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The Effect of Structural and Conformation Modifications, Including Backbone Cyclization, of Hydrophilic Hexapeptides on their Intestinal Permeability and Enzymatic Stability

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Abbreviations: SIPR, structure-intestinal permeability relationships; TEER, transepithelial electrical resistance; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; NEAA, nonessential amino acids. CR, colorimetric response; NPSA, non-polar part of the surface area; P_app, Permeability coefficient; BBMVs, brush border membrane vesicles; PAMPA, Parallel Artificial Membrane Permeation Assays
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Abstract:

A library of 18 hexapeptide analogs was synthesized including sub-libraries of N- or C-methylation of the parent hexapeptide Phe-Gly-Gly-Gly-Gly-Phe as well as backbone cyclized analogs of each linear analog with various ring sizes. N- or C-methylation as well as cyclization (but not backbone cyclization) have been suggested to improve intestinal permeability and metabolic stability of peptides in general. Here we aimed to assess their applicability to hydrophilic peptides.

The intestinal permeability (Papp) of the 18-peptide library was in the range of 0.2-6.8×10⁻⁶ cm/sec. Based on several tests we concluded that the absorption mechanism of all tested analogs is paracellular, regardless of the structural or conformational modifications. In all cases, backbone cyclization increased Papp (5-fold) in comparison to the linear analogs due to the smaller 3D size and also dramatically decreased peptide proteolysis by brush border enzymes. N- or C-methylation did not enhance the permeability of the linear analogs in this series.

Key words: Hydrophilic peptides; paracellular intestinal transport; backbone cyclization; metabolic stability
Introduction

The high selectivity and often unique mode of action of peptides makes them an increasingly important class of potential drugs. However, the poor oral bioavailability limits their usage as therapeutic agents in the clinical setting\(^1\). This comes from either lack of solubility, rapid enzymatic degradation by the intestinal proteolytic enzymes, or poor intestinal permeability. Therefore, there is a substantial need to clearly identify pharmaceutical and chemical approaches that may enhance oral bioavailability of peptides while maintaining their pharmacological activity.

It has been suggested that oral bioavailability of drug candidates can be predicted by the “Rule of 5” originally formulated by Lipinski et al.\(^2\) and later revisited by Veber et al.\(^3\). These rules, while valid for most small drug-like molecules, are usually not applicable to peptides, thus, emphasizing the need to reveal the structure-intestinal permeability relationships (SIPR) of these important compounds.

Unlike di- or tri-peptides, that are absorbed from the gut by an active transporter, PEPT1, larger peptides are absorbed mainly via passive diffusion due to concentration gradient. This passive permeability across the intestine occurs either via the transcellular pathway (i.e., through the enterocyte membrane) or via the paracellular pathway (i.e., between the enterocyte cells) or via both pathways.

Many of the bioactive peptides are hydrophilic in nature and their site of action is extracellular. For example, many peptides are known (or suspected) G-protein coupled receptor (GPCR) ligands\(^4\) such that they do not need to penetrate the biological membrane in order to exert their pharmacologic/physiologic effect. The low membrane permeability of such peptides could indicate that following oral administration, their intestinal permeability would be limited to the paracellular
pathway. This absorption mechanism has been demonstrated for several known peptidic drugs, including octreotide\textsuperscript{5,6}, vasopressin\textsuperscript{7} and salmon calcitonin\textsuperscript{8}. While the main features affecting peptide transport via the paracellular route are molecular size, charge and hydrophobicity\textsuperscript{9}, the transcellular route is mainly affected by the energy of desolvation of the solute that controls the entry of the peptide to the hydrophobic interior of the membrane\textsuperscript{10}. Conradi \textit{et al.} have shown that the reduction of the number of potential hydrogen-bonds of the peptide backbone through N-methylation leads to a significant improvement in transcellular transport\textsuperscript{10}. In addition, Okumu \textit{et al.} have shown that cyclization of a series of peptides led to improved permeability. This phenomenon could be attributed to enhanced transcellular permeability resulting from elevated lipophilicity and the reduced H-bonding potential characteristic of the cyclic peptides\textsuperscript{11}. It has also been suggested that cyclization restricts transcellular permeability of peptides\textsuperscript{12} and that several physicochemical properties are dominators of peptide permeability including LogD, LogP, polar surface area (PSA), and the non polar portion of the PSA (NPSA)\textsuperscript{13}. Unfortunately, the data currently available on peptide intestinal permeability is limited and is not directly related to the underlying absorption pathway(s). Consequently, rationally designing better orally available peptide drug candidates is hampered.

The purpose of this study was to explore the effect of structural and conformational modifications of hydrophilic peptides on their intestinal permeability and metabolic stability in order to learn how to improve their oral bioavailability. This was achieved by constructing a library of hexapeptides with the general sequence Phe-Gly-Gly-Gly-Gly-Gly-Phe (that contained positive charge at the N terminus) in which successive Gly $\rightarrow$ Ala substitutions were made. Additional structural modifications that have been suggested to enhance intestinal stability and permeability were included. Our peptides
differed by their topology (i.e., linear versus backbone cyclic), their ring size, and by the number and positions of methyl groups along the peptide backbone (i.e., the degree of N- or C-methylation). Imposing conformational constraints through backbone cyclization allowed us to focus on each structural parameter individually, allowing us to examine its effect on intestinal permeability and metabolic stability.

Backbone cyclization was chosen since it allows cyclization without manipulating the side chains, thus retaining the biological activity of the parent peptide. In backbone cyclization two amide nitrogens in the peptide are interconnected via a bridge consisting of alkyl groups and an amide or a disulfide bond. Moreover, backbone cyclization combines two structural modifications that were suggested to improve peptide metabolic stability and intestinal permeability, namely N-alkylation and cyclization\textsuperscript{10, 11}

Backbone cyclization allows a variety of cyclic structures to be prepared for any given peptide sequence (parent peptide). These can differ in the position, size and chemistry of the ring\textsuperscript{14}. From such a library, a desired biologically active analog can be identified using the appropriate bioassay.

The intestinal permeability characteristics of the hexapeptide libraries were investigated by both \textit{in-vitro} and \textit{ex-vivo} models\textsuperscript{15, 16} to overcome the possibility of model-dependent results. In addition, the main transport route of the peptides was characterized by screening the permeability across a Parallel Artificial Membrane Permeability Assay (PAMPA) which consist of a phospholipid vesicle based barrier\textsuperscript{17, 18}. In addition, the mechanism was also assessed by comparing the permeability rate following chemically induced enhancement of tight junctions pore size. In addition we used also a novel approach combining colorimetric and advanced fluorescence
spectroscopy techniques, employing probes incorporated within the phospholipid bilayer in lipid/polymer vesicles.\textsuperscript{19, 20}

No active transport was shown to be involved in the permeability mechanism by comparing the apical to basolateral permeability (A to B) to the basolateral to apical permeability (B to A) for cyclic versus linear peptides in the Caco-2 model.

The stability of the synthesized peptides to enzymatic degradation by intestinal enzymes was determined in enriched brush border membrane vesicles (BBMVs) prepared from rat intestine.\textsuperscript{21}

Results

Peptide design and synthesis:

Three sub-libraries of backbone cyclic hexapeptides with the general sequence \(c(\text{Phe(Cm)-Xaa-Xaa-Xaa-Xaa-(Nn)Phe-NH}_2)\), and two sub-libraries of linear analogs with the general sequence \(H-\text{Phe-Xaa-Xaa-Xaa-Xaa-Phe-NH}_2\) were synthesized, to give a total of 18 peptides (see Figure 1 and Table 1a). The molecular weights and product purity of all the synthesized peptides were determined by HPLC-MS and are presented in Table 2.

Peptides 1-4 were designed to investigate the effect of ring size on intestinal permeability (group 1; see Table 1a for the classification of peptides into the different groups). Peptides of groups 2 and 3 were designed and synthesized to study the effect of successive methylation on intestinal permeability. Successive N-methylation of a parent peptide is known to have two effects: it reduces the hydrogen bonding potential of the peptide and also increases its hydrophobicity. In order to distinguish between these two effects a second series of peptides (group 2; peptides 5-8) was synthesized
in which successive Gly → Ala substitutions were made. Such substitutions are expected to exert the same effect as N-methylation with respect to the hydrophobicity of the peptide without changing its hydrogen bonding potential. Thus, by comparing the results obtained for groups 2 and 3, it should be possible to distinguish between effects of hydrophobicity and hydrogen bonding. Group 4 was synthesized to investigate the contribution of backbone cyclization on intestinal permeability compared to linear and pre-cyclic analogs. Group 5 investigated the effect of N-methylation of linear analogs on intestinal permeability.

**Ex-vivo permeability study:**

Permeability coefficients (Papp) obtained from group 1 (cyclic ring size effect), show a Papp with a mean of 5.34±1.0×10^{-6} cm/sec with no statistically significant difference among the peptides. Groups 2 and 3 (Ala and NMe-Gly substitutions) show Papp mean values of 5.06±0.8×10^{-6} cm/sec and 4.75±0.9×10^{-6} cm/sec, respectively, with no statistically significant difference within each group. Peptide 8 (backbone cyclic) in comparison to peptide 13 (pre-cyclic) and peptide 14 (linear) have Papp values of 5.63±0.8, 0.67±0.1 and 0.56±0.1×10^{-6} cm/sec, respectively, p<0.01 (see Figures 2 and 4). Group 5 (linear analogs differing by degree of N-methylation) have a Papp with a mean of 0.91±0.1×10^{-6} cm/sec, with no statistical significance among the peptides, Figure 2.

**In-vitro Caco-2 permeability:**

Permeability coefficients (Papp) obtained from group 1 (backbone cyclic ring size effect) show a Papp of 3.71±0.4×10^{-6} cm/sec with no statistically significant difference among the peptides. Groups 2 and 3 (Ala and NMe-Gly substitutions) show a mean Papp of 2.7±0.8×10^{-6} cm/sec and 2.3±0.8×10^{-6} cm/sec, respectively, with no significant difference within the groups. The peptides in group 4 (except Peptide 15)
show a mean Papp of $0.48 \pm 0.1 \times 10^{-6}$ cm/sec with no significant difference within the group, Figure 3. It thus, should be noted that there were significant differences between the permeability rate constant for the cyclic derivatives in comparison to non-cyclic peptides (Figures 2 and 4).

**Assessment of the intestinal permeability mechanism:**

There are several methods to evaluate the mechanism of transport via the intestinal wall. Based on our hypothesis that the current transport mechanism is predominantly the paracellular pathway, we chose to study their transport mechanism via several independent methods.

The first method used was to enhance the paracellular route by using palmitoyl carnitine, a specific modulator of the tight junctions in the Caco-2 monolayer. We observed enhanced transport rates of mannitol (240% in the Papp value), as well as the permeability of two selected representative peptides, peptide 9, and its linear counterpart, peptide 17. The Papp values of peptides 9 and 17 were raised from 2.2 to $7.58 \times 10^{-6}$ cm/sec (340%) and from 0.58 to $1.74 \times 10^{-6}$ cm/sec (340%), respectively, Figure 5.

The second method is a novel colorimetric mixed-vesicle assay that evaluates whether the peptides have any interaction with a bilayer membrane. The colorimetric platform comprises an aqueous solution of mixed bilayer vesicles containing phospholipids [such as dimyristoylphosphatidylcholine (DMPC), used herein] and polydiacetylene (PDA), as described before by Kolusheva *et al.* Specifically, the DMPC domains interspersed within the PDA matrix serve as the biomimetic membrane layer, while the polymer acts as a colorimetric reporter. When the test compound interacts with the bilayer membrane it causes structural alterations of the pendant side chains of the
polymer at the vesicle interface that is expressed by a change in color from blue to red as well as a modification of the fluorescence quenching. The results are depicted in Figure 6 in comparison to cyclosporine, which served as a marker for transcellular transport. Cyclosporine had a 25% blue to red transition, whereas peptides 1 (backbone cyclic), 15 (linear), 9 (backbone cyclic) and 17 (linear) had no effect on color transition, Figure 6a. Fluorescence quenching was assessed with DMPC/NBD-PE tests that showed a 62% decrease in fluorescence with cyclosporine compared to a 40% decrease of peptides 1 and 15 (cyclic versus linear). Peptides 9 and 17 (cyclic versus linear) showed a decrease of 35%, a result similar to the control (quencher alone), Figure 6b.

To further validate the results obtained from this novel method, peptide permeability was studied with an artificial membrane using the established method, PAMPA Lechitin. None of the peptides tested by PAMPA Lechitin permeated the phospholipid bilayer. Testosterone and antipyrine, serving as transcellular markers, were found to permeate the barrier while paracellular markers such as mannitol and amoxicillin did not permeate the artificial membrane (Table 3). The permeability through Caco-2 cells of representative peptides 9 and 17 was evaluated from basolateral to apical (Figure 7) to assure that no active transporters are involved in absorbing the tested peptides. The results clearly demonstrate that the transport rate of these peptides from apical to basolateral (A to B) is similar to the B to A transport rate.

Metabolic stability:

The metabolic stability of the backbone cyclic peptides and their linear analogs was studied in brush border membrane vesicles (BBMV), which are known to have broad enzymatic degradation activity, and compared to an enzyme-free buffer (MES 50 mM, pH 7.4). Two cyclic peptides, 1 and 8, and two linear peptides, 14 and 13 (pre cyclic),
were tested. The cyclic analogs showed significant resistance to metabolic degradation after 90 min in BBMVs (100% recovery) in comparison to the fast and significant decay of the linear analogs (30% recovery) under the same experimental conditions, Figure 8.

**Physiochemical Properties:**

Experimental (LogD pH 7.4) values and calculated PSA of the backbone cyclic peptides and their linear analogs are presented in Table 4. No correlation was found between the permeability coefficients and these physiochemical properties.

Furthermore, considering the importance of the hydrodynamic volume when macromolecules pass through a narrow pore, *i.e.*, in the paracellular route, we experimentally studied the effective size of the molecule and its impact on restricted pore size permeability. We choose high-resolution size exclusion chromatograph as a model.

The capacity factor, $K'$, was determined for the peptides by high-resolution size exclusion chromatography. As depicted in Table 5, the backbone cyclic peptides were found to have a smaller molecular hydrodynamic volume than their linear and precyclic analogs.

**NMR Studies:**

Nuclear magnetic resonance measurements were performed on three representative peptides from the different groups; 1 and 2 as representative cyclic peptides and 15 as a linear control. Both cyclic peptides showed two conformations with a ratio of approximately 1:0.2 at 25°C in both water and DMSO. The conformations showed pairs of amide interactions in the TOCSY spectra$^{22}$ indicating structural exchange on
the timescale of the NMR measurement. Further temperature dependence experiments on the cyclic peptides, ranging between 25°C and 40°C, also indicated a change in the ratio of the amide peaks from 1:0.2 to 1:0.4. The two conformations of the linear peptide were evident but unresolved, apart from one glycine, presumably Gly5 adjacent to the N-methyl Phe6 at the carboxy terminus, which can have both cis and trans conformations.

No structural information could be determined from the NOESY or ROESY spectra\textsuperscript{23}, which gave no interactions apart from those expected due to covalent bonds. This is most likely due to the size of the ring and the fact that the glycine residues can give no side chain-to-backbone or side chain-to-side chain interactions. This left us with ambiguously assigned spectra, although the residue identity of the peaks was made (Table 6).

**Discussion:**

There is a wide range of bioactive peptides that are hydrophilic in nature. In most cases their site of action is on the outer surface of cells (e.g., GPCR receptor ligands) that do not require membrane permeability. In general, high intestinal permeability leading to enhanced oral bioavailability is desired for developing peptide-based drugs. While lipophilic compounds tend to permeate the cell membrane by the transcellular mechanism and to yield high intestinal absorption characteristics (e.g., cyclosporine), hydrophilic compounds are transported through biological membranes via active transporters or by passive diffusion via paracellular pathways. To date, the mechanism of membrane permeability of the peptides was not investigated when structure-permeability relationships were studied, despite the meaningful implications of this information. In this work we focused on investigating the transport mechanism(s) of
hydrophilic hexapeptides and the possible effect of certain structural modifications. The structural and conformational modifications that we tested were previously suggested in the literature to improve membrane permeability and could, in theory, convert the absorption mechanism (at least in part) from the paracellular to the transcellular pathway. Our working hypothesis was that the hydrophilic hexapeptide analogs investigated in this work would permeate the intestine only by the paracellular pathway.

The main finding of this work is that the structural modifications that were originally suggested to improve intestinal permeability of peptides were found not to affect the intestinal permeability of the investigated hexapeptides, except for the impact of backbone cyclization that will be detailed below. Specifically, the degree of N-methylation in our study was consistent (i.e., for both backbone cyclic and linear peptides) and found not to affect permeability by hexapeptides. This finding is unique in light of previous reports suggesting N-methylation as a structural modification that improves permeability due to the reduced H-bond potential, thus improving the ability of the peptide to traverse the transcellular pathway. Moreover, increasing the peptide methyl content (through Gly → Ala modifications), and thereby increasing lipophilicity, did not improve permeability. This fact is consistent with our hypothesis that the tested hexapeptides were predominantly transported via the paracellular pathway which is not expected to be enhanced by increased lipophilicity. The Papp values obtained from the Caco-2 model (Figure 3) were lower than those obtained in the diffusion cell model (Figure 2), further corroborating our hypothesis. These findings are in accord with the known differences in the tight junction pore size between the two models. We used PAMPA_{Lec} to further verify the hypothesis that the permeability occurs predominantly via the tight junction space. This artificial
membrane screens for molecules that tend to permeate by the passive transcellular mechanism. The negligible permeability of the tested peptides through the PAMPA\textsubscript{Lechitin} also confirms the hypothesis of predominate paracellular rather than transcellular transport.

Palmitoyl carnitine, which increases permeability via the paracellular route, enhanced permeability of peptides 9 and 17, similar to the effect seen for mannitol (Figure 5).

A novel colorimetric assay was used to assess whether the peptides tend to interact with a bilayer membrane of the liposomes\textsuperscript{19}. The results (Figure 6) suggest that both the backbone cyclic peptides studied in this work and their linear analogs do not to interact with the liposome membrane, indicating that they are also unable to penetrate through the model bilayer and supporting a predominately paracellular rather than transcellular transport.

The similar permeability rates observed for the A to B and B to A (Figure 7) indicate there are no active transport processes (\textit{i.e.} influx/efflux) involved in the permeation process of the peptides. Thus, the only driving force for permeation is diffusion due to the concentration gradient.

Taken together, the results obtained in this study substantially verify the hypothesis that the molecular compositions of the studied hexapeptides direct the transport mechanism towards the paracellular pathway.

Although the paracellular area is relatively small compared to the transcellular area: \(0.01\%\)\textsuperscript{25} to \(0.1\%\)\textsuperscript{26}, the intestinal epithelium has a surface area of over \(2\times10^6\) \(\text{cm}^2\)\textsuperscript{27}, while the corresponding values of the paracellular surface ranges from 200 to 2000 \(\text{cm}^2\)\textsuperscript{28}. This surface area should not be underestimated, since even the absorption of minute quantities (pM-nM) of a peptide drug may be sufficient to exert its required pharmacological effect due to the high potency of bioactive peptides. Several peptide
drugs such as octreotide, vasopressin analogs, and desmopressin are believed to be absorbed by this route.\textsuperscript{29}

The improved intestinal permeability of the backbone cyclic peptides via the paracellular route is of interest in view of previous reports suggesting that cyclization enhances peptide permeability by shifting it towards transcellular transport.\textsuperscript{11} The improved permeability via the inter-enterocyte space obtained by cyclization (as demonstrated in Figures 2 – 4) can be explained by the conformational constraint imposed, which results in a much more constrained three-dimensional geometry. This conformational rigidity is seen in the distribution of molecular conformations computed by molecular dynamic simulations. The backbone cyclic peptides spent 99\% of the time in a single conformation whereas their linear analogs span a much broader conformational space significantly populating several conformations.

The NMR studies that were carried out in the attempt to define three-dimensional conformations were not sufficiently resolved for this purpose. The fact that backbone cyclization imposes a more strict conformation and therefore a smaller molecular volume, can be shown empirically by a molecular size study utilizing high-resolution peptide size exclusion chromatography and is also evident in the resolved $\alpha$ protons of the glycines and bridge methylenes relative to the linear analogs (Table 6). High-resolution peptide size exclusion chromatography enables discrimination among peptides based on size (\textit{i.e.}, larger peptides are associated with a shorter retention time indicated by a smaller capacity ratio). As shown in the results (Table 5), higher capacity ratios ($K^\prime$) were found for the cyclic peptides compared to linear and precyclic analogs. This indicates a reduced conformational space of the cyclic peptide as compared to that of the pre-cyclic and linear analogs. Reduced dimensions induced by increased conformational constraints contributes to enhanced paracellular transport,
as shown in the "sieving theory" proposed by Watson et al\textsuperscript{30}. This theory suggests that hydrodynamic volume and conformation are the main factors dominating paracellular transport. The transport of a molecule across the paracellular path contains two components combining restrictive and non-restrictive pores where the restrictive path serves as a sharp molecular size cut-off and the non-restrictive path for this size of molecules provides transport for larger molecules.

Enzymatic stability of peptides in the gut lumen and the brush border is a major factor dominating the oral bioavailability of peptides, as these regions are abundant with proteolytic enzymes that considerably reduce the ability of intact peptides to reach the systemic circulation following intra intestinal administration. For tetra- and higher peptides, over 90% of the proteolytic activity is in the brush border membrane, whereas for tri-peptides it is 10-60%, and for di-peptides only 10%\textsuperscript{31}. Our results comparing the metabolic stability of backbone cyclic, pre-cyclic and linear hexa-peptides in the brush border membrane vesicle model, (Figure 8) show that cyclization of peptides significantly increases their stability towards degradation by peptidase enzymes that are bound to the brush border membrane. This is probably due to the drastic decrease in the ability of the peptide to attain the linear, flexible conformation necessary for its interaction with the catalytic site of the proteolytic enzymes\textsuperscript{32}. These results are in agreement with previous reports in our laboratory demonstrating significant metabolic stability of backbone cyclic peptides in various media such as serum and kidney homogenate\textsuperscript{33, 34}. It should be noted that the peptides are exposed to the brush border enzymes regardless of the permeability pathway. Thus, the peptides investigated in this study could be available to brush border degradation regardless of the mechanism of transport.
In conclusion: This study presents a systematic investigation into the effect of various factors previously suggested to affect peptide intestinal permeability and stability. We conclude that backbone cyclization significantly improves paracellular intestinal permeability and stability compared to that of linear analogs. Structural modifications that have been suggested to affect peptide intestinal permeability (e.g., N-methylation) do not seem to have a significant influence when the transport mechanism is paracellular. The current investigation highlights that when permeating the intestinal wall via the paracellular pathway, conformational rigidity and molecular space (e.g., hydrodynamic volume) play the most important role in intestinal permeability.

Experimental section:

Chemicals: Unless otherwise specified, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water, methanol and acetonitrile were purchased from J.T. Baker (Holland).

Animals: Male Sabra rats weighing 250-300 gr were used for the ex-vivo permeability study. The project adhered to the principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). All animals deprived of food but not water 12 hrs prior to the experiments.

Peptide synthesis:

Backbone cyclic analogs tested in this study were synthesized by solid phase, multiple parallel synthesis, using Fmoc chemistry as previously described, which was adapted to a 96-well format. Syntheses were performed on an ACT 396 synthesizer (Advanced ChemTech, Louisville, KY, USA) equipped with a Lab Tech 4 (Advanced ChemTech, Louisville, KY, USA) for heating. The backbone cyclic building units were protected on their γ-carboxy or γ-amine by the Allyl/Alloc protecting group, which was
removed before on resin cyclization. The synthesis scale was 6 µmol Rink amide MBHA resin, which resulted in approximately 5 mg crude product/well. Amino acid coupling to MBHA-Resin or to peptidyl-Resin was carried out with Fmoc-protected amino acid (5 eq. excess to resin) preactivated for 10 min. with HBTU/HOBT (5 eq.) and diisopropylethyl amine (10 eq.) in N-methylpyrolidinone at room temperature. Coupling to the N-alkylated amino acid moieties of the peptidyl-Resin, was performed according to Falb et al. Briefly: Fmoc amino acid and bistrichloromethylcarbonate were dissolved in dioxane or 1,3-dichloropropane, cooled in ice water and collidine was added carefully. The mixture was added to the resin and heated to 50°C for 1 hr. After washing the Fmoc group was removed by 25% piperidine in N-methylpyrolidinone for 20 min (X2). At the end of the assembly, Allyl/Alloc deprotection on the bridging arms was performed with tetrakis (triphenylphosphine)-palladium (0). Cyclization was performed on the resin using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate. Cleavage from the resin was carried out by a reagent mixture (92.5% trifluoroacetic acid, 2.5% triisopropyl silane, and 2.5% ethane dithiol, 1 h). The crude peptides were partially purified by C18 Sep-Pak chromatography using 50% acetonitrile in water as the eluent. The molecular weight and purity of the peptides was determined by LC-MS using Waters Millenium LC-MS instrument equipped with Micromass ZQ detector, Waters 600 Controller gradient pump and Waters 717 auto-sampler. The analysis was conducted using Xterra MS C18 column 2.1×150 mm (Table 2). Large scale synthesis was carried out according to procedures described previously.

**Ex-vivo animal permeability study:**

Permeability experiments were performed in a modified Ussing chamber system (Physiological Instruments Inc. San Diego, CA, USA). Following a midline incision
to the rat, 25 cm of small intestine was removed and placed in ice-cold Ringer bicarbonate buffer (NaCl 6.54 gr, KCl 0.37 gr, CaCl₂ × 2H₂O 0.18 gr, MgCl₂ × 6H₂O 0.24 gr, NaHCO₃ 2.1 gr, Na₂HPO₄ 0.23 gr, NaH₂PO₄ 0.05 gr in 1000 mL). All buffer solutions were freshly prepared and equilibrated to pH 7.4, osmolality 290 mOsm. The jejunal portion of the small intestine (10-15 cm distal to the pylorus) was used. Peyer patches could be easily identified visually, and sections containing them were not used in these studies. The individual segments were obtained and underlying muscularis was removed from the serosal side of the tissue before mounting. The exposed tissue surface area was 0.5 cm² and fluid volume in each half-cell was 5 mL. The system was preheated to 37°C. Modified Ringer buffers were added to the serosal and the mucosal sides (mucosal modified Ringer buffer contained 10 mM mannitol and serosal modified Ringer buffer contained 8 mM D-glucose and 2 mM mannitol). The tissue oxygenation and the solution mixing were performed by bubbling with 95% O₂ – 5% CO₂. The system was equilibrated for 30 min. The permeability experiments continued for 180 min, samples were withdrawn at predetermined times. The sampled volume was replaced by blank (non-compound containing) buffer to maintain sink conditions. The integrity of the epithelial tissue was monitored by measuring the transepithelial electrical resistance (TEER) throughout the experiment. Viability was assessed by applying 1 µM forskolin at the end point (180 min.) and an increase or decrease of above 5% in Isc was observed. Any tissue with values < 30 Ωcm² was discarded before the start of the experiment. Generally, TEER values were 70-130 Ωcm² and remained steady throughout the experiment.

**In-vitro permeability study**

**Growth and maintenance of cells:** Caco-2 cells were obtained from ATCC and then grown in 75 cm² flasks with approximately 0.5×10⁶ cells/flask at 37°C in 5% CO₂
atmosphere and at a relative humidity of 95%. The culture growth medium consisted
of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-
inactivated fetal bovine serum (FBS), 1% nonessential amino acids (NEAA), and 2
mM L-glutamine. The medium was replaced twice weekly.

**Preparation of cells for transport studies:** For the transport studies, cells in a
passage range of 60-66 were seeded at a density of $25 \times 10^5$ cells/cm$^2$ on untreated
culture inserts of polycarbonate membrane with 0.4 µM pores and surface area of 1.1
cm$^2$. The culture inserts containing Caco-2 monolayer were placed in 24 transwells
plates 12mm, Costar$^\text{TM}$. The culture medium was changed every other day. Transport
studies were performed 21-23 days after seeding, when the cells were fully
differentiated and the TEER values were stable (300-500 Ω×cm$^2$).

**Experiment protocol:** Transport study (apical to basolateral, A to B) was initiated by
removing the medium from both sides of the monolayer and replacing it with apical
buffer (550 µL) and basolateral buffer (1200µL), both warmed to 37°C. The cells were
incubated for 30 min at 37°C with shaking (100 cycles/min). After the incubation
period the buffers were removed and replaced with 1200 µl basolateral buffer on the
basolateral side. Test solutions were warmed previously to 37°C and added (600 µl) to
the apical side of the monolayer. 50 µL samples were taken from the apical side
immediately at the beginning of the experiment, leaving a 550 µL apical volume
during the experiment. For the period of the experiment the cells were kept at 37°C
with shaking. At predetermined times (30, 60, 90, 120, 150 and 180 min), 200 µL
samples were taken from the basolateral side and replaced with the same volume of
fresh basolateral buffer to maintain a constant volume.

For the basolateral to apical study (B to A), compounds were placed in the basolateral
chamber, followed by sampling the apical side similar to with the A to B protocol.
Physiochemical Properties:

**Lipophilicity:**

Log $D_{oct/7.4}$ Shake-flask was determined by the shake-flask method. Octanol and MOPS buffer were pre-saturated prior to the experiment. The vials were shaken overnight in a Vortex Genie 2 (Freid, Israel). Both phases (octanol and MOPS buffer) were analyzed by HPLC. The final $D_{oct/7.4}$ value was calculated by dividing the areas corresponding to the compound in the two phases ($D = A_{octanol} / A_{buffer}$), while $A$ corresponds to the area of the peak.

**Molecular size:**

The average hydrodynamic volumes of the backbone cyclic peptides and their linear analogs were estimated by high-resolution size exclusion chromatography performed in 0.02 M phosphate buffer, pH 7.4, with 0.25 M NaCl using a superdex peptide 30/100 HR column (10×100 mm, Pharmacia Biotech, Uppsala, Sweden). The peptide solution (50 µL ~ 1 mg/mL, in running buffer) was injected on the column and solutes were detected with a UV detector at $\lambda=254$ nm.

**Data Analysis**

Permeability coefficient ($P_{app}$): The $P_{app}$ for each compound was calculated from the linear plot of drug accumulated vs. time, using the following equation:

$$P_{app} = \frac{1}{(C_0 \times A)} \times \frac{dQ}{dt}$$

Where $dQ/dt$ is steady state appearance rate of the drug on the receiver side, $C_0$ is the initial concentration of the drug on the donor side, and $A$ is the exposed tissue surface area, 0.5 cm² tissue area and 1 cm² in the Caco-2 experiments.

**Interaction with the liposome bilayer**

**Vesicle preparation:**
All lipid constituents were dissolved in chloroform/ethanol (1:1, v/v) and dried in vacuo to a constant weight. All lipids were suspended in deionized water, followed by probe sonication on a Misonix Incorporated sonicator (Farmingdale, NY, USA), applying an output power of ~100 W. Vesicles containing lipid components and PDA were sonicated at 70°C for 3-4 min. The vesicle suspensions were then cooled to room temperature, incubated overnight at 4°C, and polymerized by irradiation at 254 nm for 20-30 s, resulting in solutions with an intense blue color. Vesicle suspensions were allowed to anneal for 30 min and centrifuged for 15 min at 6000 g to remove titanium particles.

**UV-Vis measurements:**

Peptides at concentration of 30 µM were added to 60 µL of polydiacetylene (PDA) containing vesicle solutions consisting of ~0.2 M phospholipids in 25 mM Tris-base (pH 8.0). Following addition of the peptides, the solutions were diluted to 1 mL and spectra were acquired at 28°C, between 400 nm and 700 nm, on a Jasco V-550 spectrophotometer (Jasco Corp., Tokyo, Japan), using a 1-cm optical path cell.

To quantify the extent of blue-to-red color transitions within the vesicle solutions, the percentage colorimetric response (% CR) was defined and calculated as follows:

\[
\text{% CR} = \left( \frac{PB_0 - PB_I}{PB_0} \right) \times 100
\]

Where \(PB = A_{\text{blue}}/(A_{\text{blue}} + A_{\text{red}})\), and \(A\) is the absorbance at 640 nm (the 'blue' component), or 500 nm (the 'red' component). The colors 'blue' and 'red' refer to the visual appearance of the material, not the actual absorbance. \(PB_0\) is the blue/red absorption ratio of the control sample before the induction of a color change, and \(PB_I\) is the value obtained for the vesicle solution after the colorimetric transition occurred.

**Fluorescence quenching measurements:**
NBD-PE was added to lipids from a 1 mM chloroform stock solution, yielding a final concentration of 4 µM, and then dried together by vacuum sonication (see Vesicle preparation above). Samples were prepared by adding peptides, at a bound concentration of 30 µM, to 60 µL of vesicle solutions at ~0.2 mM total lipid concentration in 25 nm Tris-base (pH 8.0). The quenching reaction was initiated by adding sodium dithionite from a 0.6 M solution, prepared in 50 mM Tris base (pH 11.0) buffer, to a final concentration of 0.6 mM. The decrease in fluorescence was recorded for 210 s at 28°C using 468 nm excitation and 538 nm emissions on an Edinburgh FL920 spectrofluorimeter. The fluorescence decay was calculated as a percentage of initial fluorescence measured before the addition of sodium dithionite.

**PAMPA\textsubscript{Lechitin}**

Stock solutions (2.5-5 µM) of each peptide were made by first dissolving the peptide in pure DMSO and then diluting the DMSO solution with PBS to achieve a concentration of 5% DMSO. The stock solution was used as starting donor well solutions for the PAMPA\textsubscript{Lechitin} (MultiScreen-IP hydrophobic plate, cat. no. MAIPN4510/Millipore). A 1% solution of lecithin in dodecane was then added to each filter well at 5 µL per well. Immediately after adding the lipid membrane, donor solutions were added to the wells. Incubation times for all peptides were 16 h, after which the acceptor was sampled and analyzed using LC-MS. The permeability values (presented as Pe) for each peptide were obtained and compared to standards. Pe was calculated according to the following equation\textsuperscript{38}:

\[
P_e = C \times \left( -\ln \left( 1 - \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}} \right) \right)
\]
\[ C = \frac{V_D \times V_A}{(V_D + V_A) \times \text{Area} \times \text{Time}} \]

Where \( V_A \) is the acceptor side volume, \( V_D \) the donor side volume, Area, the effective area of the membrane exposed for diffusion (cm\(^2\)), and Time, the incubation time (sec).

**Preparing brush border membrane vesicles (BBMVs):**

BBMVs were prepared from combined duodenum, jejunum and upper ileum of rats by a Ca\(^{2+}\) precipitation method\(^{39}\). The intestines of five rats, 200-250 gr, were rinsed with ice cold 0.9% NaCl and freed of mucous; the mucosa was scraped off the luminal surface with glass slides and put immediately into buffer containing 50 nM KCl and 10 mM Tris-HCl (pH 7.5, 4°C) and the mixture homogenated by Polytron (Polytron PT 1200, Kinematica AG, Switzerland). CaCl was added to a final concentration of 10 mM. The homogenate was left shaking for 30 min at 4°C and subsequently centrifuged at 10,000 g for 10 min. The supernatant was then centrifuged at 48,000 g for 30 min and additional two purification steps were performed by suspending the pellet in 300 mM mannitol and 10 mM Hepes/Tris (pH 7.5) and centrifuged (24,000 g, 1 hr). Purification of brush border membranes was assayed using the brush border membrane enzyme markers GGT, LAP and alkaline phosphatase. During the course of these studies, enrichment in brush border membrane enzymes varied between 13- and 18-fold.

The enzymatic reaction was performed as follows: 2 \( \mu \)M stock solutions of the peptides were diluted with purified BBMV's solution to a final 0.5 \( \mu \)M. The solution was incubated at 37°C and sampled at time 0, 10, 20, 30, 60 and 90 min. The enzymatic reaction was stopped by adding 1:1 v/v of ice-cold acetonitrile and centrifuged (4000 g, 10 min) before analysis.
Conformational Analysis:

Sampling the conformational space of peptides 1-18 was achieved through mixed-mode molecular dynamics (MD) simulations using the AMBER force field and GBSA water as implemented in the MacroModel molecular modeling package. Simulations were equilibrated for 1 ns at 27°C using a 1 fs time step and then continued for an additional 10 ns using the same temperature and time step. Representative structures were sampled every 10 ps (a total of 1000 structures) and subjected to the surface area calculations described below.

Surface Area Calculations:

ZZ Vega 2.0.1 program was used to calculate the surface area of each conformer. The atomic van der Waals radii used were the following: sp² carbons 1.94 Å, sp³ carbon 1.90 Å, oxygen 1.74 Å, nitrogen 1.82 Å, electro-neutral hydrogen 1.50 Å, hydrogen bonded to oxygen 1.10 Å and hydrogen bonded to nitrogen 1.125 Å. The polar surface area (PSA) was defined as the area occupied by nitrogen and oxygen atoms, plus the area of the hydrogen atoms attached to these heteroatoms. The positively charged N-terminus was introduced as NH₂⁺. The NPSA was defined as the total surface area minus the PSA. The percentage of the surface area occupied by polar groups was also calculated.

NMR Measurements

The NMR experiments were performed on a Bruker Avance 600 MHz DMX spectrometer operating at the proton frequency of 600.13 MHz using a 5mm selective probe equipped with a self-shielded xyz-gradient coil. The transmitter frequency was
set on the HDO signal and the spectra referenced to this position at 4.77 for the water peak at 25°C. The residual water resonance was suppressed using a Watergate sequence \(^{445}\) for TOSCY \(^{454}\) experiments and by low power continuous wave irradiation during the relaxation delay and the mixing time of the NOESY \(^{23, 46, 47}\) experiments. Two-dimensional homonuclear spectra were acquired in the phase-sensitive mode with 4K complex data points in \(t_2\) and 512 \(t_1\) increments. The spectral width was 12 ppm and the relaxation delays were set to 1.5 s and 2 s in the TOCSY and NOESY experiments, respectively. A range of temperatures between 4 and 45°C was examined to find optimal conditions for the NMR measurements that balance minimal amide proton exchange with maximal spectrum spread. TOCSY spectra were recorded using the MLEV-17 pulse scheme for the spin lock at mixing periods of 45-75 ms with 48 scans per \(t_1\) increment \(^{45, 48}\). The NOESY experiments were investigated with mixing times ranging from 50 to 300 ms and ROESY experiments were taken with a mixing time of 150 ms.

Spectra were processed and analyzed with the XWINNMR and Viewer software packages (Bruker Analytische Messtechnik GmbH), and SPARKY (provided by Goddard T. D. and Kneller D. G., SPARKY 3, University of California, San Francisco) on a Silicon Graphics Indigo2 R10000 workstation. Zero filling in the \(t_1\) dimension and data apodization with a shifted squared sine bell window function in both dimensions was applied prior to Fourier transformation. The baseline was further corrected in the \(F_2\) dimension with a quadratic polynomial function.

Resonance assignment was based on the TOCSY and NOESY spectra measured under identical experimental conditions.

**Acknowledgments:**
This paper is part of Shmuel Hess PhD dissertation.

We would like to thank Dr. P. Artursson for its support in PSA calculations.
References:


11. Okumu, F. W.; Pauletti, G. M.; VanderVelde, D. G.; Siahaan, T. J.; Borchardt, R. T., Effect of restricted conformational flexibility on the permeation of model


### Table 1a

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical modification</th>
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<tr>
<td>1</td>
<td>Ring size</td>
<td>1-4</td>
</tr>
<tr>
<td>2</td>
<td>C-methylation (Gly-Ala)</td>
<td>1,5-8</td>
</tr>
<tr>
<td>3</td>
<td>N-methylation</td>
<td>1,9-12</td>
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<tr>
<td>4</td>
<td>Cyclic vs. Pre-cyclic vs. Linear</td>
<td>8,13,14</td>
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<tr>
<td>5</td>
<td>Backbone cyclic vs. Linear</td>
<td>1,6,9,10,15-18</td>
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### Table 1b

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Permeability$^a$ x10^6 [cm/sec]</th>
<th>Permeability$^b$ x10^6 [cm/sec]</th>
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<tbody>
<tr>
<td>Pep 1</td>
<td>c(Phe(C2)-Gly-Gly-Gly-Gly-Phe(N2)NH₂)</td>
<td>ND</td>
<td>5.43±1.32</td>
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<tr>
<td>Pep 2</td>
<td>c(Phe(C3)-Gly-Gly-Gly-Gly-Phe(N2)NH₂)</td>
<td>4.2±0.27</td>
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<td>Pep 3</td>
<td>c(Phe(C4)-Gly-Gly-Gly-Gly-Phe(N2)-NH₂)</td>
<td>5.02±1.8</td>
<td>1.5±0.5</td>
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<td>Pep 4</td>
<td>c(DPhe(C5)-Gly-Gly-Gly-Gly-Phe(N2)-NH₂)</td>
<td>6.8±1.09</td>
<td>ND</td>
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<td>Pep 5</td>
<td>c(Phe(C2)-Ala-Gly-Gly-Gly-Phe(N2)-NH₂)</td>
<td>3.89±0.51</td>
<td>1.9±0.24</td>
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<td>Pep 6</td>
<td>c(Phe(C2)-Ala-Ala-Gly-Gly-Phe(N2)-NH₂)</td>
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<td>3.22±0.9</td>
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<td>3.47±1.4</td>
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<td>c(Phe(C2)-Ala-Ala-Ala-Ala-Phe(N2)-NH₂)</td>
<td>5.63±0.75</td>
<td>1.47±0.44</td>
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<td>Pep 9</td>
<td>c(Phe(C2)-MeGly-Gly-Gly-Gly-Phe(N2)-NH₂)</td>
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<td>2.22±0.57</td>
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<td>c(Phe(C2)-MeGly-MeGly-Gly-Gly-Phe(N2)-NH₂)</td>
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<td>2.45±1.1</td>
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<td>c(Phe(C2)-MeGly-MeGly-MeGly-Gly-Phe(N2)-NH₂)</td>
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<td>Pep 13</td>
<td>Phe(C2)-Ala-Ala-Ala-Ala-Phe(N2)-NH₂ (pre-cyclic)</td>
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<td>Pep 14</td>
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<td>Pep 16</td>
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<td>0.99±0.33</td>
<td>0.87±0.18</td>
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<td>Pep 17</td>
<td>MePhe-MeGly-Gly-Gly-Gly-MePhe-NH₂</td>
<td>0.75±0.06</td>
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<td>MePhe-MeGly-MeGly-Gly-Gly-MePhe-NH₂</td>
<td>1.0±0.32</td>
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<td>mannitol</td>
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<td>5±1.1</td>
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<td>Pep 3</td>
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<td>Pep 4</td>
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<td>98</td>
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<th>Compound</th>
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<tr>
<td>antipyrine</td>
<td>1.18 ± 0.39</td>
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<tr>
<td>acyclovir</td>
<td>0.01 ± 0.001</td>
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<tr>
<td>amoxycilin</td>
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</tr>
<tr>
<td>Peptide 3</td>
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<tr>
<td>Peptide 7</td>
<td>ND</td>
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Table 3

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<th>PSA/(\text{NPSA})</th>
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<td>Pep 1-Phe(C2)-Gly-Gly-Gly-Gly-Phe(N2)</td>
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<td>ND</td>
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<td>Pep 8-Phe(C2)-Ala-Ala-Ala-Ala-Phe(N2)</td>
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<td>144</td>
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<td>Pep 14-NMePhe-Ala-Ala-Ala-Ala-NMePhe</td>
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<td>Pep 9-Phe(C2)-NMeGly-Gly-Gly-Gly-Phe(N2)</td>
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<td>172</td>
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<td>Pep 17-NMePhe-NMeGly-Gly-Gly-Gly-NMePhe</td>
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* Determined by the shake-flask method in octanol-buffer pH 7.4

ND- not determined
Table 4

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<td>Pep 13-Phe(C2)-Ala-Ala-Ala-Ala-Phe(N2) (pre-cyclic)</td>
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<td>Pep 14-NMePhe-Ala-Ala-Ala-Ala-NMePhe (linear)</td>
<td>0.45</td>
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Table 5

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<tr>
<th>Residue identity</th>
<th>HN protons</th>
<th>α protons</th>
<th>β protons</th>
<th>Others (ppm)</th>
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<td><strong>Peptide 15 NMePhe-Gly-Gly-Gly-Gly-NMePhe</strong></td>
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<td>Phe</td>
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<td>NH₂</td>
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<td>Amides 7.10, 7.52</td>
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<td>Amides 7.01, 7.50</td>
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</tbody>
</table>
Figure 1

\[ c(\text{Phe}(\text{Cm})-\text{Xaa}-\text{Xaa}-\text{Xaa}-\text{Xaa}-\text{Phe}(\text{Nn})-\text{NH}_2) \]
Figure 2

A

Papp x 10^{-6} cm/sec

Pep2, Ring(C3)  Pep3, Ring(C4)  Pep4, Ring(C5)

B

Papp x 10^{-6} cm/sec

Pep5, ala(1)  Pep6, ala(2)  Pep7, ala(3)  Pep8, ala(4)

C

Papp x 10^{-6} cm/sec

Pep9, N-Me(1)  Pep10, N-Me(2)  Pep11, N-Me(3)  Pep12, N-Me(4)
Figure 3

A

![Bar graph for Pep1, Ring(C2), Pep2, Ring(C3), and Pep3, Ring(C4) showing Papp x 10^{-6} cm/sec.]

B

![Bar graph for Pep5, ala(1), Pep6, ala(2), Pep7, ala(3), and Pep8, ala(4) showing Papp x 10^{-6} cm/sec.]

C

![Bar graph for Pep9, N-Me(1), Pep10, N-Me(2), Pep11, N-Me(3), and Pep12, N-Me(4) showing Papp x 10^{-6} cm/sec.]

Figure 4
**Figure 5**

1. **Papp [cm/sec]**
   - Mannitol
   - Mannitol + PC

2. **Papp [cm/sec]**
   - Pep #9
   - Pep #17
   - No treatment
   - PC, 0.3 mM

3. * Indicates statistically significant difference.
Figure 6

A

Cyclosporine  Backbone cyclic (1)  Linear (15)

B

% Intensity (535 nm)

Time sec

% CR

0 5 10 15 20 25
0 50 100 150 200 250 300

90.0
80.0
70.0
60.0
50.0
40.0
30.0

ACS Paragon Plus Environment
Figure 7:

![Bar Chart]

Figure 8

![Line Graph]
Legends:

**Table 1:** A) Classification of the peptides into groups. B) Structures of peptide derivatives investigated in this study and permeability coefficients (Papp). All experiments \( n \geq 3 \pm \text{SEM.} \)

\( ^a \) results obtained from excised tissue, \( ^b \) results obtained from Caco-2

**Table 2:** The calculated and found molecular mass of the synthesized peptides and their purity as determined by HPLC-MS.

**Table 3:** Permeability of members of the library peptides in the PAMPA model in comparison to transcellular and paracellular markers

**Table 4:** Comparison of cyclic (1, 8, 9) and linear (15, 14, 17) physiochemical properties.

**Table 5:** Capacity factors (K’) obtained by size exclusion chromatography, indicating the relative molecular size of backbone cyclic peptides compared to their linear and pre-cyclic analogs.

**Table 6:** Proton chemical shifts of the major conformations of cyclic (2) and linear (15) peptides in 10% D2O in water at 25°C.
**Figure 1**: General structure and nomenclature of the backbone cyclic peptides. Xaa denotes an amino acid (for both backbone cyclic and linear peptides), which can be Gly, Ala, or MeGly. The ring (labeled with two dotted circles) connects two backbone nitrogens, namely those of the N-terminal and C-terminal Phe residues. The abbreviated name of the cyclic peptide contains the prefix c for cyclic and the sequence in parentheses. Cm and Nn (for backbone cyclic and pre-cyclic peptides) represent the number of methylenes in the ring forming arms; m is the number of methylenes in the arm bearing the CO and n is the number of methylenes in the arm bearing the NH.

**Figure 2**: Side-by-side diffusion chamber: Effect of the cyclic ring size on intestinal permeability (A); Effect of the degree of Ala content on backbone cyclic permeability (B); Effect of degree of N-methylation on cyclic peptide analogs (C), all experiments n ≥ 3 ± SEM.

**Figure 3**: Caco-2 cell culture model: Effect of the cyclic ring size on intestinal permeability (A); Effect of the degree of Ala content on backbone cyclic permeability (B); Effect of degree of N-methylation on cyclic peptide analogs (C), all experiments n ≥ 3 ± SEM.

**Figure 4**: Permeability coefficients of backbone cyclic peptides compared to pre-cyclic and linear analogs in the Caco-2 model (A); Permeability coefficients of backbone cyclic peptides compared to additional linear analogs in the caco-2 model (B); Permeability coefficients of backbone cyclic peptides compared to pre-cyclic and linear analogs in the side-by-side diffusion chamber (C); Permeability coefficients of backbone cyclic peptides compared to additional linear analogs in the side-by-side diffusion chamber (D); all experiments n ≥ 3 ± SEM.

* Statistical difference between cyclic, pre-cyclic and linear analog, P<0.05
Figure 5: The effect of paracellular transport enhancer (palmitoyl carnitine) on the permeability of mannitol, peptide 9 and its linear analog, peptide 17, in the Caco-2 model. n ≥ 3 ± SEM. * Statistical difference compared to the non-treated cells, P < 0.05.

Figure 6: Colorimetric transitions induced by peptides in DMPC vesicles. The percentage of colorimetric response (%CR, see Materials and Methods) induced by tested peptides and cyclosporine as a positive control (A).

Time-resolved fluorescence quenching of NBD-PE. Decay of the fluorescence (5358 nM) of NBD-PE dye induced by sodium dithionite-induced quenching of fluorescence emission after adding peptides relative to the control (no peptides added) (□) control, (●) cyclic analog peptide 1, ( ■) cyclic analog peptide 9, (▲) linear analog peptide 15, (○) linear analog peptide 17, (x) cyclosporine A (B), all peptides were added at a concentration of 30 µM.

Figure 7: Permeability coefficients of backbone cyclic peptide (Pep9) and its linear counterpart (Pep17) in apical to basolateral (A-B) and basolateral to apical (B-A) in Caco-2 cells; n ≥ 3 ± SEM.

Figure 8: Metabolic stability in brush border membrane vesicles (BBMV), (■) linear analog, peptide 14, (x) pre-cyclic analog, peptide 13, (▲) backbone cyclic analog, peptide 8, (●) backbone cyclic analog, peptide 1, n ≥ 3 ± SD.
The Effect of Structural and Conformation Modifications, Including Backbone Cyclization, of Hydrophilic Hexapeptides on their Intestinal Permeability and Enzymatic Stability

Shmuel Hess, Oded Ovadia, Deborah E. Shalev, Hanoch Senderovich, Bashir Qadri, Tamar Yehezkel, Yoseph Salitra, Tania Sheynis, Raz Jelinek, Chaim Gilon, Amnon Hoffman

\[(\text{Phe}(\text{Cm})-\text{Xaa}-\text{Xaa}-\text{Xaa}-\text{Xaa}-\text{Phe}(\text{Nn})-\text{NH}_2)\]

\[(\text{N-(Amino-(alkyl) } n) = (\text{Nn})\]

\[(\text{N-(Carboxy-(alkyl) } m) = (\text{Cm})\]