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The effect of backbone cyclization on PK/PD properties of bioactive peptide-peptoid hybrids: The melanocortin agonist paradigm

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ABSTRACT

A peptide–peptoid hybrid (peptomer) library was designed and synthesized, based on the sequence Phe-D-Phe-Arg-Trp-Gly. This sequence was previously found to specifically activate the melanocortin-4-receptor (MC4R) which participates in regulation of energy homeostasis and appetite. The library of peptomers included a peptoid bond in the Phe and/or D-Phe position and consisted of linear and backbone cyclic analogs, differed in their ring size. While the linear peptides rapidly degraded in serum and in brush border membrane vesicles (BBMV's), the linear peptomers were more stable. Backbone cyclic peptomers were also stable under the same conditions. Backbone cyclization significantly increased the intestinal permeability of two peptomers compared to their linear counterparts, in the Caco-2 model. Pharmacological evaluation revealed that two linear and one backbone cyclic peptomer, were found active towards MC4R at the nanomolar range. Thus, the conformational constrains imposed by these local and global modifications affect both the pharmacokinetic and pharmacodynamic properties of the parent peptide. This study demonstrates the potential of imposing backbone cyclization on bioactive peptomers as a promising approach in developing an orally available peptidomimetic drug leads.

1. Introduction

Active peptides have a high potential as therapeutic agents for the treatment of various diseases due to their high selectivity and limited side effects. Yet, their poor metabolic stability and low intestinal permeability limit their oral administration and hence their wide usage as drugs is restricted.[1]

Various strategies have been proposed in order to develop peptidomimetic drugs which will, on one hand, improve their oral bioavailability by improving their enzymatic stability and/or intestinal permeability and on the other hand, will preserve their biological activity. These strategies include structural modification that preserve the side chains of the parent bioactive peptides such as cyclization,[2,3] N-methylation,[4] D-amino acids,[5,6] aza peptides,[7,8] peptoids and peptomers (peptide–peptoid hybrids).[9–11]

Most peptides are absorbed by passive diffusion, either across the entrocytes membrane (i.e., transcellular) or through the hydrophilic channels between the enterocytes (i.e., paracellular). Most active peptides are hydrophilic, form hydrogen-bonding interactions with the aqueous surrounding, and as a result the paracellular pathway is their main absorption route.[12] Yet most of the intestinal surface area is consisted of lipidic membrane (99%) giving the transcellular pathway an advantage as a route of achieving improved permeability.[13] Therefore, most of the chemical modifications suggested to improve permeability of peptides are aimed to decrease their hydrophilic nature by either decrease their number of H-bonds or increase their hydrophobicity or both (e.g., in the N-methylation approach, methyl groups replace hydrogens on the N^{α}). In peptoids, the side chain of the alpha-carbon is moved to the nitrogen (Fig. 1) eliminating the polar N–H bond of the peptide while preserving the side chain. This chemical modification elevates the lipophilicity of the molecule and therefore may improve the likelihood permeation of the peptoid across the cell membrane.[14]

Following oral administration of peptides they encounter proteases along their absorption pathway, within the intestinal lumen and at the brush border of the gastrointestinal wall, as well as in the plasma and in organs (i.e., liver, kidney etc.). Reduced susceptibility of the peptidic amide bond to the peptidase activity, as in the cases of cyclization and peptoids, enables improved resistance to proteolytic enzymes.[15–17]

The available data regarding the pharmacological potential of peptoids is so far derived from linear peptoids. Very limited

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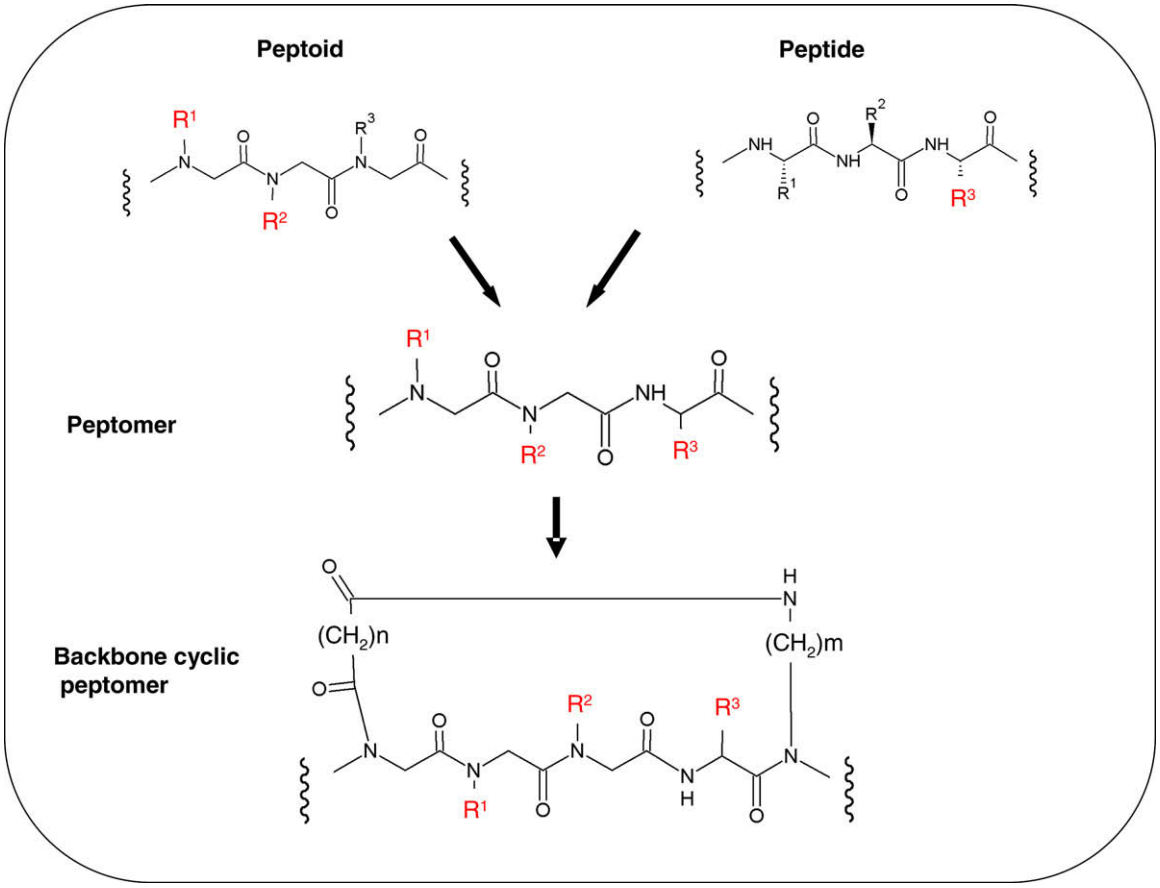

Figure 1. General structure of peptides, peptoids, peptomers and backbone cyclic peptomers.

information is currently available on cyclic and/or backbone cyclic peptoids.[18] Cyclization of peptides was found to impose conformational constraint which can potentially enable selective molecular recognition, enhanced metabolic stability and intestinal permeability.[19–21] Backbone cyclization (BC)[12,22] is a general method by which conformational constraint is imposed on peptides.[23] In backbone cyclization, a peptidomimetic compound is constructed by covalently interconnecting atoms in the backbone (N and/or C) of a target linear peptide to form a ring. A significant advantage of BC is the fact that unlike the usual cyclization techniques (e.g., side chain to side chain and side-chain to amino or carboxy ends) the bridge/interconnection is formed between backbone atoms and not side chains, which are generally essential for biological activity. This method has been used on various bioactive peptides and has been shown to improve the pharmacological selectivity of a given peptide as demonstrated for substance P[24] and somatostatin analogs.[25] It has also been shown to dramatically enhance the metabolic stability of peptides to intestinal and serum proteases as well as to improve their paracellular permeability.[17,24]

To our knowledge, there are no reports in the literature that consider the backbone cyclization of peptoids and/or peptomers. The aims of this work were (1) to investigate the pharmacologic impact of incorporating a peptoid bond into a bioactive peptide (2) to investigate whether backbone cyclization of peptomers may lead to a synergistic effect of improved enzymatic stability and intestinal permeability as well as to affect receptor selectivity. In this work, a peptomer library was synthesized, based on the sequence Gly-Phe-D-Phe-Arg-Trp-Gly-NH_2. The library of peptormers included peptoid bond in the Phe and/or D-Phe position and included linear and backbone cyclic peptoids which varied in their ring size (Fig. 3 and Table 1).

Table 1
Chemical structures of the backbone cyclic peptomers

Peptomer	*m*	*n*	Phe position	D-Phe position	Mass calculated	Exact mass analysis	Yield (%)
1 (PLY1-1)	–	–	+	–	810.35	810.405	36
2 (PLY1-2)	–	–	–	+	810.35	810.406	34
3 (PLY1-3)	–	–	+	+	810.35	810.406	41
4 (PLY2-1)	2	2	–	+	835.41	836.420	22
5 (PLY2-2)	2	2	+	–	835.41	836.420	32
6 (PLY2-3)	2	2	+	+	835.41	836.420	24
7 (PLY3-1)	3	2	–	+	849.41	850.437	25
8 (PLY3-2)	3	2	+	–	849.41	850.438	31
9 (PLY3-3)	3	2	+	+	849.41	850.437	24
10 (PLY4-1)	4	2	–	+	863.41	864.453	33
11 (PLY4-2)	4	2	+	-	863.41	864.452	27
12 (PLY4-3)	4	2	+	+	863.41	864.452	24

Melanocortins are peptide hormones which affect various physiological processes including pigmentation, adrenal steroid production, grooming behavior, nerve regeneration, inflammation and body weight regulation.[26] Five receptors exist that mediate the effects of melanocortins. We have previously synthesized a library of backbone cyclic melanocrtin-4-receptor (MC4R) agonist peptides based on the sequence Gly-Phe-D-Phe-Arg-Trp-Gly-NH_2, derived from the endogenous hormone, melanocyte-stimulating hormone (αMSH) which was found to cause a decrease in food intake and to elevate energy utilization upon binding to the MC4R[17] (chemical structure is depicted in Figure 2A). The peptide library was designed in order to develop a peptide based drug which could be orally administered for treatment of obesity. The library of peptides differed in their ring size and chemistry. This diversity was found to influence both pharmacological activity and also pharmacokinetic parameters. One analog, BL3020-1 (Fig. 2B) was found to cause decrease in food intake and reduce weight gain following oral administration to mice.[17]

The peptomers were evaluated for their drug-like properties as followed: intestinal permeability was assessed using Caco-2 cells model, a well established in vitro model. In addition, a novel colorimetric assay[27] was used to assess whether the peptoids tend to interact/penetrate a synthetic bilayer liposome as a model of the cell membrane. The permeable peptomers were also evaluated for their transcellular permeability properties using Parallel Artificial Membrane Permeability Assay (PAMPA).

Members of the peptomer library were assessed for their stability in rat serum and purified brush border membrane vesicles (BBMV's), representing the enzymes expressed in the intestinal enterocytes.[12]

In addition, the degree of agonistic activity of the synthesized compounds on melanocortin receptors was assayed using HEK293 cells, stably expressing mouse MC1R, MC3R-MC5R, transfected with the CRE/β-galactosidase reporter gene as previously described.[17]

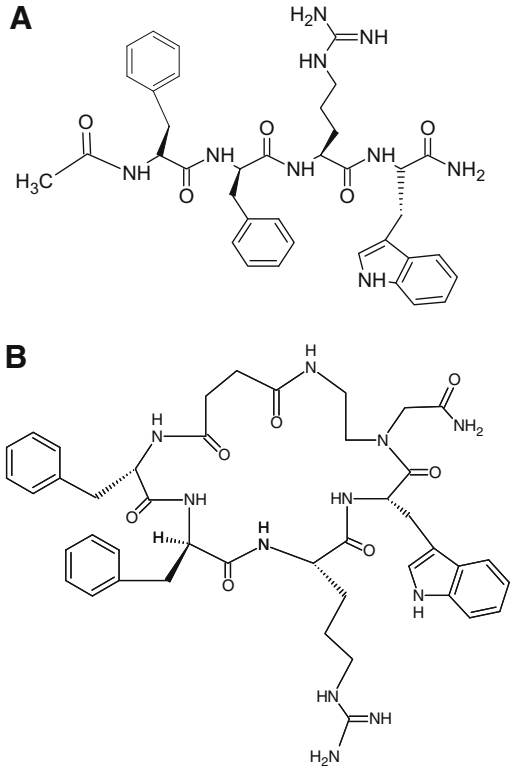

Figure 2. Chemical structure of the template linear (A) and backbone cyclic (B) peptide analogs.

2. Results

2.1. Enzymatic stability

Four groups of peptomers were synthesized, that differ in the bridge chemistry (Table 1). The metabolic stability of the peptomers from each group was evaluated in rat serum and in purified BBMV's.

As a first step, several peptomers were evaluated for stability in rat serum (Fig. 5A) and compared to their counterpart linear peptide. As can be seen, the linear peptide was rapidly degraded in rat serum, after 1 h incubation, the peptide could not be detected in the serum. The linear peptomer tested, compound **1** (PLY1-1) was also found to be degraded in serum, but is a slower rate. After 1 h nearly 50% of the peptomer was degraded, and 3 h later only less than 10% of the peptomer was detectable in serum. On the other hand, all the BC peptomers were practically stable over 1 h (±5% of initial concentration). The stability of the tested BC peptomers was found not to be uniform. While 2 BC peptomers, compounds **7** (PLY3-1) and **10** (PLY4-1) were partially degraded (>40% degradation) after 4 h, compound **4** (PLY2-1), having the smallest ring size was found to be completely stable at the time points examined.

The relative metabolic stability in serum seen for peptomers was also found when incubated with BBMVs which include the variety of peptidases expressed in the intestine (Fig. 5B). As can be seen in Figure 5B, in general, all peptomers tested display good stability up to 90 min while the linear peptide degraded considerably faster. In addition, the two tested linear peptoids, compounds **1** and **2** (PLY1-2) were less stable in comparison to the backbone cyclic peptoids. Yet, while the backbone cyclic peptomers were found to be completely stable (±5% of initial concentration), a noticeable degradation was evidenced for the linear peptoids, and their concentrations at that endpoint, were reduced by about 20%.

2.2. Intestinal permeability

The permeability coefficient (Papp) of all tested peptomers is presented in Figure 6. As can be seen the range of the Papp values was significantly lower than Papp of testosterone, a marker for transcellular permeability. The Papp of all linear peptomers was lower than the Papp of mannitol, a marker for paracellular transport. Two of them exhibited very low permeability (i.e., Papp $<1 \times 10^{-7}$ cm/sec). Most of the BC peptomers demonstrated higher permeability rate compared to mannitol. Papp of compound **7** and **9** (PLY3-3) was almost threefold higher than the Papp of mannitol, indicating improvement in their intestinal permeability properties.

2.3. Biological activity

In order to examine the effect of modifying the active peptide into peptomers on their pharmacological properties, the peptomers were analyzed for the degree of activation of the different melanocortin receptors (Table 2). The changes in the ring size and chemistry led to significant changes in potency of activating of the melanocortin receptors and hence displayed selectivity of the peptides to the different MC4s. The peptomers were found to have diverse potency. Two of the linear peptomers, compounds **2** and **3** (PLY1-3) exhibited the highest potency to MC4 and MC3 receptors (8–16 nM). The BC peptomers were found to be less active towards MCRs, the most active peptomer (compound **10**) was almost sixfold higher than that of **2** and exhibited the highest activation of MC4R, while they had the same affinity to MC5R. The stimulation/agonist potency of all other peptomers was higher than 100 nM for all tested melanocortin receptors.

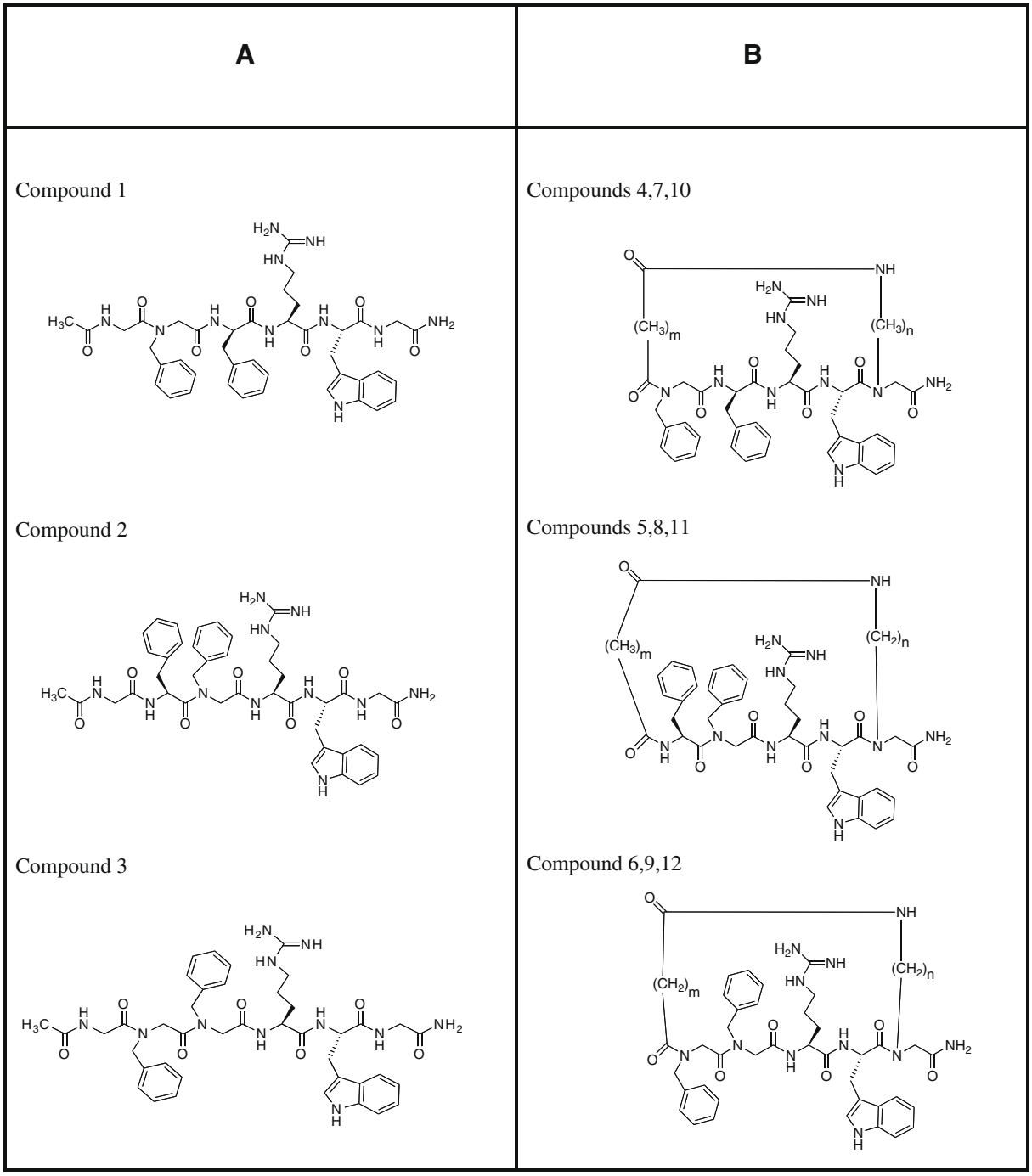

Figure 3. Chemical structure of the backbone cyclic peptoids (A) Linear sublibrary, based on the peptide sequence Phe-D-Phe-Arg-Trp. Peptoid residue introduced either on the Phe (compound **1**), D-Phe (Compound **2**) or both positions (compound **3**) (B) templates of the backbone cyclic peptomer library. Each sublibrary included 3 peptomers, as described for the linear peptomers. The sublibraries differed in their ring size (2–4 methyl groups).

2.4. Interaction with the liposomal bilayer

Membrane interactions of peptomers were analyzed by measuring chromatic transitions of the lipid/PDA vesicle system.[27,28] The biomimetic platform consists of mixed vesicular constructs containing phospholipids and polydiacetylene (PDA).[27,28] Interactions of membrane-active species with the phospholipid domains of the mixed assemblies induce rapid blue-to-red color change within the polymer moieties.[27,29,28] As depicted in Figure 7 most of the peptomers interact poorly with lipid bilayer (i.e., <30% colorimetric response). Among the linear peptomers, **1** was found to have the highest membrane activity (31% colorimetric response). While most of the BC peptomers show impaired association with model membrane, compound **7** exhibits intensive membrane activity (89%). Compound **9** showed intermediate extent of interaction with lipid bilayer.

3. Discussion

One of the most versatile approaches in the development of peptidomimetic based lead libraries is peptoids (N-substituted glycines) and peptomers (peptide–peptoid hybrids). These are

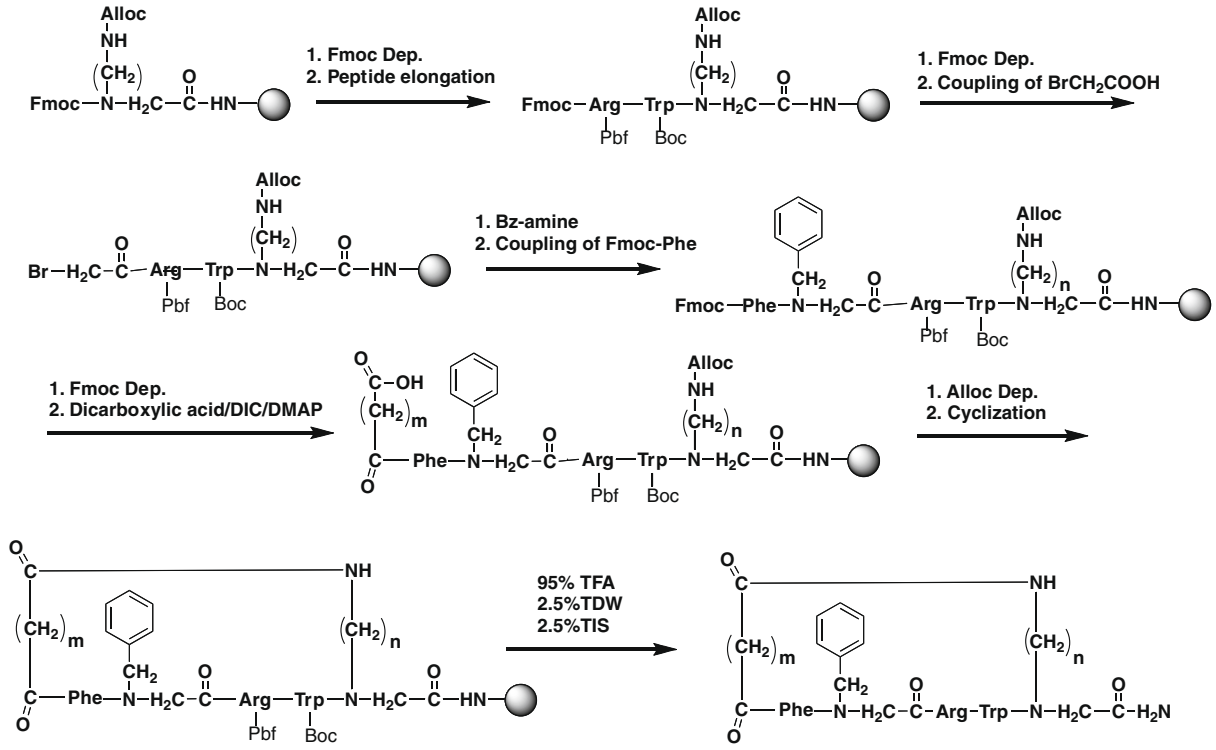

Figure 4. A scheme describing the steps involved in the synthesis of the BC peptomer library.

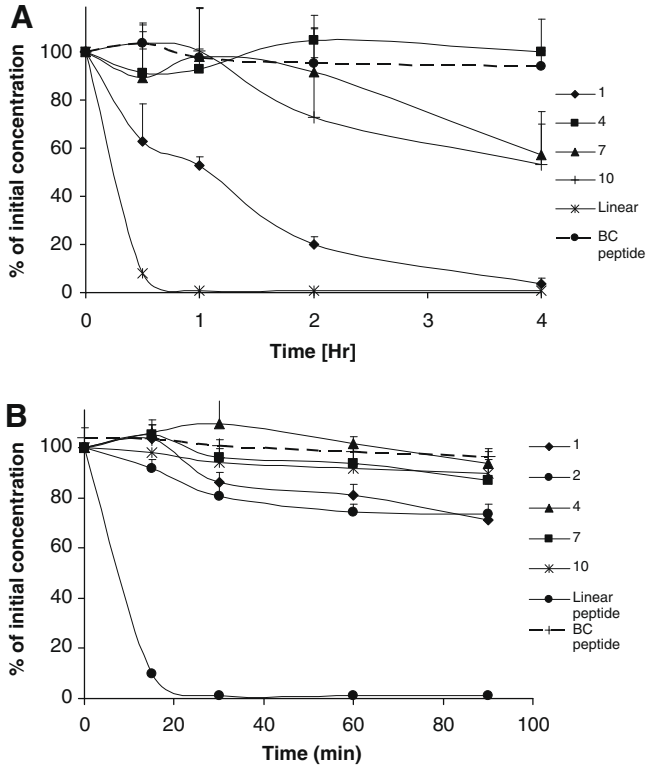

Figure 5. Metabolic stability of linear and cyclic peptoids. (A) Stability in rat serum, (B) stability in BBMVs. Samples ($n \geqslant 3$) were taken at time 0 and after several time points. The samples were diluted 1:2 with ice-cold methanol, centrifuged (10,000*g*, 10 min, 4 °C) and transferred to analysis.

attractive candidates for development of orally available peptidomimetics since they are generally found to be resistant to proteolytic enzymes[16,15], and were suggested to hold improved intestinal permeability compared to peptides.[30] In addition, several potent bioactive peptoids have been discovered during screening of peptoid libraries.[31–33] The conformational restriction achieved by replacing peptide with peptoid residues, often diminishes the biological activity while altering the physico-chemical properties, leading to improved PK properties. The challenge facing the development of peptoid based drug is to screen for analogs which incorporate preservation or even enhancement of PD properties, accompanied by improved PK properties.

The peptomer approach can be used in the conversion of bioactive peptides, into an active analog with reduced transformations in the essential amino acids comprising the lead motif, compared to peptoids, therefore may probably enhance the chances to discover analogs with improved PK/PD properties.

Cyclization of peptides has been widely used in order to generate conformationals constraints that provide peptide analogs with improved pharmaceutical properties. Backbone cyclization of peptides is a useful tool for cyclization of bioactive peptides which was found to enhance metabolic stability and increase intestinal permeability, without affecting the amino acid side chains, thus conserving biological activity. We have recently synthesized a library of MC4R agonist backbone cyclic peptides that maintain the parent sequence Phe-D-Phe-Arg-Trp-Gly-NH_2 and have conformational diversity. Changes in the ring chemistry of these peptides caused variations in the selectivity and affinity of the peptides to the MC4R.[17] A bioactive library member (BL3020-1, Fig. 2B) was found to have oral bioavailability (5%) in rats.

In effort to expand the range of structural motifs that can enable increased molecular diversity for selection of drug leads with desired drug-like properties, a peptomer library was synthesized, based on the sequence Phe-D-Phe-Arg-Trp-NH_2. The library members included linear and BC peptomers differed in their ring size, which included a peptoid bond on the Phe, D-Phe or on both residues. In order to elucidate the potential of these modifications in improving the drug-like properties of these analogs, the library

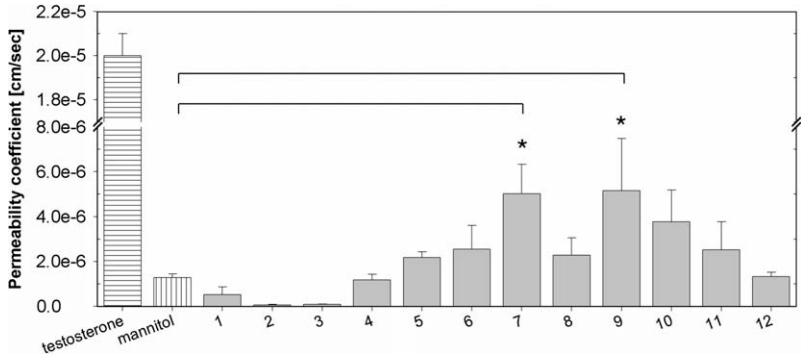

Figure 6. Permeability coefficient (P apparent, P_{app}) of cyclic peptoids compared to the linear analog and to known standards. Caco-2 cell monolayers were incubated with the tested molecules at 37 °C, added to the apical side and detected at the basolateral side for 150 min. $n \geqslant 3 \pm$ SEM. * $P < 0.05$.

Table 2
Summary of functional activity (EC50) at the mouse melanocortin receptors

	mMC1R (nM)	mMC3R (nM)	mMC4R (nM)	mMC5R (nM)
αMSH	0.008 ± 0.001	0.063 ± 0.004	0.097 ± 0.016	0.078 ± 0.008
Linear sequence[a]	503 ± 100	11,900 ± 1800	70.6 ± 13.8	143 ± 5
1	11800 ± 1900	>100	9820 ± 500	3430 ± 840
2	220 ± 76	>100	8.8 ± 2.7	16.2 ± 4.2
3	240 ± 33	40% at 100 μM	10.5 ± 2.1	8.1 ± 0.9
4	380 ± 27	>100	1370 ± 360	1650 ± 590
5	2810 ± 150	>100	3830 ± 330	3960 ± 1100
6	620 ± 190	>100	1960 ± 590	2950 ± 960
7	220 ± 21	>100	640 ± 14	310 ± 80
8	1000 ± 60	>100	25% at 100 μM	2320 ± 530
9	1680 ± 210	>100	2250 ± 14	350 ± 42
10	120 ± 36	7170 ± 21	49 ± 16	8.1 ± 2.0
11	1170 ± 355	>100	3300 ± 960	485 ± 45
12	990 ± 100	>100	1700 ± 210	320 ± 75

The concentration of compound at 50% maximum receptor stimulation (EC50), or the % of receptor stimulation at the highest concentration of compound tested (relative to control). The indicated errors represent the standard error of the mean determined from at least three independent experiments. NA denotes that no agonist activity was observed at up to 100 mM; PA– potent antagonist.

[a] First published by Holder et al.[37]

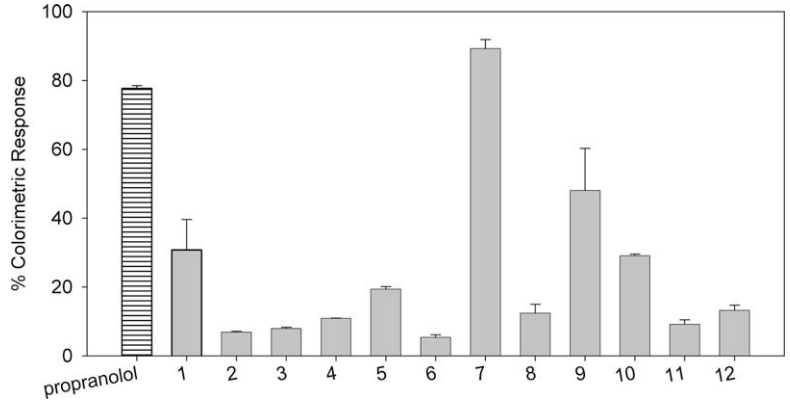

Figure 7. Interaction of the peptomers with a colorimetric membrane assay Interactions of linear peptomer analogs (**1**–**3**) and backbone cyclic analogs (**4**–**12**, differ in their ring size) with mixed DMPC/PDA chromatic assemblies (expressed as colorimetric response, %CR) are compared to propranolol, marker for passive transcellular permeability.

was screened for enzymatic stability in serum and in BBMV's, representing the intestinal milieu, and also evaluated for intestinal permeability in Caco-2 model. In addition, the activity and selectivity of the library members was also investigated.

3.1. Pharmacokinetics aspects

The current evidence regarding the stability of peptoids and peptomers, is based on studies using serum or selected set of enzymes.[16,15] We tested the linear and backbone cyclic peptomers in rat serum and also used the BBMV's model, which includes all enzymes expressed on the brush border enterocytes and therefore is a valid model to test the stability in the gut. As can be seen in Figure 5A, the impact of insertion of a peptoid bond is obvious when comparing the peptide sequence (linear1) to its peptomer counterpart in which one amino acid (Phe) was replaced by a peptoid. While no traces of the linear peptide were detected after 1 h incubation, the linear peptoid was detected after 2 h incubation

(>20% of its initial concentration). There is also a distinguished difference in the stability of the linear peptoid compared to the backbone peptoids which were found to be completely stable under the assay conditions.

There is a clear difference between the linear and BC peptomers when tested for their stability to BBMV's (Fig. 3B). While all BC peptomers were found to be stable under the study conditions, the linear peptomers were less stable, loosing ~20% of their initial concentration. Insertion of a peptoid bond, as expected, enhanced the enzymatic stability, as can be seen by the rapid degradation of the linear peptide. Combining backbone cyclization and peptomer resulted in the most stable form. It appears that the conformational restrictions imposed by backbone cyclization prevent recognition by the proteases. The rationale for these findings is that the intramolecular bridge reduces molecular flexibility for interconversion. The constraint imposed by the cyclization significantly reduces the number of conformations that can be attained by the BC analogs in comparison to the linear compounds. This conformational restriction leads to reduced affinity to the active site(s) at the peptidase and diminished efficacy of the enzymatic degradation. These conformational restrictions may also alter the efficacy and selectivity to the receptor, as will be discussed later on.

Very little information is available on the intestinal and cellular permeability of peptoids. Kwon and Kodadek[30] have reported that peptoids are more cell permeable than their corresponding peptides. It was suggested that reduction in H-bond potential increased the peptoid lipophilicity and enabled movement across the cell membrane.

The intestinal permeability of the linear peptomers as denoted by the Papp values for permeation in the Caco-2 model was found to be poor (Fig. 5, lower than the permeability of mannitol, a marker for passive paracellular permeability). The permeability of all BC peptomers was higher than mannitol. Among the backbone cyclic peptomers, the permeability varied without clear linkage to ring size, site or number of peptoid bonds. Two peptomers, compounds **7** and **9** were found to have significant increase in their permeability compared to mannitol. This improved permeability may indicate a possible shipment by the transport mechanism, which can include improved transport through the paracellular pathway, active transport and/or shipment towards transcellular permeability.

Transcellular transport of molecules includes a passive interaction of the molecule with the hydrophobic membrane followed by crossing the membranes (i.e., the apical and basolateral membranes) to reach the blood circulation. In order to assess possible shipment of the permeability towards the transcellular permeability, we used a novel colorimetric model.[27] This platform comprises vesicles of phospholipids and the chromatic lipid mimetic polymer polydiacetylene. The polymer undergoes visible, concentration-dependent blue-red transformations induced through interactions of the vesicles with the molecules examined.

The results (Fig. 7) indicate that while all linear peptomers and most of the backbone cyclic peptomers poorly interact with the liposomal bilayer, two peptomers, **7** and **10**, the most permeable peptomers in the Caco-2 model, were found to hold enhanced interaction (50% and 90% colorimetric response, respectively).

In a previous study[17] we have shown that backbone cyclic peptides were generally more permeable than their linear counterparts through the paracellular pathway, probably due to their conformational rigidity and hydrodynamic volume. This finding could also be attributed to shipment towards transcellular permeability resulting from elevated lipophilicity and the reduced H-bonding potential characteristic of the backbone cyclic peptomers. Since there are fewer intramolecular H-bonds in peptomers their enhanced lipophilicity is expected to cause more interactions with the cell membrane and higher transcellular permeability.[14]

When analyzing the contribution of peptoid bond to the interaction with the liposomal model, no correlation between the number of peptoid bonds and colorimetric values could be revealed. For example, compound **7** has only one peptoid bond (Table 1) yet its interaction with the liposome is considerably higher than compound **12** (PLY4-3), which has 2 peptoid bonds. This may suggest that for these peptomers, hydrogen-bonding potential is not a major determinant in dictating their permeability. As can be seen in Figures 5 and 6, there is a correlation between the predicted intestinal permeability and the colorimetric reaction with the liposomal model, mainly for compounds **7** and **9**, which were found to relatively have the highest permeability values. This may suggest on a possible partial absorption through the transcellular route.

The interaction compound-membrane is a mandatory but not exclusive condition to cross the membrane. Therefore, these peptomers were further tested for their passive transcellular permeability using $PAMPA_{Lechitin}$.[14] In this non-cell based method, an artificial membrane is coated with a lecithin, mimicking the cellular bilayer.

The peptomers which with the highest permeability in the Caco-2 study, were screened through PAMPA. While cyclosporine, known to permeate by transcellular mechanism, showed high permeability in this assay (Pe 3.5×10^{-6} cm/sec), none of the peptomers was permeable. These results together with the fact that both **7** and **9** transport in similar rate from apical to basolateral and vice versa (data not shown) indicate that the peptomers main transport route was the passive paracellular pathway.

3.2. Pharmacological aspect

The transformation of a biologically active MC4 agonist peptide into peptoid analogs while retaining biological activity is known to be a challenge due to several considerations[34] including: (i) the flexibility of a peptoid bond may increase the entropic energy associated with ligand binding to receptors; (ii) reduction in hydrogen binding potential may diminish the capability of forming regular structural motifs.

Several studies have suggested the usage of peptoids or peptomers as potent melanocortin agonist.[34,35] Most studies are aimed at finding selective MC3R and MC4R agonists due to the participation of these receptors in weight and energy balance.[36] Kruijtzer et al.[35] have reported that an introduction of a peptoid moiety in an MC4 receptor peptide resulted in a decreased affinity, yet several peptomers were still active in a sub micro molecular concentrations.

The activity of the linear sequence Phe-D-Phe-Arg-Trp-NH_2, which was the parent sequence in this study, was previously tested[37] and was found to be potent towards MC4R and MC5R (EC_{50} 70 ± 14 and 143 ± 5nM, respectively).

Introduction of a peptoid moiety resulted in remarkable changes in the peptomer activity (Table 2). When the peptoid was introduced in the Phe position (compound **1**), the peptomer lost completely its biological activity. Yet, replacement of the D-Phe (compound **2**) caused an enhancement in a magnitude of order in the activation of MC4R and MC5R, compared to the peptide counterpart.

We have previously shown that chemical changes in BC peptides (i.e., ring size) have changed the activity and selectivity of backbone cyclic melanocortin agonist peptides.[17]

Backbone cyclization of the parent linear sequence resulted in several selective analogs towards MC4R. The most potent analog, BL3020-1 (EC50 4 ± 0.6 nM).

In this study we have imposed backbone cyclization on the linear peptomer sequences. It is interesting to note that the peptomer analogs with identical ring size as BL3020-1 (compounds **4**, **5** and **6**, Table 1) have lost completely their potency and selectivity

towards the melanocortin receptors. It was found that all backbone cyclic peptomers except for **10** were not selective towards the melanocortin receptors. Compound **10** was the most potent peptomer, yet with poor selectivity towards MC4R (Table 2). These results suggest that, in most cases the introduced constraint resulted in an unfavorable conformational arrangement. Yet, as mentioned, several peptomers were found to activate the MC4R. When analyzing the results, it appears that the Phe position is crucial for activity since peptoid residue in the Phe position, diminished the activity towards MC4 (**1**, Table 1). The same rationale leads to the estimation that the D-Phe residue is less important for MC4 activation. Peptoid residue in this position did not circumvent the MC4 activation. However, when the ring size was modified, the effect was opposite as depicted for **10**. Peptoid residue in the Phe position of the BC-peptomer analog increased the activity towards MC4R whereas peptoid residue in the D-Phe position of the BC-peptomer analog diminished it. This is probably due to the fact that BC had a global conformational effect while a single peptoid modification has a local effect.

Interestingly, all peptomers found to activate the MC4R also activated the MC5R. This could be explained by the fact that these two receptors share very high sequence homology.[38]

The difference between the affinity of the linear peptomers and their BC analogs to the melanocortin receptors is evident and in our view derives from the same rationale of conformational constrains provided above for the metabolic stability properties.

Drug design based on the three-dimensional structures of G-protein-coupled receptors (GPCRs) is barely feasible for those GPCRs whose structures have not been solved. Global restricted conformation, caused by backbone cyclization combined with local constraint by incorporation of specialized amino acids, such as peptoids, can narrow down the conformational space and thus lead to analogs that either fit the receptor/enzyme site or not, thereby yield higher degree of selectivity. In previous studies we have demonstrated this pharmacodynamic selectivity produced by BC to somatostatin derivatives,[39] and in the same fashion to MC4 peptide agonist.[17]

4. Summary

Assessment of the PK/PD properties for a 'drug-like' bioactive peptide derivatives is a key factor in the development of new peptide-based therapeutics. Rapid PK/PD evaluation using a panel of in vitro models may be beneficial in order to characterize and choose the most promising lead compound among a given library.

The concept of combining two approaches, namely BC and peptoids, each has been proved to be advantageous in improvement of the physicochemical and/or pharmacological properties of peptide based drug candidates, was found to be justified. Significant improvement was detected in the enzymatic stability of BC peptomers compared to their linear counterparts. Two BC peptomers were found to have improved intestinal permeability. In addition, chemical modifications in the ring chemistry generated modifications in the biological activity and selectivity of the peptomers.

Although no peptomer analog showed improvement in all features (i.e., biological activity, intestinal permeability and enzymatic stability) compared to the backbone cyclic peptide BL3020-1, it is very likely that screening a higher number of BC peptomers will lead to identify one or more candidates which may serve as a promising MC4R agonist drug therapy. Further examination of conformational and topographical space should prove fruitful in the design of more selective ligands for the melanocortin receptors since it appears that each receptor has its own special requirements.

5. Experimental section

5.1. Chemistry

Protected amino acids, 9-fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu), bromo-tris-pyrrolidone-phosphonium hexafluorophosphate (PyBrop), Rink amide methylbenzhydrylamine (MBHA) polystyrene resins, organic and supports for solid phase peptide synthesis (SPPS) were purchased from Nova Biochemicals (Laufelfingen, Switzerland). Bis(trichloromethyl)carbonate (BTC) was purchased from Lancaster (Lancashire, England). Glyoxylic acid, 1,2-diaminoethane, bromoacetic acid and benzyl amine were purchased from Merck (Darmstadt, Germany), and tetrakis (triphenylphosphine) palladium(0) was purchased from ACROS (Geel, Belgium).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX 300 MHz spectrometer. Thin layer chromatography (TLC) was performed on Merck F245 60 Silica Gel plates (Darmstadt, Germany). All peptides were of $\geqslant$95% purity as verified by analytical RP-HPLC. HPLC analysis was performed using a Vydac analytical RP column (C18, 4.6 $\times$ 250 mm, catalog number 201TP54) and was carried out on a Merck-Hitachi L-7100 pump and a Merck-Hitachi L-7400 variable wavelength detector operating at 215 nm. The mobile phase consisted of a gradient system, with solvent A corresponding to water with 0.1% TFA and solvent B corresponding to acetonitrile (ACN) with 0.1% TFA. The mobile phase started with 95% A from 0 to 5 min followed by a linear gradient from 5% B to 95% B from 5 to 55 min. The gradient remained at 95% B for an additional 5 min and then was reduced to 95% A and 5% B from 60 to 65 min. The gradient remained at 95% A for additional 5 min to achieve column equilibration. The flow rate of the mobile phase was 1 mL/min. Peptide purification was performed by reversed phase HPLC (RP-HPLC; on a L-6200A pump, Merck-Hitachi, Japan) using a Vydac preparative RP column (C8, 22 $\times$ 250 mm, catalog number 218TP1022). The flow rate of the mobile phase was 9 mL/min. All preparative HPLC runs were carried out using a gradient system with solvent A corresponding to water with 0.1% TFA and solvent B corresponding to ACN with 0.1% TFA. MS characterization was performed on a Voyager-DE PRO Biospectrometry workstation using matrix assisted laser desorption ionization (MALDI) technology in the positive mode.

5.2. Solid phase synthesis of linear peptomers and backbone cyclic peptomers

The synthesis was performed in a reaction vessel equipped with a sintered glass bottom, following general Fmoc chemistry protocols which was described by Hess et al.[17] and Linde et al.[40] Peptoid oligomers were synthesized using the submonomer approach outlined by Zuckerman et al.[41] Peptide–peptoid hybrids were synthesized using a suitable combination of (1) standard Fmoc solid-phase peptide synthesis for amino acid residues and (2) the submonomer approach for N-substituted benzyl glycine residues (NPhe[41]). In the next step, a mixture of 10 equiv bromoacetic with 10 equiv of DIEA dissolved in DMF and added to the free amine for 1 h at rt. After coupling of bromoacetic acid, the resin was shaken for 3 h at room temperature with a mixture of 10 equiv benzyl amine and 10 equiv of DIEA in DMF. Backbone cyclic peptomers were synthesized using combination of Fmoc peptide synthesis, the submonomer synthesis[41] and backbone cyclization, as depicted in Figure 4.[17,40]

Cleavage from the resin and removal of side chain protecting groups were carried out simultaneously using a pre-cooled mixture of 95% TFA, 2.5% TDW, and 2.5% triisopropylsilane (TIS). After the resin was added, the mixture was agitated for 30 min in an ice

bath, and then was shaken for 2.5 h at rt. The combined TFA filtrates were evaporated to dryness by a stream of nitrogen. The oily residue was triturated three times with cold ether to remove the scavengers, and the ether was removed by centrifugation. The dry crude peptide was dissolved in ACN/H_2O (1:1) and lyophilized. Exact mass analysis was performed on LTQ orbitrap XL (Thermo scientific).

5.3. Preparation of brush border membrane vesicles

Brush border membrane vesicles (BBMV's) were prepared from combined duodenum, jejunum, and upper ileum by a Ca^{2+} precipitation method.[42,43] The intestines of five male Wistar rats, 200–250 g, were rinsed with ice cold 0.9% NaCl and freed of mucus; 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 then homogenated (Polytron PT 1200, Kinematica AG, Switzerland). $CaCl_2$ was added to a final concentration of 10 mM. The homogenate was left shaking for 30 min at 4 °C and then centrifuged at 10,000*g* for 10 min. The supernatant was then centrifuged at 48,000*g* for 30 min and an additional two purification steps were undertaken by suspending the pellet in 300 mM mannitol and 10 mM Hepes/Tris (pH 7.5) and centrifuging at 24,000*g*/h. Purification of brush border membranes was assayed using the brush border membrane enzyme markers gamma-glutamyl transpeptidase (GGT), leucine amino peptidase (LAP) and alkaline phosphatase. During the course of these studies, enrichment in brush border membrane enzymes varied between 13- and 18-fold.

5.3.1. Stability studies

The tested molecule was mixed with purified BBMV's and incubated in 37 °C for 90 min. Duplicate samples were taken at time 0 and after 15, 30, 45, 60 and 90 min. The samples were diluted 1:1 with ice-cold methanol, centrifuged (7500*g*, 10 min, 4 °C) and sent to HPLC analysis. Time points for stability in serum were 0, 1, 2 and 5 h.

5.4. Functional bioassay

HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described.[44] Briefly, 5000–15,000 post-transfection cells were plated into 96 well Primera plates (Falcon) and incubated overnight. Forty-eight hours post-transfection, the cells were stimulated with 100 μL of peptide (10–4–10–12 M) or forskolin (10–4 M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated and 50 μL of lysis buffer (250 mM Tris–HCl, pH 8.0 and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96 well plate for relative protein determination. To the cell lysate plates, 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM MgCl2, 10 mM KCl, 5 mM β-mercaptoethanol, and 200 mg ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD 405 nm, was measured using a 96 well plate reader (molecular devices). The relative protein was determined by adding 200 μL of 1:5 dilution BioRad G250 protein dye: water to the 10 μL cell lysate sample taken previously, and the OD 595 nm was measured on a 96 well plate reader (molecular devices). Data points were normalized both to the relative protein content and non receptor-dependent forskolin stimulation. The pA_2 [$(K_i) = -\log pA_2$] values were generated using the Schild analysis method.[45]

5.5. In vitro permeability study

5.5.1. 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 (1×10^6 cells/flask at 37 °C in 5% CO_2 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, and 2 mM L-glutamine. The medium was replaced twice weekly. All medium supplements were purchased from Biological Industries, Beth-Haemek, Israel.

5.5.2. Preparation of cells for transport studies

For transport studies cells in a passage range of 52–60 were seeded at density of 25×10^5 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 monolayers were placed in 24 transwell plates, 12 mm, Costar™. The culture medium was changed every other day. Transport studies were performed 21–22 days after seeding, when the cells were fully differentiated and the TEER (trans epithelial electrical resistance) values were stable (300–500 ohm/cm^2).

5.5.3. Experimental protocol

Transport study 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 a 30 min period at 37 °C with shaking (100 cycles/min). After the incubation period the buffers were removed and replaced with 1500 μL basolateral buffer at the basolateral side. Test solutions were warmed previously to 37 °C and added (600 μL) to the apical side of the monolayer. Fifty microliters of 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 duration 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.

5.5.4. Data analysis

The permeability coefficient (Papp) for each compound was calculated from the linear plot of drug accumulated versus time, using the following equation:

$$\text{Papp} = dQ/dt/(C_o \cdot A)$$

where dQ/dt is the steady state rate of appearance of the drug on the receiver side, C_0 is the initial concentration of the drug on the donor side, and A is the surface area, 1.1 cm^2.

5.6. Analytical analysis

Were performed using a HPLC-MS Waters Millenium instrument equipped with Micromass ZQ detector, Waters 600 Controller gradient pump and Waters 717 auto-sampler. Nitrogen flow was 500 L/h; source temperature was 400 °C; the cone voltage was 40 V; the column a Xterra MS C18 2.1 × 150 mm (Waters). The mobile phase at 0.25 mL/min was 25% acetonitrile, 0.1% formic acid with a linearity range of 0.025–1 μg/mL.

5.7. Interaction with the liposome bilayer

Vesicles consisting of DMPC/PDA (2:3 molar ratio) were prepared by dissolving all lipid constituents in chloroform/ethanol

(1/1) and drying together in vacuo to constant weight. The lipid films were suspended in deionized water by probe sonication at 70 °C for 3 min, yielding total lipid concentration of 1 mM. The vesicle suspension was cooled to room temperature, incubated overnight at 4 °C, and polymerized by irradiation at 254 nm for 30 s, resulting an intense blue appearance of the vesicles solutions. UV–vis measurements were performed by addition of peptides from stock solutions (0.4 mg/mL) to 60 μL vesicle suspensions consisting of 0.5 mM total lipids in 25 mM Tris-base (pH 8), dilution to 200 μL by deionized water and spectra acquisition on an Analytical ELISA-reader (Jena, Germany), using a 96 wells microplate. All measurements were performed in duplicates.

To quantify the extent of blue-to-red color transitions within the vesicle suspensions, the colorimetric response (% CR), was defined and calculated as follows[46] $\%CR = [(PB_0 - PB_I)/PB_0] \times 100$, where $PB = A_{blue}/(A_{blue} + A_{red})$, and A is the absorbance at 640 nm, the 'blue' component of the spectrum, or at 500 nm, the 'red' component ('blue' and 'red' refer to the visual appearance of the material, not actual absorbance). PB_0 is the blue/red ratio of the control sample before induction of a color change, and PB_I is the value obtained for the vesicle solution after the colorimetric transition has occurred. More reddish appearance of the vesicle suspensions indicates higher CR values. Previous studies have shown that peptides interact selectively with phospholipid domains of the mixed phospholipid/PDA assemblies and chromatic transitions of 100% PDA constructs are minimal.[29]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.010.

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