyclic peptides.

substitution at the 4-position of its proline (Fig. 8), with a methyl- (**50**, MIC = $0.6 \mu\text{g mL}^{-1}$), fluoro- (**51**, MIC = $0.67 \mu\text{g mL}^{-1}$), dimethyl- (**52**, MIC = $0.22 \mu\text{g mL}^{-1}$) or cyclohexyl- (**53**, MIC = $0.06 \mu\text{g mL}^{-1}$) substituent. This change slightly improved stability and plasma exposure in rats: (**50**) (oral bioavailability = 47%); (**51**) (oral bioavailability = 55%); (**52**) (oral bioavailability = 59%); and (**53**) (oral bioavailability = 89%) [258]. The improvement in oral bioavailability for (**53**) results from improved stability and higher volume of distribution in vivo, attributed to increased hydrophobicity provided by the cyclohexyl substituent, which may enhance passive permeability. A crystal structure of (**49**) bound to DnaN showed two intramolecular hydrogen bonds, but no solution structure was reported, so it is unknown if this

compound class adopts the same or a different conformation in solution [258,260].

Another example of modifying a natural product is the large disulfide-bonded cyclic peptide (**54**), engineered through insertion of hydrophobic amide acids into a loop of a conotoxin from the cone snail *Conus victoriae*. It showed efficacy (despite <1% oral bioavailability) in a rat CCL-1 model of neuropathic pain, with similar efficacy to gabapentin at a 100-fold lower dose [261]. An example of a further smaller cyclic peptide example based on a designed lead compound was the design and development of a non-permeable arginine-containing cyclic hexapeptide ligand of CXCR7 ligand into an orally-bioavailable analogue (**55**) (Rat, 10 mg kg^{-1} : oral bioavailability =

18%). This involved incorporating unnatural amino acids until potency and physicochemical properties (ClogP, experimental polar surface area (EPSA), permeability) were optimized, as determined in MDCK cells [262]. Replacing a positively charged arginine and two tryptophan residues reduced polar surface area and enhanced permeability, while incorporating two fluorine atoms reduced metabolic sites, although metabolism still limited oral bioavailability. Another success in maintaining biological activity whilst improving oral bioavailability, came through assessing 30 *N*-methylated analogues of the Verber-Hirshmann cyclic hexapeptide for binding the somatostatin receptor and examining its permeability in Caco-2 monolayers. PK measurements in rats showed that (**56**) had an oral bioavailability of 10% [263].

Another approach to promote permeability of cyclic peptides is to append CPPs [264]. These are often rich in arginine or lysine and are amphipathic in nature. Common examples are TAT, penetratin, and (Arg) 6–8 [265]. Cyclic CPPs have emerged in a strategy in the last decade to improve both uptake and stability [266], compound (**57**) being an example of a cyclic hexapeptide CPP with some oral bioavailability in mice (4%) [267]. Alternatively, to convert highly polar cyclic peptides bearing charged amino acids into cell permeable compounds with improved oral bioavailability, prodrug carbamate analogues of arginine aspartic acid and lysine (**58**) [268,269] were inserted and increased oral bioavailability (e.g. 0.6–43.8% for **59**). The increased hydrophobicity of the prodrug also changed the permeation route across epithelial monolayers from paracellular to transcellular. An intriguing prospect that has yet to be fully realised is the incorporation of bioactive sequences into known orally bioavailable scaffolds of intrinsically prodrug-protection of polar side chains. Also, use of prodrug forms of cyclic peptides with low or limited oral bioavailability could be pursued.

8.4. Oral cyclic peptides: lessons for future translation

Until recently, most focus on improving oral bioavailability of peptides has been directed to increasing permeability across membranes. While cyclisation certainly protects peptides from digestion in the GI tract and it enables more peptide to remain intact so as to present at the enterocyte epithelial membrane, the polar nature of cyclic peptides is still a major limitation for oral absorption. Several approaches have been successful to some extent in increasing membrane permeability of cyclic peptides, including (i) covalent tethering to, or incorporation of CPPs, (ii) increasing hydrophobicity of amino acids in the cyclic peptide to enhance lipid-water partitioning, (iii) *N*-methylation of amides to reduce the number of hydrogen bond donors, (iv) designing cycles to encourage intramolecular and transannular hydrogen bonds that direct polar groups away from water and inside the macrocycle, and (v) shielding polar atoms and hydrogen bonds from solvent by using branched hydrophobic amino acids or hydrophobic straps. However, achieving high membrane permeability does not necessarily lead to enhanced oral bioavailability.

Following intestinal absorption, other factors can dramatically affect oral bioavailability. The first of these is metabolism, with ~60 membrane-associated cytochrome P450 enzymes in the intestinal lining, liver, lung, kidney, notably in their mitochondria and endoplasmic reticulum. They metabolize amino acid side chains, which reduces circulating concentrations of intact macrocycle and can also alter affinity and function at target peptide receptors. CsA is a classic example of how the fraction taken up by enterocytes (F_{abs} : 0.86) is not reflected in oral bioavailability owing, in part, to GI and hepatic metabolism. Clearance from the circulation is also a major problem, with high polarity and presence of *D*-amino acids promoting renal and hepatic clearance, while high hydrophobicity promotes partitioning into and trapping by lipid membranes. For these reasons, peptides typically have high clearance rates, with only low systemic concentrations available to reach their molecular targets that mediate therapeutic effects. This has been circumvented for some cyclic peptides by covalent attachment of lipids that promote albumin binding and maintain higher drug

levels in the circulation, and for other cyclic peptides by increasing receptor residence time so that maintenance of high plasma levels is not a necessity for efficacy [232].

A comparative study of over 100 orally absorbed cyclic peptides recently concluded that the Rule-of-5 restrictions on oral bioavailability for small molecule drug-like compounds do not apply to the same extent to cyclic peptides [193]. However, low numbers of hydrogen bond donors, conformational rigidity through fewer rotatable bonds, and decreased polar surface areas all contributed to cyclic peptide oral bioavailability. While *N*-methylation and depsi-peptides can be used to reduce polarity, they can affect conformation and reduce biological activity, while ester bonds appear to be prone to metabolism. Increasing conformational flexibility can increase aqueous solubility, but it does not necessarily enhance membrane permeability and often promotes metabolic instability, thereby reducing oral bioavailability. Intramolecular hydrogen bonds can be used to localise some polar components in the interior of a cyclic peptide, stabilizing a receptor-binding bioactive conformation, while hydrophobic and branched side chains on the exterior surface of the macrocycle can further shield the polar peptide backbone. In summary, while proteins use packing forces and hydrophobic collapse to direct hydrophobic groups to their interiors with exterior polar components conferring water solubility, the opposite is required for oral bioavailability of exogenous cyclic peptides. This is because there is often a need for hydrophobic exteriors to permeate lipid membranes. One or two polar side chains can sometimes be tolerated for passive permeability and can serve to promote water solubility and an amphipathic surface. Connecting hydrophobic surfaces together to present a lipophilic patch appears to be important for many cyclic peptides to increase cellular uptake and oral bioavailability, denying access of water to the peptide backbone and of metabolic enzymes to vulnerable sites in the macrocycle. Currently, Lipinski's rules for oral development are the template against which cyclic peptides are largely being mapped. There are retrospective examples of the successful development of molecules where reliance on Rule-of-5 principles proved limiting [270], but such examples do not seem to relate to oral cyclic peptides.

Finally, there are a number of areas where it is anticipated that there may be advances for oral macrocycle delivery. These include better understanding of transport mechanisms by elucidating the pathways that cyclic peptides can be taken up across lipid membranes. There are both passive and active transport mechanisms that the above design strategies have only just started to exploit. Secondly, use of compounds known to be transported by carriers can be used to conjugate hydrophilic and hydrophobic cyclic peptides; this may be a fertile area not just for oral delivery of cyclic peptides, but also for targeting them to cellular receptors on tissues. Thirdly, prodrug approaches appear to be promising for masking polar amino acids for long enough timeframes for cyclic peptides to be absorbed, but short enough for rapid conversion to their bioactive form at a target receptor. Fourthly, the combination of chemically-optimized macrocycle peptides with delivery methods including nanoparticles, ultrasound, microneedle patches, and microchip technologies is expected to be the most effective way to realise their therapeutic potential.

9. Conclusions and future perspectives

Since the beginning of ADDR in 1987, oral peptide delivery has been a regular theme of the Journal. The field has gone through long periods of failure in its efforts to achieve the promise of platform technologies based on PEs and nanotechnologies. Finally, a 20-year old permeation enhancer, SNAC, was the key to success for the oral semaglutide formulation approved for humans despite an oral bioavailability of only 0.4–1.0%. We have therefore learned that a potent and reasonably stable peptide can be presented in a traditional oral dosage form, even if 99% of the peptide is lost in journey to the systemic circulation. Additionally, the 2.5-fold variation

in delivered dose for this approach suggests that it will not be applicable for peptides having a narrow therapeutic window. There are likely to be just a few niche peptide products that can withstand such constraints. There are new PEs in preclinical research that seem to offer much larger increases in oral bioavailability in rat jejunal instillation studies, the most notable example being what is classified as an ionic liquid (or deep eutectic solvent) forged from choline and geranate [271]. Research also continues on hi-jacking endogenous epithelial transporters for pathogens, molecular approaches to specifically opening TJs, and in HIP formulations to see if oral bioavailability can be increased beyond the current threshold of ~1%.

Medicinal chemistry will be at the heart of future progress towards orally bioavailable peptide. It is currently producing small, potent stable peptides macrocycles and prodrug structures of lower MW and with higher stability and greater potential for oral formulation, a more promising scenario than larger injectable peptides with inherently unsuitable physicochemical properties. However, this field is still immature and factors that influence permeability, metabolism, pharmacokinetics and pharmacodynamics of peptides are still to be fully revealed and addressed experimentally. Future studies of peptides, cyclic peptides, and other macrocycles that more comprehensively integrate knowledge of three-dimensional structure with key properties of water versus lipid solvation, intestinal absorption, metabolic stability, clearance, as well as protein-binding and tissue distribution are expected to lead to more effective oral drugs. The level of oral bioavailability needed for efficacy of macrocycles will vary between compounds and the diseases being targeted. Realistically, most macrocycles will not demonstrate sufficient intrinsic oral bioavailability, so formulation may still be required to increase it.

The current focus of nanotechnology is on simple and scalable processes, but it is hampered by a lack of understanding of mechanisms that restrict permeation through mucus membranes *in vivo* and the controversy over whether endocytosed particles can negotiate their way to the systemic circulation at sufficient rates to be pharmaceutically acceptable and commercially viable. The surface charge and particle size dilemma over how a nanoparticle can both permeate mucus and adhere to enterocytes has been explored with creative particle designs. Targeted nanoparticles have an added level of complexity that involves reproducibility of these complex structures. It is unclear if such approaches can demonstrate biologically significant efficacy compared to untargeted nanoparticles and GRAS-based PE-based systems to be cost-effective. Similarly, devices based on physical methods (microneedles, patches, and ultrasound) designed to abrogate the epithelium have produced exciting early stage data in animals suggesting that oral bioavailability of >10% can be achieved, but questions around manufacturability, toxicology, and cost may limit acceptance. If the loading, scale-up, and toxicity questions can be addressed, peptide-device combination oral products are set to move into a new phase in translating oral peptides, and perhaps eventually even for proteins and monoclonal antibodies.

Declaration of competing interest

DB and DF act as consultants to Pharma researching oral peptides. Past collaborative research in DB's lab has been funded by Sanofi and Novo-Nordisk. RJM was the scientific founder of Trinity Biosystems, examining CD91-mediated oral delivery and he has also provided scientific guidance to Syntoxin Ltd. on the development of technology to target FcRn receptors.

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