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The Effect of Multiple N-Methylation on Intestinal Permeability of Cyclic Hexapeptides

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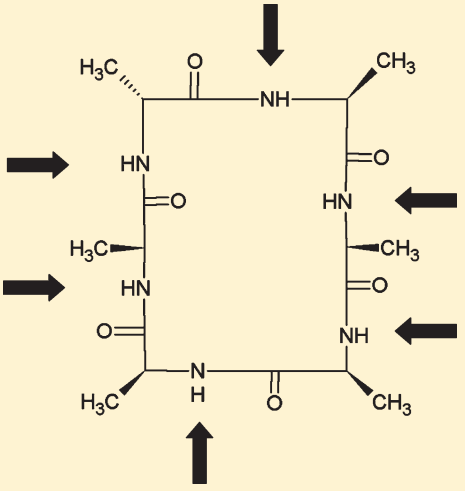
ABSTRACT: Recent progress in peptide synthesis simplified the synthesis of multiple N-methylation of peptides. To evaluate how multiple N-methylation affects the bioavailability of peptides, a poly alanine cyclic hexapeptide library (n = 54), varying in the number of *N*-methyl (*N*-Me) groups (1−5 groups) and their position, was synthesized. The peptides were evaluated for their intestinal permeability *in vitro* using the Caco-2 model. Further evaluation of the transport route of chosen analogues was performed using rat excised viable intestinal tissue, a novel colorimetric liposomal model and the parallel artificial membrane permeability assay (PAMPA). While most members were found to have poor permeability (permeability coefficient, $P_{app} < 1 \times 10^{-6}$ cm/s, lower than mannitol, the marker for paracellular permeability), 10 analogues were found to have high Caco-2 permeability, ($P_{app} > 1 \times 10^{-5}$ cm/s, similar to testosterone, a marker of transcellular permeability). No correlation was found between the number of N-methylated groups and the enhanced permeability. However, 9/10 permeable peptides in the Caco-2 model included an *N*-Me placed adjacently to the D-Ala position. While the exact transport route was not fully characterized, the data suggests a facilitated diffusion. It can be concluded that multiple N-methylation of peptides may improve intestinal permeability, and therefore can be utilized in the design of orally available peptide-based therapeutics.



KEYWORDS: cyclization, intestinal permeability, N-methylation, peptides

1. INTRODUCTION

Development of orally available peptides, either by improving their intestinal transport and/or by enhancing their stability to enzymatic degradation, has become a primary challenge for research groups in the past decade.[1] One of the strategies suggested to improve intestinal permeability is N-methylation. This chemical modification, in which the amide proton is replaced by an *N*-methyl group, is known to naturally occur in a variety of peptides[2−5] including cyclosporine.[2] N-Methylation has been used in order to produce peptides with improved intestinal and cellular permeability.[6] Moreover, incorporation of N-methylated amino acids into the sequence of peptides was found to affect important pharmacological properties, including binding affinity.[3] However, it has been shown that, when the conformation of the N-methylated analogue matches the bioactive conformation, the eminent biological activity can be retained.[1,7,8]

While multiple N-methylation is often found in natural peptides,[5,9] it has been negligibly employed in synthetic drugs or peptides owing to difficulties in synthesis,[10] subsequent problematic couplings and unpredictable conformational effects.[11] Recent work in our lab focused on a library of multiple N-methylated cyclic peptides.[12] In this context we simplified the N-methylation process of peptides on solid support and the preparation of N-methylated amino acids in solution leading to the fast synthesis of N-methylated peptide analogues.[10,13] In a recent study, a complete library of a multiple N-methylated hexapeptidic somatostatin analogues was investigated. It was found that one of the multiple N-methylated peptides, which contained three *N*-Me groups in distinct positions, still had high biologic activity in the nanomolar range. It was also considerably more permeable through Caco-2 monolayers, compared to the non-N-methylated analogue. In addition, this analogue was found to have 10% oral bioavailability in rats.[6,11] Furthermore, this analogue was found to have selective binding affinity toward the sst2 and sst5 somatostatin receptor subtypes. The pattern of three N-methylated amide groups in a hexapeptide was also used to increase integrin receptor subtype selectivity.[14]

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In order to cross the intestinal barrier and reach the circulation, a molecule must be transported through the GI wall. The molecules may be absorbed either through the aqueous pores created by the tight junctions (i.e., paracellularly, between the cells) or by the transcellular pathway (i.e., through the cells). The transcellular absorption may include carrier-mediated, active and/or passive transport.[16] Various physicochemical parameters have been recognized as discriminators which participate in dictating the transport route of peptides.[8,15] Most peptides are transported by the passive paracellular pathway due to their small size and hydrophilic nature. In order to be transported by the transcellular pathway, which consists of crossing both apical and basolateral lipophilic membranes, certain physicochemical properties, particularly lipophilicity, are required.

In order to design peptides with improved intestinal absorption, it is essential to identify what impact the introduction of certain chemical modifications will have on the transport route of the tested peptides, as performed in this study. Therefore, we combined several models for permeability assessment as a well-recognized synergistic tool.[16−18]

The Caco-2 cell culture model, originating from human colonic adenocarcinoma, was used for the characterization of the peptide's intestinal permeability.[19] In order to overcome the possibility of model-dependent results, selected peptides were further evaluated for their permeability through rat excised viable intestinal tissue using a side by side diffusion chamber (Ussing chamber).[20] Using the excised rat tissue allows a researcher to recognize regional dependent active efflux and/or transport processes involved in the absorption of a compound.[21] The excised tissue also includes several parameters missing in the Caco-2 model, such as the pH microclimate in the close perimeter of the epithelium and the complexed structure of the tissue layers. The peptides were also screened in the PAMPA model, which uses phospholipids as an artificial cell membrane[16] and is aimed to predict passive transcellular permeability. In addition, a novel colorimetric assay[22] was used to determine whether the analogues tend to interact with and/or penetrate a synthetic liposomal bilayer representing the cell membrane.

We postulated that multiple N-methylation of peptides could enhance their intestinal permeability and therefore may be a key player in designing orally available peptide-based drugs.[11] In order to test this concept, a series of N-methylated cyclic hexapeptides was synthesized. All peptides participating in this study are purely constructed of the simplest chiral amino acid alanine which was chosen with the intention of minimizing any effects from side chain functionalities and stronger steric effects on the cellular uptake. The hexaalanine sequence in common to all peptides included an alanine in the D-configuration as opposed to the rest, which were all in the natural L-configuration. Head-to-tail cyclization was performed on all the peptides. The library peptides differ only in the number and position of the *N*-methyl groups (see Figures 1 and 2). The single D-alanine was inserted in order to enforce distinct conformations in the cyclic hexapeptide structure (Figure 1) (see an analogous approach for cyclic pentapeptides in ref 12). The key findings of this study emphasize the potential of multiple N-methylation as a chemical modification which could be utilized in the design of peptides with improved drug-like properties. Ten peptides were found to have a high permeability rate similar to the permeability of testosterone, a passive transcellular permeability marker. Nine of them included an *N*-Me placed adjacently to the D-ala position. It was found that a change in the position of an *N*-methyl group caused significant changes and a high variability in the peptide's permeability. These results indicate the significance of the *N*-methyl position on dictating the permeability performance of the peptides.

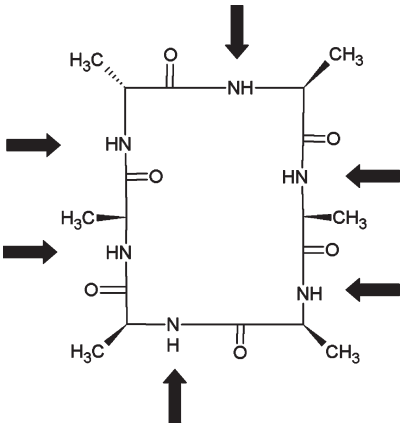

Figure 1. The chemical structure of the key peptide used as a template to synthesize the library of hexapeptides. The peptides differ in the number and position of the *N*-methyl groups. Arrows present optional positions for N-methylation.

2. EXPERIMENTAL SECTION

2.1. Chemistry. Out of the 62 possible peptides, we succeeded in synthesizing 54 compounds. All of these peptides were synthesized on solid support according to the procedure published by Carpino and Barlos for Fmoc-protected amino acids on TCP resin.[23,24] N-Methylation of Fmoc-protected alanine was performed in solution according to the conditions described by Freidinger et al.[25] While synthesizing the peptides on solid support, peptide couplings were performed using HOBT and TBTU or HATU and HOAT for couplings after N-methylated alanines. Treatment of the resin with 20% HFIP in DCM yielded the linear peptide. Cyclization was carried out using DPPA and $NaHCO_3$ in DMF to give the crude cyclic peptide which was purified via RP-HPLC.

2.2. *Ex Vivo* Animal Permeability Study. This study was performed as previously described.[26] Briefly, permeability experiments were performed in a modified Ussing chamber system (Physiological Instruments Inc., San Diego, CA, USA). Rat intestine segments were obtained and underlying muscularis was removed from the serosal side of the tissue before mounting. The permeability experiments continued for 150 min, and samples were withdrawn at predetermined times. The sampled volume was replaced by blank (non-compound-containing) buffer to maintain sink conditions. The integrity of epithelial tissue was monitored by measuring the transepithelial electrical resistance (TEER) throughout the experiment. Any tissue with values <30 Ω cm^2 was discarded before the start of the experiment. Generally, TEER values were in the range of 70−130 Ω cm^2 and remained steady throughout the experiment.

2.3. *In Vitro* Permeability Study. *Growth and Maintenance of Cells.* Caco-2 cells were obtained from ATCC (Manassas, VA, USA) and then grown in 75 cm^2 flasks with approximately 0.5 × 10^6 cells/flask at 37 °C in 5% CO_2 atmosphere 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

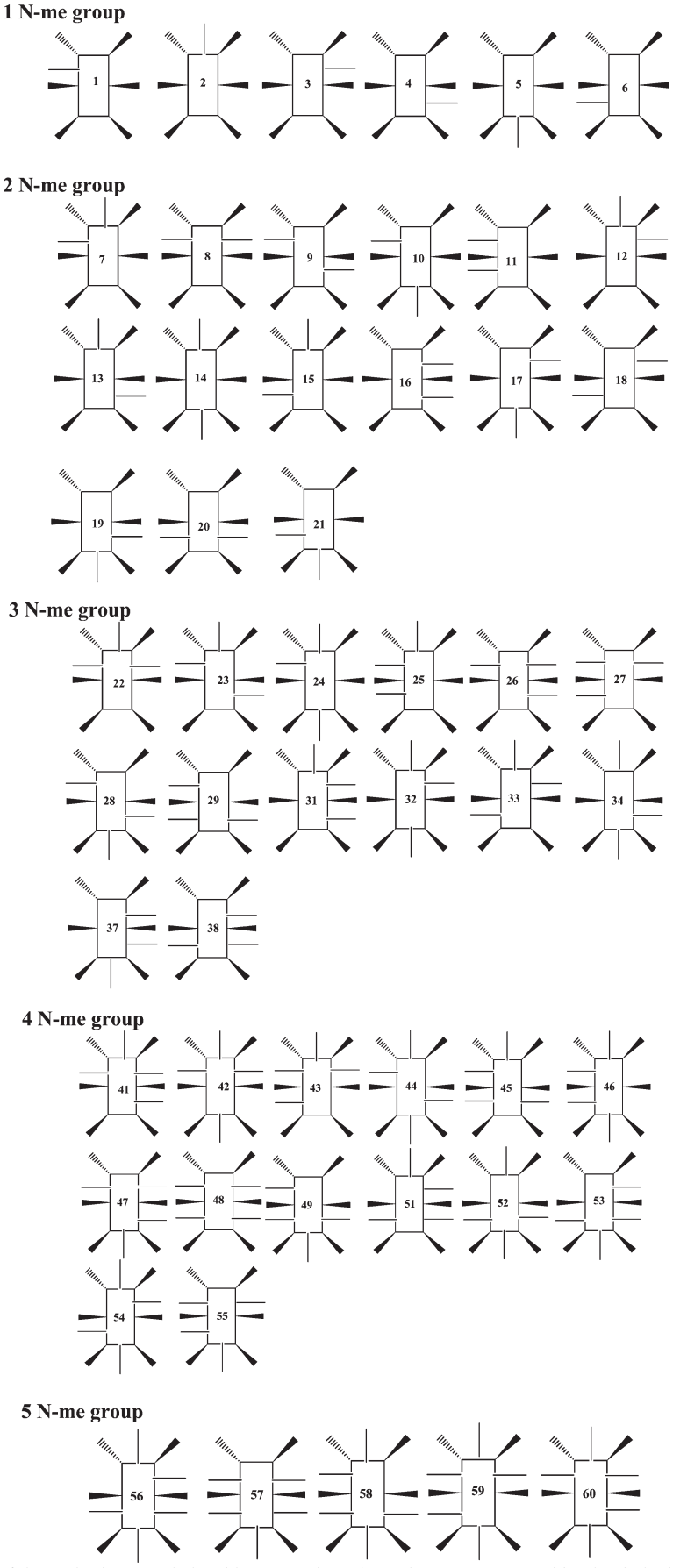

Figure 2. Chemical structures of the multiple N-methylated hexapeptides. The D-alanine is presented by the dashed line to the alpha methyl group.

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amino acids, 2 mM L-glutamine, 2 mM sodium pyruvate and 2 mM penicillin−streptomycin solution.

Preparation of Cells for Transport Studies. For the transport studies, cells in a passage range of 52−60 were seeded at a density of 25 × 10^5 cells/cm^2 on untreated culture inserts of polycarbonate membrane with 0.4 μm pores and a surface area of 1.1 cm^2. The culture inserts containing the Caco-2 monolayer were placed in 24 transwell plates 12 mm, Costar .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 medium removal from both sides of the monolayer and replacement with apical buffer (600 μL) and basolateral buffer (1500 μL), 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 1500 μL of basolateral buffer at the basolateral side. Test solutions were preheated 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, resulting in 550 μL apical volume during the experiment. For the period of the experiment, cells were kept at 37 °C with shaking. At predetermined times (30, 60, 90, 120, and 150 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. A mass-balance was performed for each tested compound in order to detect instability and/or non specific binding of the peptides.

For the basolateral to apical study (B to A), compounds were placed in the basolateral chamber, followed by sampling of the apical side, in the same manner as the A to B protocol.

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

$$P_{app} = \frac{dQ/dt}{C_0 \cdot A}$$

where dQ/dt is the steady state rate of appearance of the peptide or drug on the receiver side, C_0 is the initial concentration of the drug on the donor side, and A is the surface area.

2.4. PAMPA Assay. 2−3 μM solutions of each peptide were made in PBS, 1% DMSO as starting donor well solutions for the $PAMPA_{letichin}$ (MAIPN4510/Millipore). A 2% solution of lecithin dissolved in dodecane was applied to each well (5 μL/well) followed by 150 μL of PBS. Donor solutions (300 μL) were added to the wells for 18 h incubation at room temperature. Next, samples were collected and transferred to analysis.

2.5. Bioanalysis. Bioanalysis was performed using a HPLC−MS Waters Millennium instrument equipped with a Micromass ZQ detector, Waters 600 Controller gradient pump and Waters 717 auto sampler (Milford, MA, USA). Nitrogen flow was 500 L/h; source temperature was 400 °C; the cone voltage was 20 V; the column used was an Atlantis MS C_{18} 2.1 × 150 mm column (Waters). The mobile phase at 0.25 mL/min was 20% acetonitrile supplemented with 0.1% formic acid.

3. RESULTS

A total of 54 peptides were synthesized. The library included 6 single N-methylated peptides, 15 were di-N-methylated,

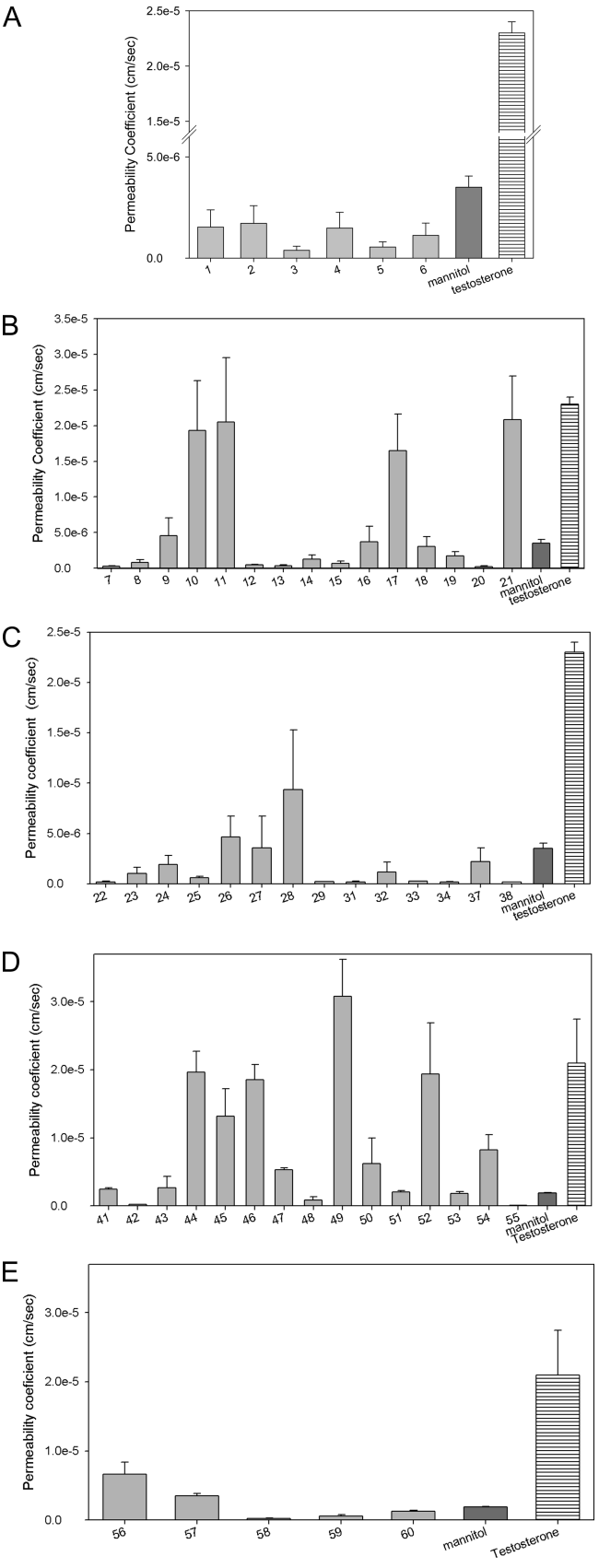

Figure 3. Caco-2 permeability of cyclic hexapeptides, differing in the degree and positions of the N-methylations, depicted as permeability coefficient, P_{app}: (A) single *N*-Me, (B) two *N*-Me groups, (C) three *N*-Me groups, (D) four *N*-Me groups, (E) five *N*-Me groups.

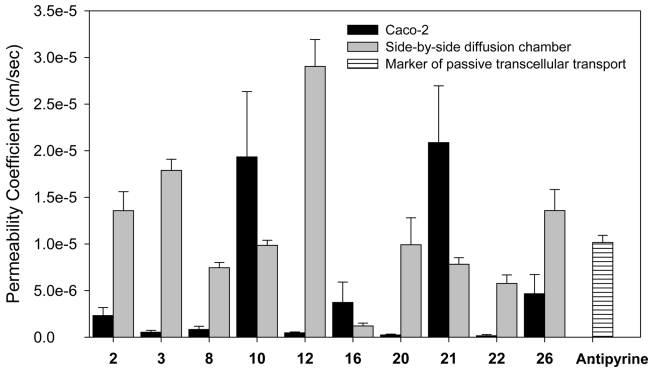

Figure 4. Intestinal permeability of selected hexapeptides in the side by side diffusion chamber model compared to their Caco-2 permeability and compared to antipyrine, the marker for passive transcellular diffusion.

14 tri-N-methylated, 14 tetra-N-methylated and 5 penta-N-methylated peptides.

All peptides were screened for intestinal permeability through a Caco-2 cell monolayer.

The permeability of the library hexapeptides is depicted in Figure 3. As can be seen for the single N-methylated hexapeptides (Figure 3A), all peptides were poorly absorbed, with permeability coefficient values lower than mannitol, the marker for paracellular permeability. Multiple N-methylation (2−5 *N*-Me groups, as depicted in Figure 3B−E) resulted in several peptides which had significant permeability, with $P_{app} > 2 \times 10^{-5}$ cm/s, in the range of the permeability of testosterone, a marker for passive transcellular transport. As seen in Figure 3B, 4 of the 15 peptides (peptides **10**, **11**, **17** and **21**) which are di-N-methylated had this exceptional permeability. On the other hand, 4 other peptides were poorly transported ($P_{app} < 1 \times 10^{-7}$ cm/s). Only one of the tri-N-methylated hexapeptides had high permeability (peptide **28**). The permeability screening of the tetra-N-methylated subgroup resulted in 5 peptides with improved permeability (peptides **44**−**46**, **49** and **52**). None of the penta-N-methylated subgroup members had enhanced intestinal permeability in the Caco-2 model.

One of the characteristics of carrier-mediated transport is the asymmetry in apical to basolateral (AP-BL) and BL-AP transport. Therefore, these differences were examined using the Caco-2 cell monolayer model (Figure 5B). AP-to-BL and BL-to-AP transport of peptides **10** and **11** were found to be identical.

Since N-methylation had clearly affected the absorption rate of the peptides, we used several additional models in order to clarify the transport mechanism of selected peptides which presented with exceptional permeability. None of the tested peptides was found to permeate the $PAMPA_{lecithin}$ artificial membrane, which abrogates the possibility that the N-methylation facilitated the peptide's transcellular permeability as a result of enhanced lipophilicity.

Chosen peptides were further tested in the Ussing model (Figure 4). Using this *ex vivo* model, additional information could be deduced regarding the transport aspects of the peptides. As can be seen in Figure 4, most of the tested peptides had moderate permeability. One peptide (**16**) was found to be poorly transported through the excised tissue. On the other hand, another peptide (**12**) had very high permeability (P_{app} 3 × 10^{-5} cm/s). In an attempt to further investigate the transport mechanism of

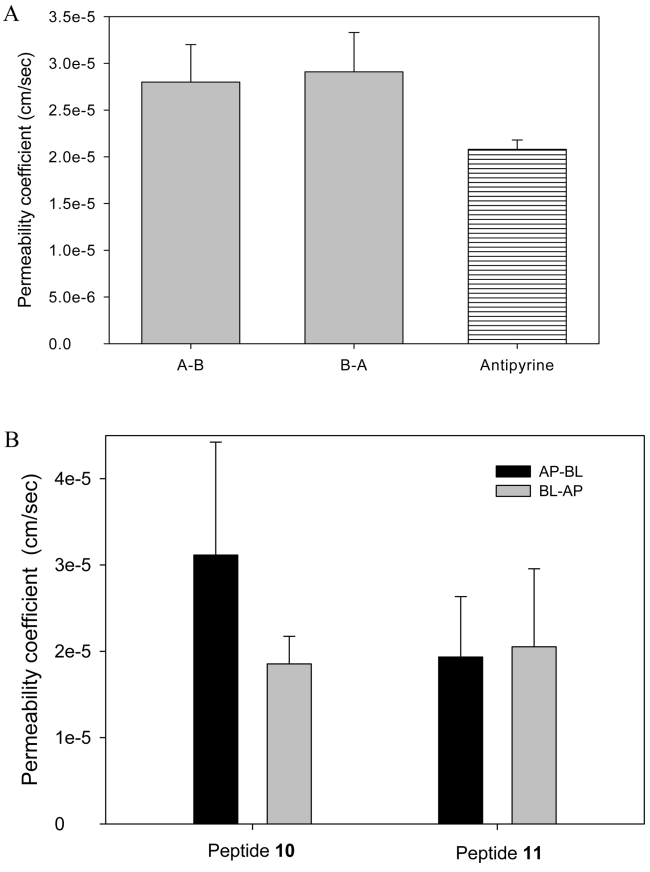

Figure 5. Apical to basolateral (AP-BL) compared to BL-AP permeability of permeable peptides, expressed as P_{app}: (A) permeability of peptide **12** in the side by side diffusion chamber model; (B) permeability of peptides **10** and **11** in the Caco-2 model.

peptide **12**, additional studies were conducted (Figures 5 and 6). As can be seen in Figure 5A, the transport in the AP−BL direction was similar to the BL−AP transport rate (P_{app} 2.8 × 10^{-5} and 2.91 × 10^{-5} cm/s, respectively). To further eliminate active transport or facilitated diffusion, including endocytosis, specific inhibitors were used (Figure 6A). Protamine is a commonly used inhibitor of endocytosis[27] while dinitrophenol (DNP) is an ATP inhibitor, and thus prevents active transport.[28] Both inhibitors had no effect on the peptide transport. An additional aspect which was investigated was the comparison of the absorption of peptide **12** in various sections of the intestine using the *ex vivo* Ussing diffusion chamber. A diversity in the expression of transporters throughout the intestine has been shown in previous studies,[29,30] therefore such a comparison provides information regarding site-specific absorption of the peptide. As depicted in Figure 6B, the transport rate of peptide **12** was in the range of 2−3 × 10^{-5} cm/s in all sections of the intestine, similar to the value of antipyrine, the marker for passive transcellular transport.

4. DISCUSSION

The preferred pharmaceutical properties for peptide drug candidates intended for oral delivery include permeability across biological membranes such as intestine and/or blood brain barrier (BBB) and resistance to degradation across the gastrointestinal (GI) tract and in the blood. Providing these "drug-like

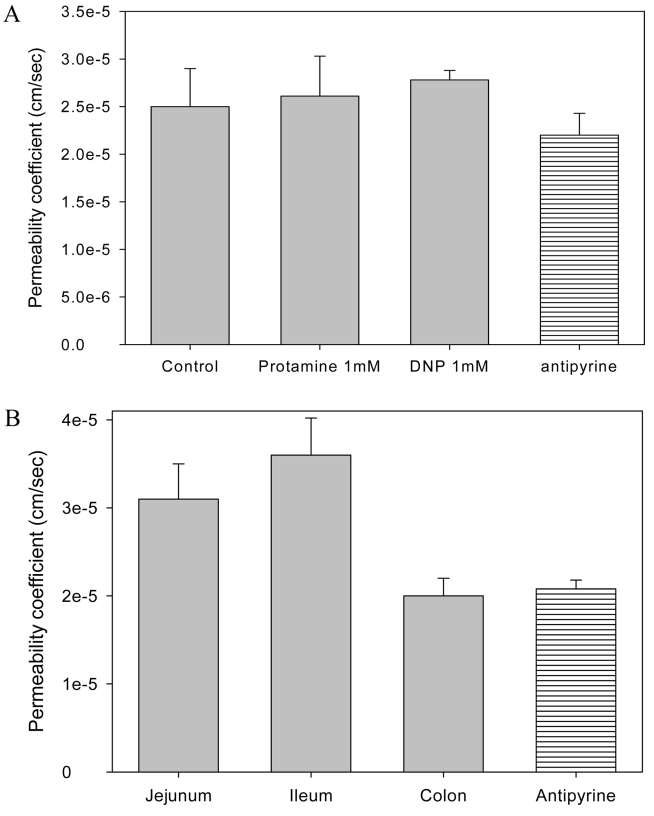

Figure 6. Evaluation of the permeability characteristics of peptide **12** in the side by side diffusion chamber model: (A) permeability following incubation with protamine, an inhibitor of endocytosis, and with dinitrophenol, an inhibitor of active transport; (B) permeability in different sections of the rat intestine.

properties" to peptides in order to generate orally available peptides is a major academic and industrial challenge. Numerous studies have been performed with the purpose of identifying those factors which may facilitate this challenge.[15,31,32]

N-Methylation generates several structural changes which are considered to affect intestinal and cellular permeability. It reduces H-bond potential and increases lipophilicity. These changes are considered to improve intestinal absorption by enabling transcellular permeability across the epithelial layer. On the other hand, the addition of *N*-Me groups increases the size of the molecule, believed to delay passive paracellular permeability.

A study which investigated the effect of multiple N-methylation on the permeability of peptides across the epithelial monolayer in the Caco-2 model demonstrated enhanced permeability which correlated with the number of *N*-methyl groups added to the peptides.[33] The observed increase in permeability was explained mainly by the decrease in hydrogen bonding potential, resulting in a decrease in the energy required for transport of the peptide from the aqueous surrounding into the cell membrane. It was also suggested that the intestinal permeability of N-methylated tripeptides could be influenced by additional factors including recognition by efflux transporters (e.g., P-glycoprotein[34]) or by absorptive transporters such as oligopeptide transporter (OPT).[11]

The findings of this study are based on the systematic screening of a unique and comprehensive peptide library. Therefore, these results may provide a general insight into the impact of multiple N-methylation on generating permeable peptides. It is evident that performing the same study on a reduced library could give rise to inaccurate conclusions regarding the effect of multiple N-methylation on a peptide's "drug-like properties".

4.1. Intestinal Permeability. Following analysis of the results achieved in this study, several major insights can be observed with regard to the permeability data acquired: no correlation could be observed between the number of *N*-Me groups and the intestinal permeability of the peptides. While 5/15 (33%) of the tetra-N-methylated and 4/15 (27%) of di-N-methylated peptides were found to have enhanced permeability, none of the single or penta *N*-Me sublibraries (Figure 3) were found to have improved permeability compared to mannitol.

In addition, no common denominator was found among the permeable analogues in terms of the position of the *N*-Me group. However, nine out of the ten permeable peptides included an *N*-Me placed adjacently to the D-ala position. Although one exceptional peptide (**17**) did not follow the common pattern, this finding may suggest that the position of the *N*-methyl group, rather than the number of *N*-methyl groups, is a governing discriminator in the permeability characteristics of the peptides. Reinforcing evidence to this insight is the fact that a change in one methyl position could have great impact on the permeability properties. For example, a shift in a single *N*-Me position, as observed for peptides **52** and **53**, completely diminished the intestinal permeability from a P_{app} value in the range of testosterone for peptide **52** to a value similar to that of mannitol for peptide **53**.

4.2. Characterization of the Transport Route. As briefly described in the Introduction, compounds may permeate the intestinal cellular barrier by several different mechanisms. The total permeation ability of a compound is governed by biophysical and biological parameters. The biologically dependent elements include involvement of active transporters and efflux systems while physically dependent elements include interaction with the liposomal bilayer for passive transcellular permeability.

The permeability coefficient values of the analogues varied from poor (i.e., lower than the passive paracellular marker, mannitol) to high (equivalent to or higher than the passive transcellular marker, testosterone).

The fact that several members of the library have been shown to have a high permeability rate may suggest that the chemical modifications which were imposed on these exceptional analogues resulted in a shipment toward a different transport pathway.

Although the Caco-2 model is commonly used as a rapid screening tool for the evaluation of oral bioavailability of peptide drugs, additional models are used for this purpose. The side-by-side diffusion chamber is an example for such a model. This *ex vivo* model allows the investigation of more aspects of intestinal absorption, including transport through the unstirred water layer, which is missing in the Caco-2 model.[35] A recent work compared these models as tools for evaluation of intestinal absorption of peptide drugs[21] and reported certain differences in the observed permeability of tested compounds using these models. The Caco-2 monolayer exhibits tighter junctions compared to the small intestine of rat and human, due to the cell's colonic origin.[36] Moreover, the transport of carrier mediated compounds can be better predicted in the excised *ex vivo* model.[37] Therefore, possible discrepancies between the models may help in understanding the transport route and mechanism.

The permeability results obtained using these models (Figure 4) were poorly correlative. Peptides which were found

to be poorly absorbed in the Caco-2 model, such as peptide **12**, were found to be highly permeable in the Ussing model. The fact that most peptides were absorbed to a higher extent in the Ussing chamber model may suggest active transport by transporters which are not expressed in the Caco-2 model and/or absorption by passive paracellular transport, which is, as previously mentioned, less restricted in rat tissue.

While poorly absorbed hydrophilic compounds are transported via the paracellular pathway, hydrophobic compounds can be absorbed rapidly by crossing the membrane by the transcellular route.[38] N-Methylation reduces the overall hydrogen bonding potential of a given peptide, leading to a reduction in the energy required to dissolve amide−water bonds (hydrogen bonds) and thus may increase membrane diffusion and transcellular permeability.[33]

Rezai et al.[39] reported the use of a novel computational model to predict membrane permeability of cyclic peptides. The computational model involved extensive conformational sampling of peptides as part of the current efforts to investigate the role of conformational flexibility in membrane permeability. The experimental data produced in the study showed a weak correlation between internal hydrogen bonding potential and passive permeability of the tested peptides. Furthermore, a study performed by Adessi et al.[40] examined a library of multiple N-methylated derivatives of a pentapeptide (1−3 *N*-Me substitutions in various positions) which was investigated for activity, stability and pharmacokinetic profiles following intravenous administration. While no direct link was observed between the number of *N*-Me groups and the compound's activity and stability, the *N*-Me position played a major role in dictating the peptide's physicochemical characteristics. These results, based on molecules with similarity to the peptides tested in our work, further support our observation regarding the limited effect internal hydrogen bonding potential can have on the passive membrane permeability of peptides.

We used two additional models in order to further shed light on the transport mechanism of the peptides. A novel colorimetric liposomal model[22] was used in order to evaluate possible interaction of the peptides with the cell membrane. This interaction is an essential but not obligatory step in order to cross the cellular phospholipids bilayer. None of the tested peptides, including the most permeable in Caco-2 and Ussing chamber, showed any interaction with this model (data not shown). Furthermore, all peptides with enhanced permeability in any of the permeability models were screened in the $PAMPA_{lecithin}$ model,[16] which was used to evaluate possible passive transcellular permeability. The screening resulted in no transport of any of the tested peptides, possibly excluding the passive transcellular pathway as the main absorption pathway. Table 1 depicts several markers used to verify the execution of the $PAMPA_{lecithin}$ experiment.

The versatile methods used in this study did not directly reveal the transport route of the permeable peptides. However, by elimination of passive and active transcellular permeability, it is possible to deduce that the transport route of the peptides is likely to be facilitated diffusion.

The term cell penetrating peptides (CPPs) refers to several peptide families which do not have an obvious common sequence or structural motif, but share the capability to efficiently move across biological membranes.[41] Although the mechanism of several CPPs was reported to be mediated by endocytosis/pinocytosis, the exact mechanisms underlying the translocation of most CPPs across membranes are still under investigation.[42] It is possible that the permeable peptides described in this study are CPPs. Additional studies are needed in order to confirm this theory.

Table 1. Permeability Coefficients of Tested Markers ($\times 10^{-6}$ cm/s) in the $PAMPA_{lecithin}$ Assay, $n \geq 4$[a]

compound	P_{app}-pampa	SEM
griseofulvin	51.4	9.7
testosterone	8.77	4.1
antipyrine	1.08	0.4
acyclovir	0.01	0.001
atenolol	0.002	
metformin	ND	
amoxycilin	ND	

[a] The P_{app} values found in the assay were in the range expected according to the transport route known for each compound (i.e. high for compounds with passive transcellular transport, and nondetectable for compounds with passive paracellular transport).

4.3. Conformational Impact of Multiple N-Methylation. N-Methylation significantly affects the conformation of the peptide backbone. For example, It has been shown that N-methylation of a peptide can cause significant structural changes, including *cis*/*trans* isomerism of the N-methylated peptide bond.[12] Therefore, N-methylated peptides are of great interest in relation to conformational studies.[43]

Cyclosporin A is a multiple N-methylated cyclic peptide, i.e., in which several of it's amino acids are N-methylated.[44] It also includes a D-alanine at position 8. It is possible that the unique chemical structure of this peptide, which involves turn-inducing building blocks (such as D-amino acids and N-methylated amino acids), was beneficial in contributing to the global restriction of the peptide conformation by generating additional local constraints, thus allowing its oral bioavailability. Since the restricted conformation can randomly generate a three-dimensional arrangement which is disadvantageous, in terms of enhanced pharmacokinetic and/or pharmacodynamic parameters, it is not evident that improved properties will be gained by restricting the conformational freedom of peptides. For example, cilengitide[6] is an integrin-targeting RGD (arginine-glycine-aspartic acid) based peptide with promising activity in the treatment of glioblastoma.[45] Although it is cyclic, contains a D-amino acid, one N-methylated amino acid, and has a relatively long half-life (2.5−4.5 h),[46] it has poor oral bioavailability. This may suggest that, in this specific case, the conformational restriction was beneficial in terms of enzymatic stability, yet its conformation was not optimal in providing enhanced permeability.

Conformational restriction has previously been shown to be an important requirement for oral bioavailability.[15,47] It is very likely that peptides which were found to have enhanced permeability were able to cross the cellular barrier as a result of their restricted conformation. However, this is not true for all peptides. When examining the conformational structures of the peptides, some of the highly permeable cyclic N-methylated alanine peptides show a relatively high variety of conformations, which exchange slowly on the NMR time scale. Therefore, it is not necessary for a peptide to be conformationally constricted in order for it to have good permeability properties. It should be mentioned that the affinity and selectivity of a certain peptide can also be affected by decreased flexibility within a peptide

backbone, as caused by N-methylation.[3,48,49] Therefore, the screening process for analogues with improved pharmacokinetic profiles should begin with screening the library peptides for the bioactive members, thus reducing the library to the derivatives with the highest potential for activity.

Recognizing the solution conformation which might be responsible for enhanced permeability of certain members of this model library can be beneficial in the rational design and optimization of orally available peptide based therapeutics. Therefore, these compounds will be further analyzed for their NMR conformations, in an attempt to form a link between conformation and intestinal permeability.

5. SUMMARY

The absorption of most peptides does not comply with the Lipinsky[50] and Veber[51] rules, which offer an estimation of the oral bioavailability of drug candidates. Therefore, new tools should be constructed in order to evaluate the absorption properties of peptides. The exclusive peptide library designed, synthesized and evaluated in this study enabled the systematic investigation of the effect of multiple N-methylation on the intestinal permeability of cyclic alanine hexapeptides.

By systematically investigating the structure−permeability relationship, the results of this study emphasize certain key issues in the design considerations of peptidomimetics with enhanced "drug-like properties". However, we were not able to identify common patterns for analogues with improved properties, probably due to the participation of multifunctional factors in dictating the properties of the peptides.

This library can be a basis for further studies searching to provide tools for optimization of peptide stability and recognition of cleavable sites. Single amino acid substitutions are an example for such a library, which is currently under investigation. This study can provide information regarding the impact of a single amino acid on both metabolic stability and permeability of multiple N-methylated peptides.

The enhanced intestinal permeability across the intestine, as shown for several members of the peptide library, suggests that by relatively minor chemical changes, a significant improvement could be achieved in the oral bioavailability of peptides. Additional *in vivo* studies, including investigation of the pharmacokinetic profile and oral bioavailability of the peptides, should be performed following high-scale synthesis of chosen peptides.

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REFERENCES

(1) Hamman, J. H.; Enslin, G. M.; Kotze, A. F. Oral delivery of peptide drugs: barriers and developments. *Biodrugs* **2005**, *19* (3), 165–177.

(2) Traber, R.; Dreyfuss, M. M. Occurrence of cyclosporins and cyclosporin-like peptolides in fungi. *J. Ind. Microbiol. Biot.* **1996**, *17* (5−6), 397–401.

(3) Dechantsreiter, M. A.; Planker, E.; Mathä, B.; Lohof, E.; Hölzemann, G.; Jonczyk, A.; Goodman, S. L.; Kessler, H. N-Methylated cyclic RGD peptides as highly active and selective alpha(V)beta(3) integrin antagonists. *J. Med. Chem.* **1999**, *42* (16), 3033–3040.

(4) Rajeswaran, W. G.; Hocart, S. J.; Murphy, W. A.; Taylor, J. E.; Coy, D. H. N-Methyl scan of somatostatin octapeptide agonists produces interesting effects on receptor subtype specificity. *J. Med. Chem.* **2001**, *44* (9), 1416–1421.

(5) Angell, Y. M.; Thomas, T. L.; Flentke, G. R.; Rich, D. H. Solid-Phase Synthesis of Cyclosporin Peptides. *J. Am. Chem. Soc.* **1995**, *117* (27), 7279–7280.

(6) Biron, E.; Chatterjee, J.; Ovadia, O.; Langenegger, D.; Brueggen, J.; Hoyer, D.; Schmid, H. A.; Jelinek, R.; Gilon, C.; Hoffman, A.; Kessler, H. Improving oral bioavailability of peptides by multiple N-methylation: somatostatin analogues. *Angew. Chem., Int. Ed.* **2008**, *47* (14), 2595–2599.

(7) Gao, J.; Sudoh, M.; Aube, J.; Borchardt, R. T. Transport characteristics of peptides and peptidomimetics: I. N-methylated peptides as substrates for the oligopeptide transporter and P-glycoprotein in the intestinal mucosa. *J. Pept. Res.* **2001**, *57* (4), 316–329.

(8) Pauletti, G. M.; Okumu, F. W.; Borchardt, R. T. Effect of size and charge on the passive diffusion of peptides across Caco-2 cell monolayers via the paracellular pathway. *Pharm. Res.* **1997**, *14* (2), 164–168.

(9) Klages, J.; Neubauer, C.; Coles, M.; Kessler, H.; Luy, B. Structure refinement of cyclosporin A in chloroform by using RDCs measured in a stretched PDMS-gel. *ChemBioChem* **2005**, *6* (9), 1672–1678.

(10) Biron, E.; Chatterjee, J.; Kessler, H. Optimized selective N-methylation of peptides on solid support. *J. Pept. Sci.* **2006**, *12* (3), 213–219.

(11) Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H. N-methylation of peptides: a new perspective in medicinal chemistry. *Acc. Chem. Res.* **2008**, *41* (10), 1331–1342.

(12) Chatterjee, J.; Mierke, D.; Kessler, H. N-methylated cyclic pentaalanine peptides as template structures. *J. Am. Chem. Soc.* **2006**, *128* (47), 15164–15172.

(13) Biron, E.; Kessler, H. Convenient synthesis of N-methylamino acids compatible with Fmoc solid-phase peptide synthesis. *J. Org. Chem.* **2005**, *70* (13), 5183–5189.

(14) Chatterjee, J.; Ovadia, O.; Zahn, G.; Marinelli, L.; Hoffman, A.; Gilon, C.; Kessler, H. Multiple N-methylation by a designed approach enhances receptor selectivity. *J. Med. Chem.* **2007**, *50* (24), 5878–5881.

(15) Hess, S.; Ovadia, O.; Shalev, D. E.; Senderovich, H.; Qadri, B.; Yehezkel, T.; Salitra, Y.; Sheynis, T.; Jelinek, R.; Gilon, C.; Hoffman, A. Effect of structural and conformation modifications, including backbone cyclization, of hydrophilic hexapeptides on their intestinal permeability and enzymatic stability. *J. Med. Chem.* **2007**, *50* (24), 6201–6211.

(16) Kerns, E. H.; Di, L.; Petusky, S.; Farris, M.; Ley, R.; Jupp, P. Combined application of parallel artificial membrane permeability assay and Caco-2 permeability assays in drug discovery. *J. Pharm. Sci.* **2004**, *93* (6), 1440–1453.

(17) Berggren, S.; Hoogstraate, J.; Fagerholm, U.; Lennernas, H. Characterization of jejunal absorption and apical efflux of ropivacaine, lidocaine and bupivacaine in the rat using in situ and in vitro absorption models. *Eur. J. Pharm. Sci.* **2004**, *21* (4), 553–560.

(18) Foger, F.; Kopf, A.; Loretz, B.; Albrecht, K.; Bernkop-Schnurch, A. Correlation of in vitro and in vivo models for the oral absorption of peptide drugs. *Amino Acids* **2008**, *35* (1), 233–241.

(19) Fogh, J. F.; Orfeo, J. M. T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer. Inst.* **1977**, *59* (1), 221–226.

(20) Polentarutti, B. I.; Peterson, A. L.; Sjoberg, A. K.; Anderberg, E. K.; Utter, L. M.; Ungell, A. L. Evaluation of viability of excised rat intestinal segments in the Ussing chamber: investigation of morphology, electrical parameters, and permeability characteristics. *Pharm. Res.* **1999**, *16* (3), 446–454.

(21) Ungell, A. L.; Nylander, S.; Bergstrand, S.; Sjoberg, A.; Lennernas, H. Membrane transport of drugs in different regions of the intestinal tract of the rat. *J. Pharm. Sci.* **1998**, *87* (3), 360–366.

(22) Katz, M.; Ben-Shlush, I.; Kolusheva, S.; Jelinek, R. Rapid colorimetric screening of drug interaction and penetration through lipid barriers. *Pharm. Res.* **2006**, *23* (3), 580–8.

(23) Carpino, L. A.; Han, G. Y. 9-Fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* **1972**, *37* (22), 3404–3409.

(24) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. 2-Chlorotrityl chloride resin. Studies on anchoring of Fmoc-amino acids and peptide cleavage. *Int. J Pept. Protein Res.* **1991**, *37* (6), 513–520.

(25) Freidinger, R. M.; Hinkle, J. S.; Perlow, D. S. Synthesis of 9-fluorenylmethyloxycarbonyl-protected N-alkyl amino acids by reduction of oxazolidinones. *J. Org. Chem.* **1983**, *48* (1), 77–81.

(26) Hess, S.; Linde, Y.; Ovadia, O.; Safrai, E.; Shalev, D. E.; Swed, A.; Halbfinger, E.; Lapidot, T.; Winkler, I.; Gabinet, Y.; Faier, A.; Yarden, D.; Xiang, Z.; Portillo, F. P.; Haskell-Luevano, C.; Gilon, C.; Hoffman, A. Backbone cyclic peptidomimetic melanocortin-4 receptor agonist as a novel orally administrated drug lead for treating obesity. *J. Med. Chem.* **2008**, *51* (4), 1026–1034.

(27) Sai, Y.; Kajita, M.; Tamai, I.; Wakama, J.; Wakamiya, T.; Tsuji, A. Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells. *Am. J. Physiol.* **1998**, *38* (3), G514–G520.

(28) Wu, D. S.; Beyenbach, K. W. The dependence of electrical transport pathways in Malpighian tubules on ATP. *J. Exp. Biol.* **2003**, *206* (Part 2), 233–243.

(29) Englund, G.; Rorsman, F.; Ronnblom, A.; Karlbom, U.; Lazorova, L.; Grasjo, J.; Kindmark, A.; Artursson, P. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. *Eur. J Pharm. Sci.* **2006**, *29* (3−4), 269–277.

(30) Stephens, R. H.; Tanianis-Hughes, J.; Higgs, N. B.; Humphrey, M.; Warhurst, G. Region-dependent modulation of intestinal permeability by drug efflux transporters: in vitro studies in mdr1a(−/−) mouse intestine. *J. Pharmacol. Exp. Ther.* **2002**, *303* (3), 1095–1101.

(31) Ovadia, O.; Greenberg, S.; Laufer, B.; Gilon, C.; Hoffman, A.; Kessler, H. Improvement of drug-like properties of peptides: the somatostatin paradigm. *Expert Opin. Drug Discovery* **2010**, *5* (7), 655–671.

(32) Mahato, R. I.; Narang, A. S.; Thoma, L.; Miller, D. D. Emerging trends in oral delivery of peptide and protein drugs. *Crit. Rev. Ther. Drug Carrier Syst.* **2003**, *20* (2−3), 153–214.

(33) Burton, P. S.; Conradi, R. A.; Hilgers, A. R.; Ho, N. F. H.; Maggiora, L. L. The relationship between peptide structure and transport across epithelial cell monolayers. *J. Controlled Release* **1992**, *19* (1−3), 87–97.

(34) Li, Y.; Yuan, H.; Yang, K.; Xu, W.; Tang, W.; Li, X. The Structure and Functions of P-Glycoprotein. *Curr. Med. Chem.* **2010**, *17* (8), 786–800.

(35) Bermejo, M.; Avdeef, A.; Ruiz, A.; Nalda, R.; Ruell, J. A.; Tsinman, O.; Gonzalez, I.; Fernandez, C.; Sanchez, G.; Garrigues, T. M.; Merino, V. PAMPA-a drug absorption in vitro model: 7. Comparing rat in situ, Caco-2, and PAMPA permeability of fluoroquinolones. *Eur. J. Pharm. Sci.* **2004**, *21* (4), 429–441.

(36) Matsson, P.; Bergstrom, C. A. S.; Nagahara, N.; Tavelin, S.; Norinder, U.; Artursson, P. Exploring the role of different drug transport routes in permeability screening. *J. Med. Chem.* **2005**, *48* (2), 604–613.

(37) Jezyk, N.; Li, C.; Stewart, B. H.; Wu, X.; Bockbrader, H. N.; Fleisher, D. Transport of pregabalin in rat intestine and Caco-2 monolayers. *Pharm. Res.* **1999**, *16* (4), 519–526.

(38) Fujikawa, M.; Ano, R.; Nakao, K.; Shimizu, R.; Akamatsu, M. Relationships between structure and high-throughput screening permeability of diverse drugs with artificial membranes: application to prediction of Caco-2 cell permeability. *Bioorg. Med. Chem.* **2005**, *13* (15), 4721–4732.

(39) Rezai, T.; Bock, J. E.; Zhou, M. V.; Kalyanaraman, C.; Lokey, R. S.; Jacobson, M. P. Conformational flexibility, internal hydrogen bonding, and passive membrane permeability: successful in silico prediction of the relative permeabilities of cyclic peptides. *J. Am. Chem. Soc.* **2006**, *128* (43), 14073–14080.

(40) Adessi, C.; Frossard, M. J.; Boissard, C.; Fraga, S.; Bieler, S.; Ruckle, T.; Vilbois, F.; Robinson, S. M.; Mutter, M.; Banks, W. A.; Soto, C. Pharmacological profiles of peptide drug candidates for the treatment of Alzheimer's disease. *J. Biol. Chem.* **2003**, *278* (16), 13905–13911.

(41) Foged, C.; Nielsen, H. M. Cell-penetrating peptides for drug delivery across membrane barriers. *Expert Opin. Drug Delivery* **2008**, *5* (1), 105–117.

(42) Jiao, C. Y.; Delaroche, D.; Burlina, F.; Alves, I. D.; Chassaing, G.; Sagan, S. Translocation and endocytosis for cell-penetrating peptide internalization. *J. Biol. Chem.* **2009**, *284* (49), 33957–33965.

(43) Moretto, A.; Crisma, M.; Kaptein, B.; Broxterman, Q. B.; Toniolo, C. N-methylation of N(alpha)-acylated, fully C(alpha)-methylated, linear, folded peptides: synthetic and conformational aspects. *Biopolymers* **2006**, *84* (6), 553–565.

(44) Demmer, O.; Frank, A. O.; Kessler, H. Design of Cyclic Peptides. In *Peptide and Protein Design for Biopharmaceutical Applications*, 1st ed.; Jensen, K. J., Ed.; John Wiley & Sons: Chichester, 2009; pp 135−136.

(45) Weller, M.; Stupp, R. Approval of new drugs for glioblastoma. *Curr. Opin. Neurol.* **2009**, *22* (6), 617–618.

(46) Friess, H.; Langrehr, J. M.; Oettle, H.; Raedle, J.; Niedergethmann, M.; Dittrich, C.; Hossfeld, D. K.; Stoger, H.; Neyns, B.; Herzog, P.; Piedbois, P.; Dobrowolski, F.; Scheithauer, W.; Hawkins, R.; Katz, F.; Balcke, P.; Vermorken, J.; van Belle, S.; Davidson, N.; Esteve, A. A.; Castellano, D.; Kleeff, J.; Tempia-Caliera, A. A.; Kovar, A.; Nippgen, J. A randomized multi-center phase II trial of the angiogenesis inhibitor Cilengitide (EMD 121974) and gemcitabine compared with gemcitabine alone in advanced unresectable pancreatic cancer. *BMC Cancer* **2006**, *285* (6).

(47) Knipp, G. T.; Vander, D. G. V.; Siahaan, T. J.; Borchardt, R. T. The effect of beta-turn structure on the passive diffusion of peptides across Caco-2 cell monolayers. *Pharm. Res.* **1997**, *14* (10), 1332–1340.

(48) Pierson, M. E.; Comstock, J. M.; Simmons, R. D.; Kaiser, F.; Julien, R.; Zongrone, J. Rosamond, J. D. Synthesis and biological evaluation of potent, selective, hexapeptide CCK-A agonist anorectic agents. *J. Med. Chem.* **1997**, *40* (26), 4302–4307.

(49) Doedens, L.; Opperer, F.; Cai, M.; Beck, J. G.; Dedek, M.; Palmer, E.; Hruby, V. J.; Kessler, H. Multiple N-methylation of MT-II backbone amide bonds leads to melanocortin receptor subtype hMC1R selectivity: pharmacological and conformational studies. *J. Am. Chem. Soc.* **2010**, *132* (23), 8115–8128.

(50) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23* (1−3), 3–25.

(51) Veber, D. F.; Johnson, S. R.; Cheng, H. Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D. Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* **2002**, *45* (12), 2615–2623.