

## Cyclic peptide oral bioavailability: lessons from the past

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**Keywords:** oral absorption, permeability, cyclic peptide

**Journal:** Biopolymers Peptide Science

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/bip.22878

## Abstract

Achieving high oral bioavailability for drugs is a key design objective in drug development. It is not surprising then that with the growing expectation of peptides as future drugs, there has also been an increasing interest in developing oral peptide therapeutics. Brought to the fore are questions such as *what* makes peptides orally bioavailable and *how* this can be achieved; questions which have inspired research into the area for decades. Early research in the area focused on linear peptides with more recent literature focusing on cyclic peptides, motivated in part by cyclic peptides like cyclosporine A that have demonstrated drug-like oral bioavailability. In this review, we take a look at research on the oral bioavailability of peptides, focusing on factors that affect passive permeability.

## 1. Introduction

As evident from recent literature on cyclic peptides<sup>1-4</sup> and the papers published in this issue,<sup>5-13</sup> there is significant interest in cyclic peptides as therapeutics. The reason for this interest is founded not only on the belief that cyclic peptides have advantages over other drug modalities but also the growing perception that they are a privileged sub-class among peptides as a whole because of their greater potential to be 'drug-like', as opposed to linear peptides for example. Supporting this trending view is the growing number of examples of cyclic peptides or analogues thereof that have oral activity or oral bioavailability.<sup>14-20</sup> Achieving high oral bioavailability is one of the most important goals in drug design, as it will result in lower doses of the administered drug, fewer side effects and, potentially, higher patient compliance.

The purpose of this review is to reflect on past and recent studies to provide perspectives on *what* affects peptide oral bioavailability. Although we will highlight some studies on linear peptides, we will focus on cyclic peptides and factors that affect their passive absorption, and briefly mention factors that affect other mechanisms of absorption towards the end of the review.

## 2. From the 'gut' to the 'blood'

Oral bioavailability is defined as the fraction of an orally administered drug that reaches systemic circulation. Once consumed, the drug must cross the enterocyte layer (in addition to other biological barriers) to reach blood vessels on the other side, as illustrated in Figure 1. Passage from one side of the cell layer to the other is referred to as cell permeability, and involves two possible pathways: the paracellular pathway, in which the drug passes through tight junctions between enterocytes; and the transcellular pathway, in which the drug passes through

enterocytes. Transcellular transport involves either active transport, which is mediated by transporters embedded in the cell membrane, or passive transport, in which the drug diffuses across the lipid membrane. It is important to distinguish between these various transport mechanisms because different factors are likely to have differing effects on peptide permeability depending on the transport pathway.

### **3. On the road of oral bioavailability**

Seminal studies by Lipinski,<sup>21</sup> Veber<sup>22</sup> and others<sup>23</sup> on the properties of drugs that are favorable for oral bioavailability have shaped much of the modern view of 'drug-likeness', but have led to the prescription that peptides are poor drugs. As these studies have been based mainly on small molecules (e.g. in Lipinski's study,<sup>21</sup> 87 peptides were excluded), the question often asked is whether these guidelines apply to peptides? In other words, are new guidelines required to determine drug-likeness of peptides?

Inspiring the quest for a more focused understanding of what governs peptide oral bioavailability is the example of cyclosporin A (CsA), a peptide drug regarded to have revolutionized human organ transplant surgery. Although there is some controversy about its discovery,<sup>24-26</sup> early studies noted that CsA was orally bioavailable,<sup>25</sup> however, the result was initially difficult to reproduce because its formulation affected the measured oral bioavailability.<sup>25-26</sup> Since the initial report of its oral bioavailability in humans of 30% (with values ranging from 10 to 60%) when administered within a chocolate drink,<sup>27</sup> subsequent studies have confirmed the importance of the delivery concoction, with reports of higher oral bioavailability (e.g. 40% for a microemulsion formulation of CsA called Neoral<sup>28</sup>) and reduced variability in absorption depending on the formulation used. Although the formulation clearly has an effect on oral bioavailability, an in-depth discussion of peptide-drug formulation is beyond the scope of this review; instead, we will focus on the structural features of peptides that affect their absorption. In this respect, CsA is distinguished from other peptides with low oral absorption by its cyclic backbone, the presence of non-canonical amino acids (e.g. N-methylated amino acids) and its conformational flexibility – specific details on these factors will be discussed in Section 4.

Figure 2 arranges selected studies on peptide oral bioavailability in a chronological display, starting with the discovery of CsA in the early 1970's and highlighting examples of cyclic peptides with moderate to high oral bioavailability. Although we made every effort to include all relevant studies, we are aware that the timeline, limited by size, is not exhaustive in its coverage. The timeline can perhaps be divided into two periods, starting with the 'dawn' of peptide oral bioavailability studies around the 1990's, which mainly encompasses works from the research groups of Burton<sup>29-33</sup> and Borchardt.<sup>34-38</sup> Although not a research article, the work by Humphrey<sup>39</sup> is highlighted in this period because its review of 20 peptides (some linear and others cyclic) and related drugs

identified factors that affect peptide absorption that are still intensely studied today. Following this period, is the 'renaissance' led mainly by the research groups of Lokey and Jacobson,<sup>16, 40-46</sup> and Kessler.<sup>15, 47-50</sup> As shown in Figure 2, these groups reported cyclic peptides with very promising oral bioavailability (e.g. a somatostatin analog with ~10% oral bioavailability<sup>15</sup> and a leucine-rich peptide with ~30% oral bioavailability<sup>16</sup>), which were surprising because they challenged the widely-held perception that peptides have poor oral bioavailability. Along with these studies, contributions from the groups of Fairlie<sup>18, 51-52</sup> and Craik<sup>17, 53</sup> and others<sup>54</sup> have progressed our understanding of what makes peptides cross membranes.

#### 4. Factors affecting passive permeability

Of the different transport pathways, a greater flux of drugs across the epithelial layer can in principle be achieved through passive transport if it is accessible to the drug. In this section, we discuss some of the key factors that affect passive permeability of peptides, including hydrogen bonds, lipophilicity, size, flexibility and structure.

##### 4.1. Hydrogen bonds

As peptides transition across a lipid bilayer, they encounter differing solvent environments, e.g. they move from an aqueous environment with a high dielectric constant to the bilayer core which has a low dielectric constant (Figure 3). In the aqueous environment, the hydrogen bond acceptors and donors of the peptide participate in hydrogen bonds with solvent water molecules, unless they are shielded from the solvent because of the local conformation or are involved in internal hydrogen bonds. These solvent interactions are disrupted upon entering the bilayer, leading to an energetic penalty that disfavors permeability.<sup>29</sup> Based on this premise, any peptide modification that reduces solvent interactions (e.g. through modification of hydrogen bond donors and acceptors<sup>15-17</sup> or by shielding them from solvent<sup>18, 45, 51</sup>) should enhance peptide permeability (Figure 3A).

Based on studies of linear peptides by Conradi *et al.*,<sup>29</sup> it was proposed that a major impediment to peptide passive absorption is the energy required to break water-peptide hydrogen bonds in order for the peptide to enter the cell membrane. A subsequent study<sup>31</sup> on N-methylated peptides found that modification of backbone amides (i.e. potential hydrogen bond donors) increased permeability, supporting the aforementioned hypothesis and distinguishing the hydrogen bond network as a determinant of peptide permeability.<sup>29, 33-34</sup> Echoing these earlier studies, more recent studies on cyclic peptides have confirmed that the hydrogen bond network also affects passive permeability of cyclic peptides,<sup>40-41, 45</sup> and N-methylation of exposed amides enhances permeability.<sup>15-17</sup> For example, White *et al.*<sup>16</sup> report, using an elegant chemical approach, the discovery of a cyclic N-methylated peptide with small-molecule-like oral bioavailability. Design of peptides with high oral

bioavailability is also possible using structure-guided approaches, such as using NMR experiments to specifically target amides for modification;<sup>17</sup> these studies highlight the utility of structural information,<sup>15-16, 18, 40, 45, 48, 51</sup> as the structure defines which amides are exposed and contribute to the energetic penalty limiting permeability. Although hydrogen bonds (both internal and external) certainly play a role in determining permeability, it has been difficult to predict permeability based on hydrogen bond counts (either internal or external) because other factors also influence permeability,<sup>53</sup> such as structure, flexibility and lipophilicity.

#### 4.2. Lipophilicity

Cyclic peptides require a degree of lipophilicity to cross a membrane – they can be neither too lipophobic nor too lipophilic. Peptides that are too lipophobic do not readily insert into the membrane, and therefore cannot cross to the other side, whereas peptides that are too lipophilic may insert too readily into the membrane, overly favouring the membrane-bound state and resulting in poor overall transport. The factors that contribute to peptide lipophilicity are varied and include hydrophobicity, which helps drive the peptide into the hydrophobic lipid bilayer core via the hydrophobic effect (a phenomenon related more to the cohesiveness between water molecules than the ‘attraction’ between hydrophobic surfaces). Another factor that affects lipophilicity is the hydrogen bond network; so, although lipophilicity is represented here as a separate factor to hydrogen bonds, the two are not completely independent of each other.

It is widely-established that lipophilicity is an important parameter for drug permeability of small molecules and peptides.<sup>35, 39, 55</sup> For cyclic peptides, there is a strong positive correlation between lipophilicity and cell permeability,<sup>42-43, 45, 53</sup> confirming that lipophilicity is also a key determinant of permeability for cyclic peptides as well; however, the trend reverses for very lipophilic compounds.<sup>53</sup> Within the positive correlation region, side-chain substitutions that increase (or decrease) lipophilicity are correlated with an increase (or decrease) in permeability.<sup>42-43, 45, 53</sup> N-methylation, which was mentioned above as a means of modifying the hydrogen bond network, also introduces additional methyl groups that increase lipophilicity, resulting in increased permeability. (In cases where lipophobic peptides that are highly N-methylated have been reported to have high membrane permeability, it is thought that those peptides are predominantly transported by mechanisms other than passive diffusion.) At high lipophilicity, however, reduced aqueous solubility and increased affinity for the membrane results in a negative correlation between lipophilicity and permeability,<sup>53</sup> suggesting that caution should be exercised at high lipophilicity. Studies on small molecules have identified that other caveats are associated with increasing lipophilicity, including higher levels of target promiscuity<sup>56</sup> and increased risks of toxicity.<sup>57</sup>

#### 4.3. Ionization

As for small molecules, it is expected that the charge state of a peptide will affect its diffusion through a lipid membrane. Specifically, an uncharged peptide would diffuse more readily through a lipid environment than a charged peptide. Indeed, increasing the content of charged amino acids in cyclic peptides is linked to reduced permeability,<sup>42, 46, 53</sup> reduced lipophilicity is also observed, indicating that charged peptides have lower lipid solubility and therefore limited diffusion through a lipid bilayer.

#### 4.4. Solubility

Poor aqueous solubility, which can arise from high lipophilicity, limits the fraction of the administered amount that is available in solution in its desired form to move across the membrane; according to Fick's Law of diffusion, the lower effective concentration (or more precisely the lower concentration gradient across the diffusion path) will result in reduced passive transport. The fraction that is not soluble will aggregate, potentially acquiring characteristics that are unfavorable for passive diffusion, such as increased size. Poor aqueous solubility also leads to variability in experimental results. For example, in early studies, very low oral absorption was sporadically observed for CsA,<sup>25-26</sup> which is very poorly soluble in water, although it is now known that CsA exhibits high oral absorption provided an appropriate formulation is used.<sup>28</sup> In *in vitro* permeability experiments, poor solubility can result in low recovery, producing permeability results that are lower than predicted<sup>42</sup> or expected based on structure-permeability correlations.<sup>53</sup>

#### 4.5. Size

Adapting the Stokes-Einstein relation to cyclic peptides, the diffusion coefficient (which represents the rate of diffusion) is expected to be inversely proportional to the molecular radius (i.e. the hydrodynamic radius if the peptide is solvated), a parameter which is in turn dependent on molecular weight. Using NMR diffusion measurements, it was observed that as molecular weight increases, so does size (in general) and shape (modulated by tertiary structure), resulting in larger radii and slower diffusion for cyclic peptides.<sup>58</sup> Attempts have been made to consider the molecular radius in computational predictions of cyclic peptide permeability<sup>42</sup> and larger cyclic peptides tend to have poor permeability.<sup>46</sup> Additionally, cyclic peptides that aggregate or self-associate will have slower diffusion because of their larger effective radii.<sup>58</sup> For example, the tendency of a cyclic penta-leucine peptide to aggregate was used to explain its relatively lower permeability.<sup>51</sup> Aside from the diffusion coefficient, other factors are also correlated to molecular size; for example, larger peptides tend to have more hydrogen bond donors and acceptors, increasing the associated effect on permeability as discussed above. So far, the largest cyclic peptide with drug-like oral bioavailability is CsA (which 11 amino acids in length); it would be interesting to discover cyclic peptides that lie far beyond this (apparent) size frontier. Towards this goal, it has been proposed that utilizing the structural features of CsA that distinguishes it from



other peptides with low oral absorption (e.g. a cyclic backbone, non-canonical amino acids, and conformational flexibility) may be the key.<sup>16</sup>

#### 4.6. Flexibility

In the discussion of the factors that affect permeability so far, it has been convenient to describe (implicitly) peptides as static systems with fixed and defined properties that determine whether they will be membrane permeable. However, even though cyclic peptides are expected to be more rigid than their linear counterparts, they can display varying degrees of dynamics depending on their chemical composition,<sup>59</sup> which would affect how they are described in terms of permeability 'factors' and how they are expected to permeate. For example, rather than attempting to characterize cyclic peptides according to discrete hydrogen bond counts, it might be more appropriate to recognize that each hydrogen bond donor (or acceptor) has varying degrees of exposure to solvent and therefore varying degrees of contribution to the thermodynamics underlying permeability.

Furthermore, the permeability process involves the movement of these dynamic systems between different solvent environments; consequently, the change in environment can induce change in peptide conformation (with the level of change dependent on how constrained the peptide is). Solvent-dependent conformational polymorphism of some membrane permeable peptides, such as CsA, led to the conformational hypothesis, which speculates that peptides that can shape-shift can negotiate into alternative energy pathways that are more favorable for permeability (Figure 3B).<sup>16, 32, 40-42</sup> In chloroform, a solvent which mimics the environment of the lipid bilayer core, CsA enters a conformational space that is different to that in an aqueous environment but one that is more compatible with its current environment by hiding most of its hydrogen bond donors and acceptors in internal hydrogen bonds.<sup>60</sup> The ability to change conformations may represent a strategy by which cyclic peptides can compensate for having other properties that are unfavorable for permeability, such as a larger size. The conformational hypothesis has been explored in more detail for cyclic hexapeptides, resulting in the finding that the change in conformation and its associated change in energy of insertion is highly predictive of passive permeability.<sup>41</sup> The benefit of conformational change has been interpreted to mean that flexibility is important, but the important distinction is that although flexibility is required for conformational change, it is more important that the peptide can 'flex' into compatible conformations without being too flexible and not sampling the 'desired conformations' with sufficient frequency. Other studies on cyclic hexa- and hepta-peptides have suggested that rigidity is another permeability-determining factor.<sup>18</sup> Although relatively rigid peptides may diffuse through membranes, it remains to be seen whether cyclic peptides of the size of CsA or larger that are rigid will be more permeable than those that can shift into energetically-preferred conformations.

#### 4.7. Structural motifs

It has been proposed that  $\beta$ -turn motifs and *cis*-peptide bonds are beneficial for peptide permeability.<sup>35, 38 48, 50</sup> The increased permeability of linear lipophilic peptides containing  $\beta$ -turn structural features has been explained by their increased lipophilicity (and improved hydrogen bond properties).<sup>35</sup> This hypothesis might explain why some structural features have been observed more frequently in membrane-permeable peptides, including CsA: they are associated with physicochemical properties that are more favorable for permeability. For example, compared to an extended linear peptide, a  $\beta$ -turn-containing peptide would have one less hydrogen bond donor and acceptor completely solvent-exposed because they are involved in an internal hydrogen bond. Other structural motifs aside from the  $\beta$ -turn might also be beneficial for peptide permeability provided that they are associated with physicochemical properties that are more favorable for permeability. As well as assisting in passive diffusion of cyclic peptides,<sup>38</sup>  $\beta$ -turn motifs have also been observed in cyclic lipophobic peptides that are not passively transported,<sup>48</sup> suggesting that  $\beta$ -turn motifs might be useful structural components for accessing other transport pathways.

#### 4.8. Cyclization

Here we attempt to link cyclization to the other factors that affect passive permeability. Compared to linear peptides of the same size, cyclic peptides sample a more restricted conformational space; if the conformations that favor permeability lie within this space and can be adequately sampled, then cyclic peptides will adopt those conformations more frequently than their linear counterparts, and therefore more frequently permeate the membrane. These conformations may be ones that contain  $\beta$ -turn motifs, exposed lipophilic atoms and side-chains, have sufficient flexibility, and/or a large proportion of internal hydrogen bonds. Indeed, early studies comparing the permeability of cyclic and linear peptides observed significantly increased permeation for cyclic peptides, which was explained by increased lipophilicity and  $\beta$ -turn motifs.<sup>38</sup> In backbone-cyclic peptides, the absence of free N- and C-termini reduce potential interactions between the peptide and solvent, which is desirable for permeability. A cyclic structure can also be more compact compared to a linear one, reducing its collision profile in solution and allowing it to diffuse faster through the membrane. Finally, cyclic peptides are typically more stable against chemical or enzymatic degradation than linear peptides, and therefore more peptide is available at each step of the transport process, resulting in greater overall amount transported.

#### 5. Other factors affecting absorption

So far, we have focused on passive absorption; however, as alluded to in Figure 1, there are many barriers that affect oral bioavailability of peptides.<sup>61-62</sup> In Figure 4, we highlight these barriers, starting with the peptide in



solution and ending with metabolism after absorption, and also identify some specific factors relevant to each barrier.

Within the gastrointestinal tract, the administered peptide is susceptible to degradation by enzymes or acidic conditions in the stomach, reducing the amount available to be absorbed. Compared to linear peptides, cyclic peptides can show enhanced metabolic stability,<sup>63-64</sup> and therefore potentially can have longer residence times within the gastrointestinal tract. It is worth noting that peptides containing cyclic structures formed by disulfide bonds may be affected by thiol-disulfide exchange reactions<sup>65</sup> that would perturb their structures and subsequently might reduce their metabolic stabilities.

Bulk flow within the gastrointestinal tract is not uniform and is limited above the epithelial layer, resulting in an unstirred water layer that is protected from convective mixing forces, slowing absorption of peptides from the bulk flow. Also on top of the enterocyte layer is the mucosal layer, which is a viscoelastic barrier that contains a large proportion of negatively-charged glycoproteins that will hinder movement of peptides due to ionic interactions. Furthermore, it has been shown that peptides >6.5 kDa have poor permeation across the mucus layer.<sup>66</sup> However a cut-off in molecular size of peptides is difficult to define, as the mucus is a very dynamic system.

Once past the mucus layer, peptides can passively diffuse across the cell layer and/or be transported actively and/or diffuse through the paracellular pathway. Paracellular transport is thought to be favored by small lipophobic peptides.<sup>36</sup> Using mirror image peptides, it was demonstrated that cyclic peptides can be actively transported across cells.<sup>49</sup> Active transporters, or more specifically efflux pumps, can also pump peptides back into the gastrointestinal lumen. For example, the amount of CsA transported across cells is affected by its binding to the efflux pump P-glycoprotein I.<sup>67</sup>

Even after a peptide crosses the cell layer, other metabolic processes can affect the amount in systemic circulation. For example, the first-pass effect can reduce the amount of active parent peptide that reaches systemic circulation after absorption because of metabolic processes in the liver. Enzymes in serum can further reduce the amount of peptide once it is in circulation. There are many obstacles preventing a peptide drug from reaching its target site of action after oral administration and to make an evolutionary analogy, it is certainly a case of “survival of the fittest”.

## ***6. Conclusions and Future Perspectives***

It is alluring to imagine a future where peptides form a significant proportion of approved drugs. For this aspiration to become a reality traditional drug-design paradigms dictate that we can easily deliver peptide drugs as oral therapeutics. Here, we have discussed some factors that affect peptide absorption, a precondition to oral bioavailability, including hydrogen bonds, lipophilicity, size, flexibility and structure. It is important to recognize that there is an interplay between many of these factors and that different factors will have differing effects depending on the transport pathway. Understanding these factors will hopefully enable the design of peptides that are orally bioavailable.

Given the intense interest in peptides amongst pharmaceutical and biotechnology companies at present we believe that the future for peptide-based drugs is bright, despite the challenges associated with oral delivery. Cyclic peptides offer particular advantages over linear peptides and represent a particularly promising class of molecules in our opinion. We hope that the information and discussion in this article will stimulate researchers to address some of the challenges in this field.

## Acknowledgements

This work was supported by an ARC Linkage Grant (LP110200213) co-funded by Pfizer and a Queensland Government Smart Futures Co-investment Grant and National Health and Medical Research Council (NHMRC) Grants (APP1076136 and APP1107403). DJC is an Australian Research Council Laureate Fellow (FL150100146).

Accepted Article

## Figure Captions

**Figure 1. Cell permeability and transport pathways.** For an orally administered peptide to be absorbed into the blood system, it must pass through several barriers as depicted here, including a layer of epithelial cells. Transport across this cell layer is called cell permeability, and includes paracellular (between cells and through tight junctions) and transcellular (through cells). Transcellular transport is divided into either active (carrier mediated) or passive transport across the membrane lipid bilayer, as illustrated in the inset.

**Figure 2. A timeline of studies on peptide oral bioavailability, highlighting selected studies.** Published studies and some of their main findings are shown and are grouped roughly according to their associated research groups. They are colored according to the decade in which they were published (the colors for each decade are shown on the arrow in the middle of the figure) for studies on cyclic peptides; articles describing mainly linear peptides are shaded with a grey background. Selected cyclic peptides reported to have interesting permeability or oral bioavailability are shown in circles with their chemical structures, reported oral bioavailability and size.

**Figure 3. Passive permeability of cyclic peptides across a lipid bilayer.** Panel A shows the surface and stick representation of a cyclic hexa-peptide (with a red background) that has poor permeability across the lipid bilayer (schematically represented as a head group with hydrophobic tails). The surface of the peptide comprises hydrophobic regions (orange), which would favor the membrane core environment, and polar regions (red and blue), which interact with solvent water molecules if they are exposed to the solvent. After N-methylation of specific amides that are solvent-exposed, the peptide (within the green box) has increased permeability. As a result of N-methylation, the surface has an increased hydrophobic surface area and reduced polar surface area, as well as reduced interactions with solvent water molecules. Panel B shows cyclosporine A undergoing conformational change as it transitions into the membrane. The change in conformation facilitates permeability.

**Figure 4. Barriers to absorption and associated factors.** After being dissolved in solution, a peptide is challenged by the contents of the gut, and then must pass through the mucus and unstirred water layer, followed by the cell membrane. Before reaching systemic circulation, a peptide will encounter the first-pass effect (i.e. it might be metabolized in the liver). Adapted from a figure by Humprey.<sup>39</sup>

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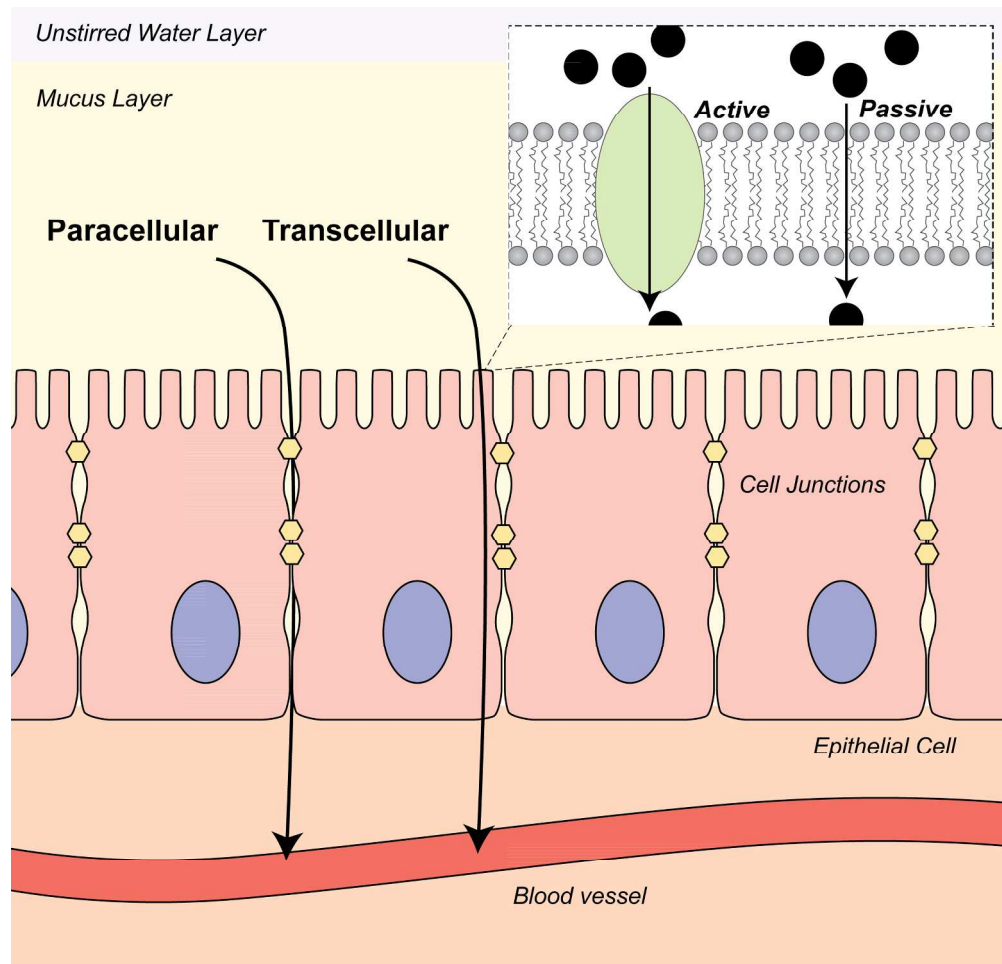


Figure 1. Cell permeability and transport pathways. For an orally administered peptide to be absorbed into the blood system, it must pass through several barriers as depicted here, including a layer of epithelial cells. Transport across this cell layer is called cell permeability, and includes paracellular (between cells and through tight junctions) and transcellular (through cells). Transcellular transport is divided into either active (carrier mediated) or passive transport across the membrane lipid bilayer, as illustrated in the inset.

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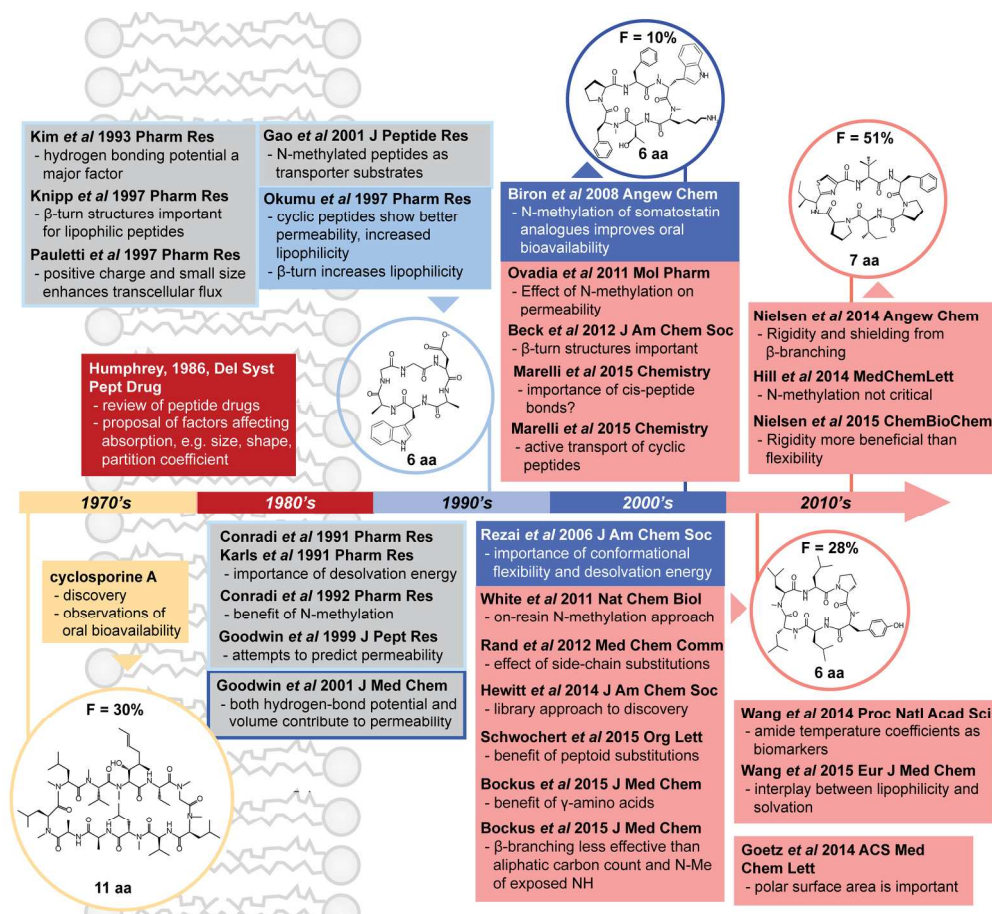


Figure 2. A timeline of studies on peptide oral bioavailability, highlighting selected studies. Published studies and some of their main findings are shown and are grouped roughly according to their associated research groups. They are colored according to the decade in which they were published (the colors for each decade are shown on the arrow in the middle of the figure) for studies on cyclic peptides; articles describing mainly linear peptides are shaded with a grey background. Selected cyclic peptides reported to have interesting permeability or oral bioavailability are shown in circles with their chemical structures, reported oral bioavailability and size.

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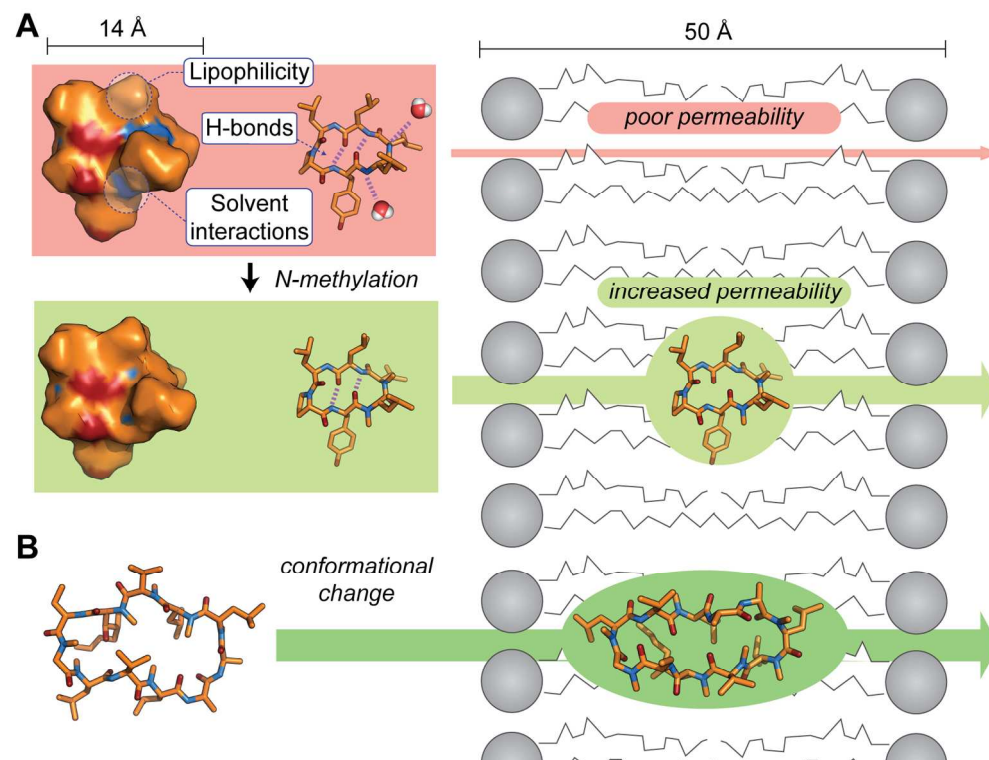


Figure 3. Passive permeability of cyclic peptides across a lipid bilayer. Panel A shows the surface and stick representation of a cyclic hexa-peptide (with a red background) that has poor permeability across the lipid bilayer (schematically represented as a head group with hydrophobic tails). The surface of the peptide comprises hydrophobic regions (orange), which would favor the membrane core environment, and polar regions (red and blue), which interact with solvent water molecules if they are exposed to the solvent. After N-methylation of specific amides that are solvent-exposed, the peptide (within the green box) has increased permeability. As a result of N-methylation, the surface has an increased hydrophobic surface area and reduced polar surface area, as well as reduced interactions with solvent water molecules. Panel B shows cyclosporine A undergoing conformational change as it transitions into the membrane. The change in conformation facilitates permeability.

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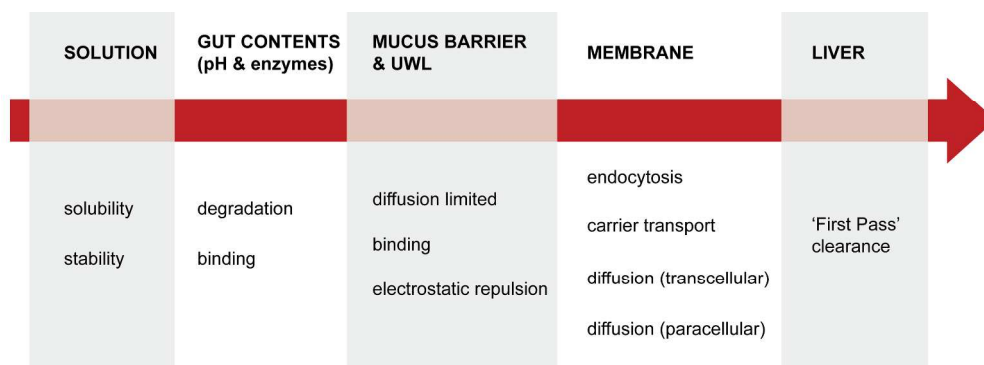


Figure 4. Barriers to absorption and associated factors. After being dissolved in solution, a peptide is challenged by the contents of the gut, and then must pass through the mucus and unstirred water layer, followed by the cell membrane. Before reaching systemic circulation, a peptide will encounter the first-pass effect (i.e. it might be metabolized in the liver). Adapted from a figure by Humprey.

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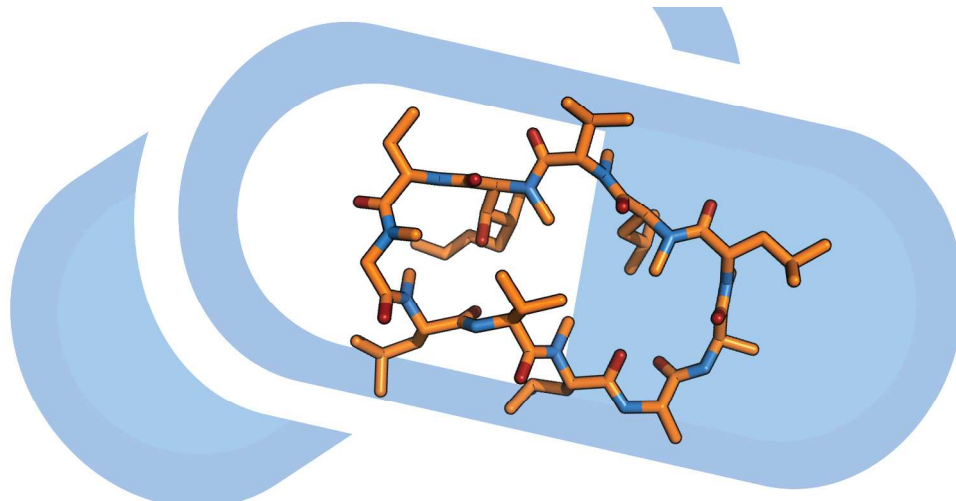


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