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β-Turn secondary structure and melanocortin ligands

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Abstract—The melanocortin pathway has emerged during this past decade as an important target area for the discovery and development of therapeutic agents related to obesity and type 2 diabetes. This peptide-G-protein coupled receptor (GPCR) pathway has evolved from peptide-based ligands to small molecules possessing a variety of different molecular scaffolds. Herein, we summarize the originating hypothesis of the importance of the reverse β-turn secondary structure for agonist ligand potency at the melanocortin receptors and how that information was utilized for the discovery of small molecules based upon this type of turn structure.

1. Introduction

The melanocortin system is comprised of five G-protein coupled receptor (GPCR) subtypes and a series of endogenous agonists and antagonists that interact and regulate the intracellular signaling of these receptors. The melanocortin receptors (MCRs) are found in a variety of tissues, and are associated with a myriad of vital functions. The melanocortin-1 receptor (MC1R) is best known for its role in skin pigmentation and hair coloration.[1,2] The melanocortin-2 receptor (MC2R) is unique among the melanocortin receptors in that it is only stimulated by the adrenocorticotropin hormone (ACTH) ligand, versus other endogenous agonist peptides (i.e., α-MSH, β-MSH, γ-MSH).[1] The MC2R is expressed primarily in the adrenal glands and in adipocytes,[1,3,4] and is involved in steroidogenesis and the body's stress response mechanism in correlation with adrenocorticotropin hormone (ACTH) through the cortisol and other stress-related pathways.[3–5] The melanocortin-3 receptor (MC3R) is expressed throughout the body, notably in the central nervous system, the pancreas, the heart, and the gastrointestinal tract.[6–8] The location of the MC3R in these tissues, as well as the phenotype of the knockout mouse, suggests a potential role in energy homeostasis, thermoregulation, and cardiovascular function.[9,10] The melanocortin-4 receptor (MC4R) is primarily expressed in the central nervous system and the brain.[11–14] The MC4R has been shown to be directly involved in feeding behavior, weight, and energy homeostasis by the changes in food intake observed after the administration of MC4R selective agonists and antagonists in mouse-feeding studies as well as the phenotype of the knockout mouse.[15–18] Additionally, the MC4R plays a role in sexual function.[19–21] The melanocortin-5 receptor (MC5R) is expressed in a plethora of tissues throughout the body.[7,22–24] Even though the MC5R remains largely uncharacterized, it has been tentatively linked to exocrine gland function and sebaceous gland lipid production.[7,22–24]

The melanocortin receptors respond to endogenous agonists derived from the proopiomelanocortin (POMC) gene transcript as well as endogenous antagonists (agouti and agouti-related protein, AGRP).[25,26] α-Melanocyte-stimulating hormone (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) is generated by enzymatic cleavage and modification of the POMC gene transcript.[27,28] α-MSH has potent nM agonist activity at all of the melanocortin receptor subtypes with the notable exception of the MC2R. ACTH is a 39 amino acid peptide that results from the first fragmentation of POMC by prohormone convertase 1 (PC1).[29–34] ACTH is cleaved by PC2 to yield the precursor for α-MSH (which is further enzymatically modified at the N- and C-termini). Both α-MSH and ACTH, as well as the other endogenous melanocortin ligands, contain a core His-Phe-Arg-Trp sequence that has been postulated to be important for molecular recognition and receptor stimulation.[35–39]

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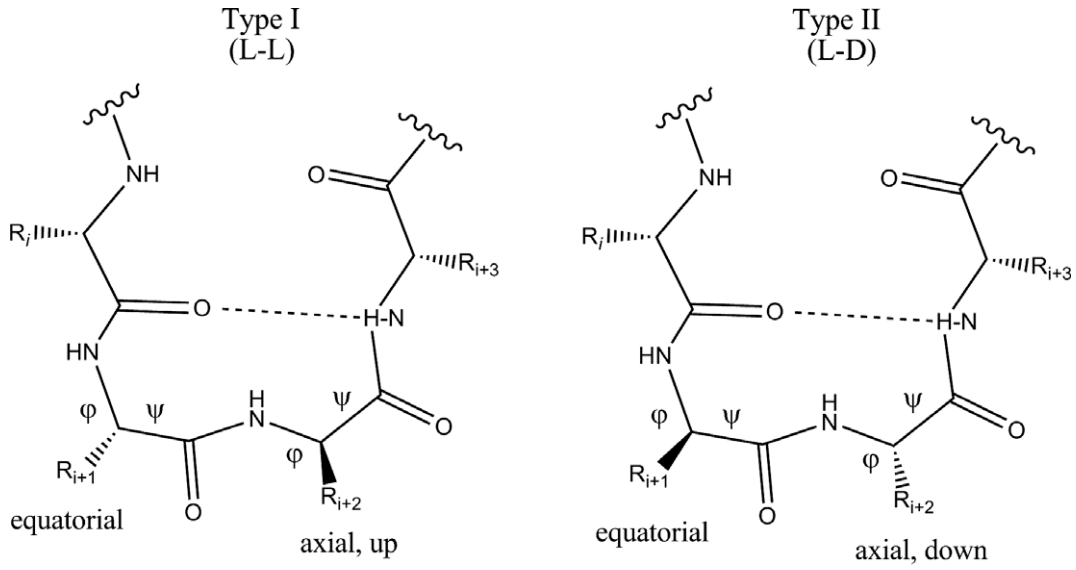
Molecular recognition of peptides by their cognate receptors has been attributed to the ligand pharmacophore, secondary structure, and chemical functionality of the amino acid side chains. One of the most common and important secondary structures found in peptide hormones that stimulate GPCRs is a reverse turn, specifically a β-turn.[40,41] A β-turn is a turn of specific orientation in which the direction of the peptide chain is reversed within four residues with the distance between the first and fourth residue defined as less than 7 Å and an α-helix secondary structure is not formed. A hydrogen bond between the carbonyl group of residue *i* and the amide of the third peptide bond (*i* + 3) stabilizes β-turns within the peptide chain.[41–43] β-turns are classified based upon the ϕ and ψ angles observed between the side chains of residues *i* + 1 and *i* + 2.[43] For a standard β-turn, the side chain of the *i* + 1 residue is oriented equatorially, while the *i* + 2 residue is oriented axially, either up or down, depending on the stereochemistry of the residue (Fig. 1).[41,43] This paper will focus on melanocortin peptides and small molecules designed to mimic type I and type II β-turns (Fig. 1).

2. Design, synthesis and characterization of melanocortin peptides

α-MSH is a linear 13 amino acid peptide (Fig. 2) that is highly flexible and can adopt a variety of conformational states depending upon local aqueous, protein, or membrane-related environments. One of the elementary concepts in peptide-related ligand design is that by reducing the degree of conformational freedom, ligands that are entropically favorable and mimic a 'bioactive conformation' could be designed by the use of conformational constraints ranging from side chain to cyclization strategies.[44–47] Studies on melanocortin agonist ligands from the 1970s to 1980s will start this perspective. Sawyer et al. conducted racemization experiments using quantitative gas chromatographic methods on α-MSH confirming that the Met[4] and Phe[7] (α-MSH numbering) residues were both racemized after heat-alkali treatment that led to the design of diastereoisomeric analogues based on the observation that after heat-alkali treatments α-MSH experiences an increase in biological function in both in vitro and in vivo experiments.[48] Subsequently, a linear 13 amino acid residue analogue of α-MSH was designed in which Met[4] was replaced by Nle[4] and LPhe[7] was stereochemically substituted by DPhe[7] [Nle[4],DPhe[7]]-α-MSH, more commonly referred to as NDP-MSH (Fig. 2).[48] NDP-MSH exhibited strong biological activity in the classical frog skin pigmentation assay without having to undergo heat-alkali treatment, unlike the linear α-MSH peptide. NDP-MSH also showed increased potency as compared to both α-MSH and Nle[4]-α-MSH, and did not degrade when exposed to serum enzymes.[49] NDP-MSH was more potent than α-MSH in activating mouse melanoma adenylate cyclase, and elicited a stronger response in the mouse melanoma cell tyrosinase assay.[48] Interestingly, a DPhe residue is postulated to stabilize a reverse turn secondary structure around the α-MSH 5–9 residues. This hypothesis resulted in the design and synthesis of an analogue in which Cys residues replaced Met[4] and Gly[10] in α-



	i+1	*i*+2
φ	-60	-90
ψ	-30	0

	i+1	*i*+2
φ	-60	-90
ψ	120	0

Figure 1. Type I and II β-turn motif. L and D refers to the stereochemistry of the side chains.

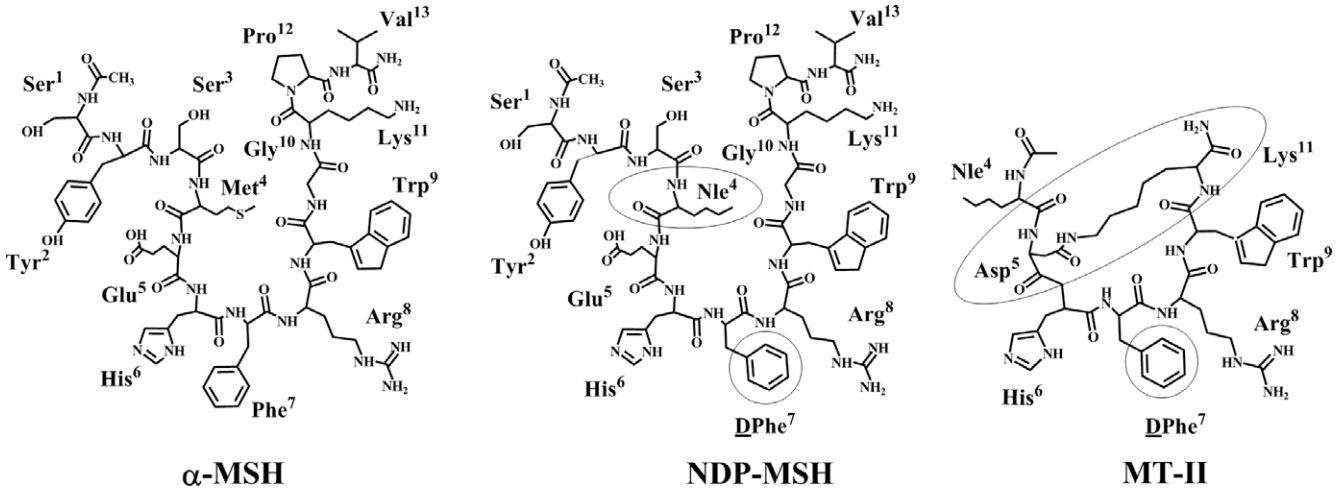


Figure 2. Structures of melanocortin ligands with circled regions indicating important structural changes.

MSH resulting in a covalently linked side chain to side chain disulfide bond. Cyclic [Cys^4,Cys^{10}]-α-MSH was shown to be more potent than α-MSH in the classical frog and lizard skin pigmentation assays and more potent in stimulating adenylate cyclase in the mouse melanoma adenylate cyclase assay.[50,51] It was during this time that mounting evidence suggested that the 'biologically active' conformation of α-MSH may be due to the presence of a β-turn found within the His-Phe-Arg-Trp sequence and that cyclizing the peptide would produce a constrained conformation similar to a reverse turn that oriented the melanocortin pharmacophore moieties optimally for molecular recognition and receptor potency.[50,51]

Knittel et al. then synthesized Ac-[Cys^4,Cys^{10}]-α-MSH$_{(4-10)}$-NH$_2$ and Ac-[Cys^4,Cys^{10}]-α-MSH$_{(4-13)}$-NH$_2$ to further the investigation of the biological role of the reverse turn within α-MSH.[51] Since prolongation of skin darkening was not observed with Ac-[Cys^4,Cys^{10}]-α-MSH$_{(4-10)}$-NH$_2$, it supported the conclusion that a reverse turn is not the only structural feature that is important for melanocortin activity. Although the potencies of both synthesized cyclic analogues are greater than NDP-MSH, the prolongation of these peptides is less than that of NDP-MSH, suggesting that potency and prolongation are directed through different structural and conformational features.[39,48,51]

The culmination of melanocortin peptide β-turns and cyclization studies was the discovery and characterization of the super potent melanocortin agonist Melanotan II (MTII) (Fig. 2) by Al-Obeidi et al. based upon structure–activity relationships, molecular dynamic calculations, and examining other agonist peptides such as NDP-MSH and Ac-[Cys^4,Cys^{10}]-α-MSH-NH$_2$.[52,53] The structural requirements for melanotropic potency at positions five and ten of α-MSH were examined.[53,54] A series of NDP-MSH based peptide analogues were synthesized using the 1–13, 4–10, and 4–13 fragment templates as core sequences. Substitutions were made in which Glu^5 was replaced by Asp and Gly^{10} was replaced with amino acids containing basic side chains. It was reported that one analogue displayed enhanced potency when compared to α-MSH and Ac-[Nle^4,D-Phe^7]α-MSH$_{(4-10)}$-NH$_2$ in the frog skin and the lizard skin assays.[53,54]

NMR studies conducted by Sugg et al. provided experimental evidence that the DPhe^7 melanocortin ligand residue appeared to stabilize the β-turn within the core tetrapeptide sequence, resulting in the conclusion relating to the structure and bioactivity of NDP-MSH and its analogues.[55] It was hypothesized based upon these NMR studies that the hydrophobic side chains of the His, DPhe and Trp residues are on the same side of the peptide in close proximity to one another, while the hydrophilic Arg side chain is oriented away from the aromatic moieties.[55] These NMR data and structure–activity relationship data of the linear α-MSH fragment analogues NDP-MSH and Ac-[Cys^4,Cys^{10}]-α-MSH resulted in the design of cyclic α-MSH analogues using the 4–10 and 4–13 fragments.[48,50,51,53,54] Al-Obeidi et al. synthesized a series of cyclic peptides with the following modifications: Ac-Nle^4-*c*[Xxx^5,DPhe^7,Yyy^{10}]-Gly^{11}-α-MSH$_{(4-13)}$-NH$_2$ and Ac-Nle^4-*c*[Xxx^5,DPhe^7,Yyy^{10}]-α-MSH$_{(4-10)}$-NH$_2$, in which Xxx was substituted for by an acidic amino acid residue and the Yyy position was substituted for by a basic residue. A lactam bridge was formed between the side chains of the Xxx^5 and Yyy^{10} residues to further constrain the molecule.[52,53] The cyclic peptide, Ac-Nle^4-*c*[Asp^5-His^6-DPhe^7-Arg^8-Trp^9-Lys^{10}]-NH$_2$, was shown to exhibit a potency equivalent to α-MSH in the frog skin assay, however, it was 90–100 times more potent in the lizard skin assay and demonstrated prolonged biological activity in comparison to α-MSH.[52,53] This cyclic, super potent compound later came to be known as Melanotan II (MTII).

Although MTII is a potent peptide, it is not a selective agonist for any of the frog, lizard, mouse, or human melanocortin receptors.[52,53,56,57] Additional NMR studies have shown that MTII exhibits a type II β-turn within the His-DPhe-Arg-Trp region.[45] Molecular modeling has further supported the separation of side chains with the His, DPhe and Trp on one side and Arg on the

opposite side.[58,59] Ying et al. synthesized a series of peptide analogues based on the NMR structure of MTII incorporating disulfide or lactam bridge macrocycles as a means of further constraining the conformation of the compounds.[59] Based on ROESY NMR data for MTII, the hydrogens bonded to the Asp^5 and Arg^8 were identified to be in close proximity, leading the authors to hypothesize that replacement of these amino acids by sulfur could be used to form sulfide bonds and introduce a conformational constraint.[59] Further modifications were made by altering the N^{α}-alkylation residue (intended to mimic the Arg^8 pharmacophore), inverting the chirality of the C atom of the residue being N^{α}-alkylated, or substitution with bulky aromatic residues.[60]

The compounds were tested for their binding affinity and adenylate cyclase activity at the hMC1,3-5R. Three peptides were reported to be antagonists that exhibited high selectivity for the hMC4R. It was hypothesized that the selectivity was due to the increased conformational constraint as compared to the predicted values for MTII.[59] The compounds containing a disulfide bond instead of a lactam bridge had higher binding efficiencies, leading to the hypothesis that the disulfide-linked peptides were better ligands because of the increased flexibility of a disulfide bond compared to that of a lactam bridge. The data that the peptides exhibited only 50% binding efficiency were interpreted to suggest the presence of an allosteric binding site at the hMC4R to which some of the synthesized peptides bound in lieu of the site used by MTII.[60]

3. Melanocortin small molecule agonists

The ease with which peptides are degraded in the body and their general lack of selectivity has led to the desire for and the generation of a new class of compounds, termed peptidomimetics, designed to copy the general shape and chemistry of the endogenous peptide ligands with the goal of increased resistance to degradation, resulting in a longer half-life in the body and greater selectivity between receptor subtypes. The advent of small molecules targeting the melanocortin receptors has also meant the development of ligands that are capable of being selective for only one receptor subtype. Since the melanocortin receptor subtypes have such diverse roles that are tissue dependent, it is important to continue to seek more potent and selective ligands for use as effective drug therapy treatments with minimal side effects.

In 1999, Haskell-Luevano et al. screened a series of small molecules based on a heterocyclic, nine membered ring structure. The focus of the experiment was to identify small molecules whose structure incorporates a β-turn within the Phe-Arg-Trp region that exhibits agonist activity at the mMC1R.[35–37,61] Two non-peptide, heterocyclic compounds were identified as low micromolar agonists at the mMC1R by the screening of this library and were the first non-peptidic β-turn mimetic ligands reported in the literature able to stimulate the melanocortin receptors.[57] While the compounds (Fig. 3, 1) did not contain a basic side chain at the Arg^8 position, they still possessed low micromolar agonist activity at the mMC1R. This data refuted the belief that the basic side chain of Arg^8 was required for activity.[35,37,57,61] The results of this study further supported the hypothesis that a β-turn secondary structure present in the Phe-Arg-Trp sequence of agonist peptides may be key to biological activity at the melanocortin receptors and showed that biological activity could be reproduced by incorporating the secondary structure into small molecule ligands.[35] The identification of small molecules with agonist activity opened the gate for future researchers to design non-peptide agonists for the melanocortin receptors.

Bondebjerg et al. synthesized a series of peptidomimetics based on the β-turn configuration of the endogenous peptide ligands. A novel thioether cyclized peptidomimetic scaffold capable of being modified at up to four different positions was used as the basis for the compounds synthesized in the described study.[62] The resulting small molecules exhibited low micromolar activity at the mMC1,3-5Rs with some compounds demonstrating moderate selectivity for the mMC1R. Additionally, the study was able to synthesize and identify one peptidomimetic (Fig. 3, 2) with higher than average activity at all four assayed receptors compared to the other synthesized compounds.[62]

In 2006, Cain et al. synthesized a series of small molecules based on a pyrrolopiperazine template (Fig. 3, 3) intended to mimic the type II β-turn configuration. Based on the observation that the majority of small molecules with activity at the melanocortin receptors contain two aromatic hydrophobic groups and a group with a basic nitrogen, the group synthesized a series of small molecules to investigate the effects of substitution of the hydrophobic residues, the orientation of the hydrophobic groups, and the significance of the basic Arg residue based on a template synthesized from LPro and D,L-Phe.[63] Many of the resulting small molecules were capable of binding to one or more of the hMCRs at nanomolar concentrations, some exhibiting selectivity for the hMC5R. Though many of the compounds were unable to stimulate a cAMP response, a new small molecule template capable of binding to the melanocortin receptors was identified.[63]

In 2007, Verdie et al. developed a method for the incorporation of benzodiazepinone templates into sequences of α-MSH and other known agonists. The synthetic route used was to insert the benzodiazepinone like an amino acid residue during solid-phase peptide synthesis.[64] Based upon the NMR studies performed by Ying et al., core sequences were selected to be used as templates for the benzodiazepinone structures incorporated into the peptides.[59] Further molecular modeling predicted that the substitution of these amino acid residues with the benzodiazepinone template would not disrupt the natural folding conformation.[64] Two consecutive amino acids, either His^6-$DPhe^7$ or $DPhe^7$-Arg^8, were then replaced by a modified 1,4-benzondiazepine-2,5-dione building block (Fig. 3, 4).[64] Originally, five α-MSH analogues were modified with this benzodiazepi-

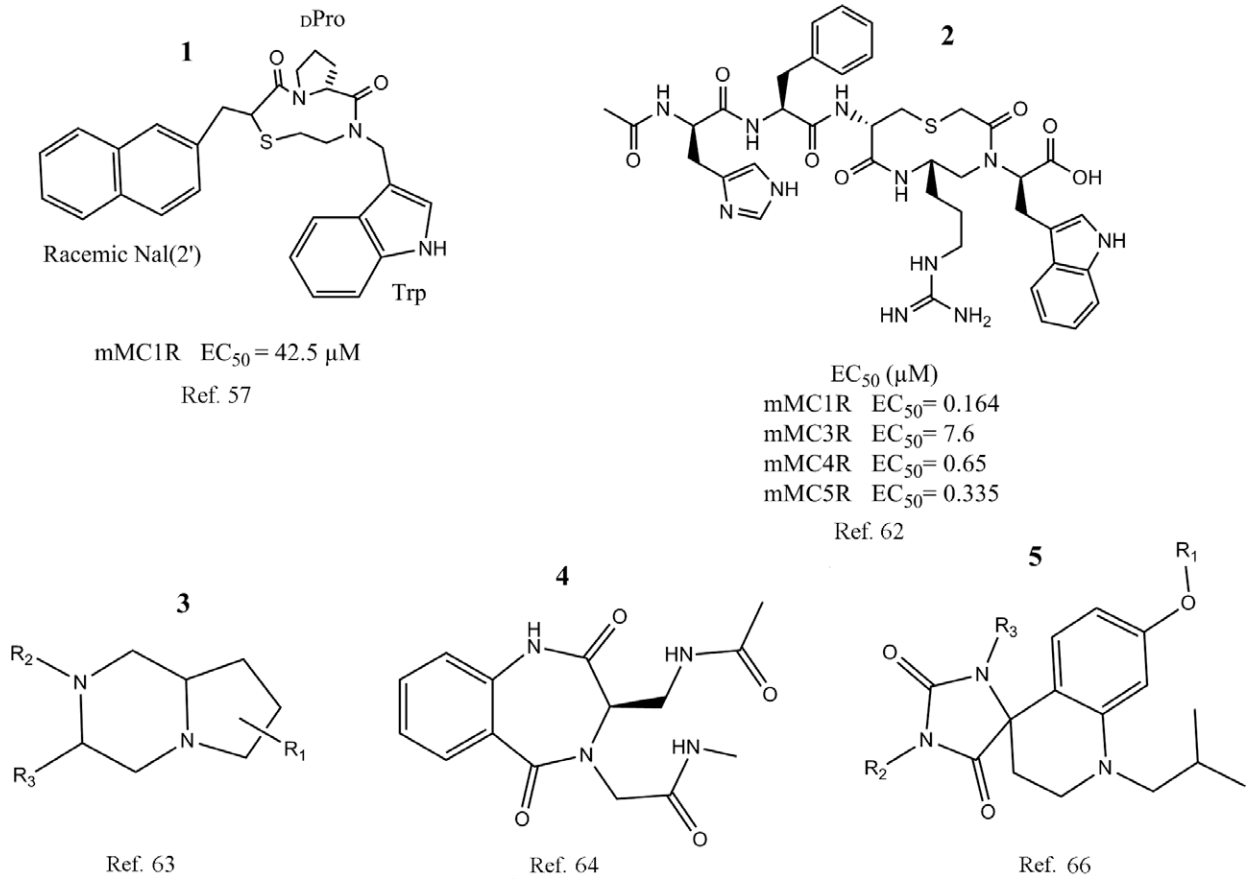


Figure 3. Structures and scaffolds of melanocortin targeting molecules incorporate a β-turn motif.

none template, with continued studies leading to the design of many additional analogues based on the primary results. The study found that when the His^6-$DPhe^7$ dipeptide sequence was replaced by the benzodiazepinone functional activity was lost, however, weak binding still occurred at the MC1R. Furthermore, compounds in which $DPhe^7$-Arg^8 were substituted with the small molecule template resulted in no binding at the receptor.[64] While the study did not result in potent agonist molecules, it did present the important discovery of the chemistry needed to substitute small molecules into a peptide chain using on-resin synthetic techniques.[64] The development of this approach which allows for the direct substitution of small molecules into a peptide will advance the field of peptide chemistry because of the ability to introduce privileged structures into known peptide ligands.

Chianelli et al. previously generated a series of ligands possessing activity for the somatostatin receptors using a novel method for the design of templates suitable for synthetic modification yielding libraries of small molecules possessing drug-like properties as well as exhibiting peptide pharmacophores.[65] This study was done to develop melanocortin non-peptide agonists to demonstrate that this approach is applicable to ligands of other receptors. Scaffolds were designed and synthesized with the goal of achieving small molecules of comparable size and shape to the β-turn sequence backbone. Three libraries were generated during this study, each based upon the results of the previous library.[66] From the first library, one compound was reported to show weak agonist activity at the MC4R. The second synthesized series produced one compound with low micromolar activity at the MC4R. In the third round generation of MC4R targeted ligands, 11 compounds demonstrated MC4R agonist activity greater than the activity shown by the compound selected from the second library as the model for modification for the third library.[66] The generation of several compounds capable of agonist activity at the MC4R demonstrates that the approach employed in the development of the compound libraries is applicable to more than just one class of GPCRs that recognize a β-turn secondary structure.[66] (Fig. 3, 5)

4. Conclusion

This perspective has reviewed the evolution of melanocortin peptide agonists that were hypothesized to contain a reverse β-turn secondary structure postulated to be important for the 'bioactive' conformation of melanocortin agonist ligands. These hypotheses were supported by biophysical studies (NMR and computational molecular modeling) and further validated by the development of small molecules that were proposed to mimic the β-turn secondary structure. It is

well recognized that an extremely large number of different molecular scaffolds have been used and identified as ligands that are able to potentially stimulate the melanocortin receptors that are not remotely related to the β-turn secondary structure, but that is outside the focus of this perspective and has been reviewed elsewhere. Since this special issue is a tribute to the groundbreaking contributions by Professor Jonathan Ellman, and the first β-turn small molecule melanocortin receptor agonist was a result of a collaborative effort between Haskell-Luevano, Cone, and Ellman, we thought it a fitting topic for this special tribute.

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