Design, Synthesis and Biological Evaluation of Ligands Selective for the Melanocortin-3 Receptor

Victor J. Hruby[*], **Minying Cai**, **James P. Cain**, **Alexander V. Mayorov**, **Matthew M. Dedek**, and **Devendra Trivedi**
Department of Chemistry, University of Arizona, Tucson, Arizona 85721

Abstract

The processed products of the proopiomelanocortin gene (ACTH, α-MSH, β-MSH, γ-MSH, etc.) interact with five melanocortin receptors, the MC1R, MC2R, MC3R, MC4R, and MC5R to modulate and control many important biological functions crucial for good health both peripherally (as hormones) and centrally (as neurotransmitters). Pivotal biological functions include pigmentation, adrenal function, response to stress, fear/flight, energy homeostasis, feeding behavior, sexual function and motivation, pain, immune response, and many others, and are believed to be involved in many disease states including pigmentary disorders, adrenal disorders, obesity, anorexia, prolonged and neuropathic pain, inflammatory response, etc. The roeianocortin-3 receptor (MC3R) is found primarily in the brain and spinal cord and also in the periphery, and its biological functions are still not well understood. Here we review some of the biological functions attributed to the MC3R, and then examine in more detail efforts to design and synthesize ligands that are potent and selective for the MC3R, which might help resolve the many questions still remaining about its function. Though some progress has been made, there is still much to be done in this critical area.

INTRODUCTION

The five melanocortin receptors and their corresponding agonist ligands which are derived from the precursor protein proopiomelanocortin (POMC) and the endogenous antagonist protein ligands agouti and agouti related protein (AGRP) have been the subject of scientific investigation for over 100 years, primarily for their function in melanopigmentation and adrenal function [1–4] and disorders related to pigmentation, stress and melanoma cancer. More recently, however, interest in both the melanotropin ligands and melanocortin receptors has greatly accelerated due to the cloning of the receptors in the 1990s, and the discovery of a number of important functions for the ligands and their receptors in a wide variety of biological activities including feeding behavior, obesity, anorexia, cachexia, sexual function and motivation, pain, immune response, cardiovascular function and many others [5,6]. And new discoveries of the involvement of this system in an ever wider variety of biological activities and behaviors including many diseases of the periphery and the central nervous system (CNS) are being made.

The major focus of this paper is on the melanocortin-3 system. The melanocortin-3 receptor (MC3R) is located on POMC neurons in the CMS, especially in the arcuate nucleus and within other areas of the brain [7] including the hypothalamus, and in several peripheral organs as well. It often is co-localized with the MC4R, but its precise function in obesity, cachexia, and related feeding behaviors is still not well understood. Initially (while still an orphan receptor) the MC3R was linked to non-insulin-dependent diabetes mellitus on human chromosome 20q

[*] Address correspondence to this author at the Department of Chemistry, University of Arizona, Tucson, Arizona 85721; Tel: (520] 621-6332; Fax: (520) 621-8407; E-mail: hruby@u.arizona.edu.

which we now know is the MC3R [8,9], and there is some evidence that the MC3R is an inhibitory autoreceptor on POMC neurons including the stimulation of food intake by peripheral administration of an MC3R specific agonist [10], and inhibition of spontaneous action of POMC neurons after application of a MC3R agonist [11]. Interestingly and paradoxically in the MC3R knockout mice an obesity syndrome is observed with reduced lean body mass and increased fat mass for both sexes, and with no apparent hyperphagia and with apparent normal energy expenditure [12,13,14]. Interestingly the MC3R knockout mice appear to be resistant to high fat diet-induced insulin resistance [14]. These and other findings suggest that development of peptide and non-peptide ligands that are selective for the MC3R may herald the arrival of exciting new classes of drugs for the treatment of a variety of disease states that involve melanotropin peptides and melanocortin receptors. In addition they can serve as valuable ligands for sorting out the detailed mechanism(s) of action of these ligands and their receptors which are poorly understood.

THE MELANOCORTIN-3 RECEPTOR (MC3R) – OVERALL CONSIDERATIONS

The MC3R is expressed in the central nervous system, peripheral tissues and immune cells, with initial studies highlighting expression in brain, gut, and placenta but none were detected in the adrenal gland or melanocytes [15], This lack of expression in the adrenal glands is not surprising given the fact that MSH peptides do not increase circulating corticosterone levels, through activation of the hypothalamic pituitary adrenal axis (HPA) [16]. As already discussed, a potential role in energy metabolism has been postulated for the MC3R since in MC3R null mice there is an increased fat mass and higher ratio of weight gain to food intake [13]. Other studies have demonstrated a central role in modulating the host inflammatory response with receptor detection on peritoneal [16–18] and knee joint macrophages [19]. Activation of MC3R in the heart has been shown to exhibit a protective effect in ischaemic-reperfusion injury [20, 21]. Thus the MC3R can be viewed as a fine tuner of specific mechanisms operating during inflammation, cardiovascular function and energy metabolism.

In the central nervous system, the melanocortin type 3 receptor (MC3R) is located on POMC neurons and other areas of the hypothalamus and often is co-localized with the melanocortin type 4 receptor (MC4R). The MC4R which regulates food intake is activated and modulated by the endogenous agonist, α-melanocyte stimulating hormone (α-MSH), a peptide processed from POMC, and also is modulated by an endogenous antagonist, agouti-related protein (AGRP) (orexigenic) [22]. Leptin, an adipocyte-derived hormone, acts on POMC and AGRP neurons in the arcuate nucleus of the hypothalamus, resulting in increased α-MSH and decreased AGRP [23]. Several lines of evidence have indicated that activation of the MC4R by α-MSH or synthetic peptide agonists reduces food intake, but that suppression of MC4R signaling by AGRP or synthetic melanocortin-4 receptor antagonists increases food intake and diminishes the hypophagic response to leptin [24,25]. Thus, the central melanocortin pathway is extremely important for normal energy homeostasis, and energy homeostasis via this pathway is highly susceptible to quantitative variations in MC3R/MC4R expression.

Melanocortin receptors belong to the superfamily of seven transmembrane G protein-coupled receptors (GPCRs). The hMCRs transduce signals by coupling to the heterotrimeric Gs protein and subsequent activation of adenylate cyclase. In many GPCRs, signaling is rapidly attenuated within minutes of agonist exposure, a process termed desensitization [26,27]. This process is associated with phosphorylation of serine/threonine residues in the third internal loop or carboxyl tail of the GPCR by G protein-coupled receptor kinases (GRKs) or second-messenger-dependent kinases [28]. There is no direct evidence for the hMC3R phosphorylation site yet. Phosphorylation by GRKs allows the GPCRs to interact with cytoplasmic proteins, the arrestins, which uncouple the GPCRs from G proteins [28,29]. For the hMCRs, arrestins target the receptor to clathrin-coated vesicles, and the receptor is sequestered into an intracellular

vesicular compartment, probably endosomes, after pinching off the vesicles from the plasma membrane by dynamin [30]. After sequestration, the GPCRs may recycle to the surface membrane after resensitization [31,32] or undergo lysosomal degradation [33]. Certain GPCRs can be targeted selectively to lysosomes leading to down-regulation of the GPCR by the same membrane pathway that mediates internalization [33]. Down-regulation of GPCRs in various neural cell types is of particular interest because this may lead to certain diseases, such as addiction [34], and opiate tolerance and dependence [35]. More generally any down-regulation/ desensitization, which occurs for GPCRs in response to agonists, leads to a decrease in drug efficacy.

Despite the central importance of the MC3R/MC4R in energy homeostasis, feeding behavior and the existence of an endogenous agonist and antagonist of the melanocortin system, highly selective MC3R agonists and antagonists, which could have important applications in treatment of cachexia and type II diabetes, and in evaluating the role of the MC3R in these conditions, are not yet available. Design of selective MC3R/MC4R agonists and antagonists thus becomes of critical importance. Our research group has been working on the design and synthesis of selective melanotropins for many years. Describing in a single review all existing approaches that have been taken to obtain ligands that target human melanocortin receptors (hMCRs) is not possible. Rather we will examine a few different approaches investigators have taken for design of selective ligands for the MC3R which reflect the current knowledge. We hope this will address the fundamental and the methodological issues which can promote rational design of more selective hMCR ligands. In this regard, understanding the molecular mechanisms underlying hMCRs function is also important in the design of ligands with the desired pharmacological profile. Knowledge of the current biostructural data is also important since it is becoming increasingly clear that stereostructural differences between the closely related receptors, such as the MC3R in relation to the MC1R, MC4R and MC5R, will be an important aspect of any successful development of a selective ligand for biological and biomedical applications [36].

GENERAL CONSIDERATIONS FOR THE DESIGN OF hMC3R SELECTIVE AGONISTS AND ANTAGONISTS

Although numerous native peptides have great potential for medical applications, and over 50% of all current drugs are peptide or peptidomimetic-based, often peptides have to be modified to enhance certain properties including metabolic stability, selectivity, and bioavailability, to be suitable therapeutic agents. Thus design of modified peptides for peptidomimetic and non-peptide applications has dramatically advanced during the last two decades [e.g. 37–42], One of the most challenging aspects of this research is rational design based on 3D-topographical features of the peptide pharmacophore. The first strategy in this approach is to identify the core structural features which are necessary for receptor/acceptor recognition. This is usually accomplished by truncation of the whole sequence; systematic single amino acid modifications of the peptide such as glycine, alanine, *D*-amino acid, etc. scans; ring size scans, bulky amino acid scans, etc. [e.g. 41,43]. Once the structure activity relationships (SAR) of the peptide ligand have been elucidated, the conformation-activity relationships have to be examined. For this purpose, a wide variety of physical/chemical methods are employed. For example, the three-dimensional arrangement of critical side chain groups and backbone conformations can be analyzed via NMR spectroscopy [44,45], X-ray crystallography [46], circular dichroism measurements [47], and computational methods [48]. The next step is to find a suitable non-peptide scaffold which can impart the same 3D topographic structural features as the peptide based on conformational analysis, computational chemistry and 3D modeling. The derived scaffold then can be used to replace the peptide drug pharmacophore and position the crucial structural elements correctly in 3D space. Finally, the

structure can be refined by evaluating the biological responses of designed ligands. The general scheme for the *de novo* design of peptide drugs has been outlined in a recent review [41]. Understanding the conformation-activity relationships of biologically active peptides can provide important guidance in the design of peptide drugs and accelerate the process from native peptides to biologically active peptide drug or to peptidomimetics and small molecules.

STRUCTURE-ACTIVITY RELATIONSHIPS OF LINEAR MSH-DERIVED PEPTIDES

The endogenous MC3R agonist: γ-MSH H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH [49] was discovered by homologous screening of the various core sequences of POMC that contain the critical tetrapeptide sequence -His-Phe-Arg-Trp- [50–51]. Biological screening demonstrated that γ-MSH was selective for the hMC3R by up to 100 fold, compared to α-MSH [49]. Grieco *et al.* used a *D*-amino acid scan of the γ-MSH sequence to obtain the first highly selective agonist for the hMC3R (H-Tyr-Val-Met-Gly-His-Phe-Arg-*D*-Trp-Asp-Arg-Phe-Gly-OH; $EC_{50} = 0.33$ nM; hMC4R/hMC3R = 300, hMC5R/hMC3R = 250) [43]. Furthermore, replacement of the oxidizable Met[3] residue with Nle increased the stability of the peptide [52], which is being widely used as a ligand for the hMC3R in a variety of ongoing pharmacological studies and in animal model studies related to the hMC3R. Interestingly, when the Phe[6] residue of γ-MSH was substituted with *D*-Nal(2′), a substitution known to convert α-MSH analogues to antagonists at the hMC3R and hMC4R [53], the resulting analogue (H-Tyr-Val-Nle-Gty-His-*D*-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH$_2$) exhibited potent hMC3R/hMC5R antagonist activity, while retaining potent full agonist activity at the hMC4R [54].

Hybridization of α-MSH with γ-MSH [52] proved to be an effective approach toward the design of selective hMC3R ligands, yielding a potent hMC3R selective antagonist (H-Tyr-Val-Nle-Gly-His-*D*-Phe-Arg-*D*-Nal(2′)-Asp-Arg-Phe-Gly-NH$_2$; $IC_{50} = 6$ nM), which also showed potent agonist activities at the hMC1R, hMC4R and hMC5R (Table 1).

GLOBAL AND LOCAL CONFORMATIONAL CONSTRAINTS IN THE DESIGN OF hMC3R SELECTIVE PEPTIDES

The conversion of the linear α-MSH into a cyclic peptide to enhance potency, metabolic stability, and to test hypotheses regarding a β-turn structure for the bioactive conformation of melanotropin ligands was an early innovation in the development of approaches to peptide ligand design. In the case α-MSH and its fragment analogues, the Cys[4],Cys[10](Cys[11]) analogues were examined over 25 years ago [55,56] and this evolved using computational methods, molecular dynamic simulations, conformational analysis via NMR and molecular modeling to the development of cyclic lactam analogues of α-MSH such as the truncated analogue Ac-Nle[4]-c[Asp[5],*D*-Phe[7],Lys[10]]α-MSH(4–10)-NH$_2$ (MTII) [57,58] which is used worldwide for the study of melanocortin 1, 3, 4 and 5 receptors for both *in vitro* and *in vivo* studies because of its high potency, metabolic stability, and excellent biodistribution properties [59]. More recently we have examined a number of other cyclization strategies to obtain melanocortin receptor selective ligands. As an earlier effort to develop highly selective and potent agonists and/or antagonists for the *h*MC receptors, a new group of linker arms and a backbone to side chain cyclization strategy were employed (Fig. 1). A key analogue was found to have the desired selectivity and potency at the *h*MC3 receptors which were implicated to play pivotal roles in energy homeostasis and other biological effects in this study. Structure-activity studies [60] have shown that replacing the succinyl linker arm of 1 by an *o*-phthalic acid group and substituting a *D*-Nal(2′)[7] residue in place of *D*-Phe[7] results in a potent antagonist 7 (Fig. 1) at the *h*MC4 receptor. Furthermore, increasing the 23-membered lactam ring of 1 by one carbon atom (succinic →glutaric acid linker) gives a highly selective and potent antagonist 9 for the

*h*MC3 receptor (Fig. 1). This cyclization strategy showed that a 23 membered ring is favored for maintaining good binding at all subtypes of melanocortin receptors [60]. Increasing the ring size or introducing an aromatic linker are likely to lead to more selective melanotropins. Thus, the selective MC3R antagonist: c[(O)C-(CH$_2$)$_3$-C(O)-His-*D*-Nal(2′)-Arg-Trp-Lys)]-NH$_2$ showed good biological activity as an antagonist with a pA$_2$= 10.6 and IC$_{50}$ = 5.6 nM (24 membered ring, (hMC4R/hMC3R = 38)) [60].

In another approach, the template of SHU-9119, Ac-Nle^4-c[Asp^5-His^6-*D*-Nal(2′)^7-Arg^8-Trp^9-Lys^{10}]-NH$_2$), a potent but non-selective hMC3R/hMC4R antagonist [53] was modified in position 6 with nonconventional amino acids (Fig. 2) [61]. The biological results of this series of compounds clearly indicate that a conformational restriction with bulky amino acids in position 6 in a cyclic peptide can be used to obtain selective antagonists for the hMC3R and the hMC4R (pA$_2$ = 9.1–9.8) [61]. Furthermore, modification of the MT-II template by aNle^4 → Xaa^4 substitutions (Xaa = aromatic amino acid) resulted in improved hMC4R/hMC3R selectivities, notably yielding a highly hMC3R-selective partial agonist PG-951 (H-*D*-Phe^4-c[Asp^5-His^6-*D*-Phe^7-Arg^8-Trp^9-Lys^{10}]-NF$_2$, IC$_{50}$ = 3.7 nM, EC$_{50}$ = 4.87 nM, 61% cAMP stimulation) (Table 1) [62].

Disulfide cyclization of the native γ-MSH around the pharmacophore sequence His-Phe-Arg-Trp provided no further leads toward the enhancement of hMC3R selectivity [54]. On the other hand, such cyclization in the α-MSH/β-MSH hybrid series yielded potent and highly selective hMC3R antagonists Ac-*c*[Cys-Glu-Pro-*D*-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH$_2$, and Ac-*c*[Pen-Glu-His-*D*-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH$_2$ (IC$_{50}$ = 31 and 3 nM, respectively) (Table 1) [63].

Introduction of the χ-constrained phenylproline analogues into the His^6 position of the MT-II template resulted in topographical changes that lead to increased hMC5R selectivity, which indicated that hMC3 and hMC4 receptors are more sensitive to steric effects and χ-conformational constraints than the hMC5 receptor [64]. Another example of using χ-constrained amino acids that lead to selective partial agonists and antagonists at the hMC3R was obtained by introducing *(o-Phe)*-Phe at the *D*-Phe position of MT-II. yielding a selective partial antagonist of the MC3R (Ac-Nle-c[Asp-His-(*o-Phe*)Phe-Arg-Trp-Lys]-NH$_2$) [65]. This is a typical example showing that introducing an alkyl or aryl group on the aromatic ring of an aromatic amino acid residue, particularly in the *ortho* position can significantly restrict its conformation in χ^2 space. The χ^2 torsional angle can be efficiently restricted by the interaction between the aryl moiety and the β-hydrogens of the amino side-chain in the *o*-substituted phenylalanine and finally increase the selectivity of melanocortin receptors.

USING NMR BASED MODELING AND COMPUTATIONAL METHODS FOR MELANOCORTIN RECEPTOR SELECTIVE LIGANDS

Computational methods in conjunction with NMR structural determination and molecular modeling are a very useful tool in peptide and peptidomimetic design considerations, with the caveat that for novel structures, current force fields might not give a completely accurate picture of the structure or energetics of the ligands. Along with the NMR structure of AGRP (the endogenous antagonist of melanotropin) (PDB: 1HYK) [66] and MTII (potent agonist) [67], the 3D pharmacophore of human melanocortin receptor ligands has been partially deciphered. Results from these biophysical and chemical studies can be crucial for designing novel selective agonist and antagonist melanotropins [52].

Recent conformation-activity relationship studies also have yielded some important structural clues for development of hMC3R-selective ligands. Thus, Ying *et al.* [67] determined that solution structures of the non-selective agonist MT-II, hMC3R/hMC4R antagonist SHU9119,

hMC4R-selective agonist VJH085 and hMC3R-selective antagonist MK-9 all feature (β-turn-like motifs spanning the His[6] and *D*-Phe/*D*-Nal(2′)[7] residues. This structural feature was hypothesized to be very important for receptor-ligand recognition and interaction. Grieco *et al.* had found that replacement of the His[6] residue in SHU9119 template with a variety of conformationally constrained amino acids leads to substantially improved receptor selectivity (Table 1) [61,68]. On the basis of these results, Grieco *et al.* suggested that the improvement in receptor selectivity was due to differences between the hMC3R and hMC4R binding pockets, which are able to accommodate amino acid residues with different conformational profiles.

Recently, our laboratories have produced several potent and selective hMC3R agonists and hMC3R/hMC5R antagonists by placing a bulky hydrophobic Nle residue next to the melanocortin pharmacophore Xaa-Phe-Arg-Trp in a cyclic γ-MSH-derived template (Fig. 3) [69]. The observed hMC3R selectivity was attributed to steric interference between the Nle[4] side chain and the Arg[7] binding space, based on MCMM-LMCS/OPLS-AA simulations, as well as relatively high rigidity of the 20-membered cyclic lactam template. Similar results were obtained when Nle was placed within the melanocortin pharmacophore, in position 6 of a cyclic α-MSH template, yielding an hMC3R antagonist cyclo (5β→10ε)-(succinyl[5]-Nle[6]-*D*-Phe[7]-Arg[8]-Trp[9]-Lys[10])-NH$_2$ (IC$_{50}$ = 84 nM) and an hMC3R/hMC5R antagonist cyclo(5β→10ε)-(succinyl[5]-Nle[6]-*D*-Nal(2′)[7]-Arg[8]-Trp[9]-Lys[10])-NH$_2$ (IC$_{50}$ = 12 and 17 nM, respectively: pA$_2$ = 8.3 (hMC3R) and 8.7 (hMC5R)) [70] These findings underscore the significance of steric factors in melanocortin receptor selectivity, and suggest that introduction of bulky residues in the direct proximity to the melanocortin pharmacophore is an effective approach to design of novel hMC3R-selective agonists and antagonists. Congruent findings were reported by Ballet *et al* for the MT-II/SHU9119 cyclic Lactam template, where the His[6]-Xaa[7] residues were replaced with the 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one-Xaa (Aba-Xaa) building blocks (Fig. 4) [71] resulting in a highly hMC3R selective antagonist Aba-2 (Ac-Nle[4]-c [Asp[5]-Aba[6]-*D*-Phe[7]-Arg[8]-Trp[9]-Lys[10])-NH$_2$, IC$_{50}$ = 50 nM) and a hMC3R/hMC5R antagonist Aba-4 (Ac-Nle[4]-c[Asp[5]-Aba[6]-*D*-Nal(2′)[7]-Arg[8]-Trp[9]-Lys[10])-NH$_2$, IC$_{50}$ = 43 and 87 nM, respectively) (Table 1). The authors hypothesized that the observed hMC3R receptor selectivity may be caused by either steric influence of the Aba block or its unique conformational features, or, possibly, both.

Deletion of His[6] from the sequence of cyclic α-MSH analogues, reported by Bednarek *et al.* [72], yielded a hMC4R-selective antagonist MBP10 (cyclo(6β->10ε)-(succinyl[6]-*D*-Nal(2′)[7]-Arg[8]-Trp[9]-Lys[10])-NH$_2$). It was suggested that the tripeptide core *D*-Nal(2′)-Arg-Trp was sufficient for high binding affinity toward the hMC4R, whereas the tetrapeptide core His-*D*-Nal(2′)-Arg-Trp is required for high binding affinity toward the hMC3R and hMC5R. Contrary to this hypothesis, our recent results [70] suggest that the tripeptide sequence *D*-Phe/*D*-Nal(2′)-Arg-Trp is sufficient for high binding affinity and agonist activity not only at the hMC4R but also at the hMC3R, as exemplified by cyclic peptides AVM75, cyclo(6β→10ε)-(2,3-pyrazinedicarbonyl[6]-*D*-Phe-Arg[8]-Trp[9]-Lys[10])-NH$_2$, and AVM76, cyclo(6β→10ε)-(2,3-pyrazinedicarbonyl[6]-*D*-Nal(2′)[7]-Arg[8]-Trp[9]-Lys[10])NH$_2$, which exhibit a potent and selective partial agonist (EC$_{50}$ = 27 nM, 70% cAMP stimulation) and antagonist (IC$_{50}$ = 23 nM) activities at the hMC3R, respectively (Fig. 5). These findings strongly suggest that the observed high receptor selectivities in 'tripeptide-pharmacophore' analogues are due to different conformational and steric properties of the linker arm, rather than different pharmacophore sequence requirements of the melanocortin receptor subtypes. Thus, manipulation of the linker structure is proving to be a powerful tool in the development of highly selective melanotropin peptides.

DEVELOPMENT OF hMC3R-SELECTIVE SMALL MOLECULE LIGANDS

Though many commercial pharmaceutical companies have been examining small molecules that can bind to the melanocortin receptors, surprisingly few of these ligands appear to have high potency and selectivity for the MC3R. It is worth noting that a number of publications of small-molecule research report little or no data for the MC3R.

One of the most popular strategies to emerge in the pursuit of small-molecule ligands for the melanocortin receptors has been the use of so-called "privileged structure" motifs, presumably biased towards GPCR activity, in combination with a *D*-tetrahydro-isoquinoline carboxyl (Tic)-*D*-*para*-chloro(*p*-Cl)-phenylalanine (Phe) dipeptide consensus sequence. A series of heterocycle-substituted cyclohexylpiperidine structures emerged from this work [73]. Interestingly, analogue 1 (Fig. 6) with *L*(*S*) stereochemistry at the *pCl*-Phe asymmetric center, has the highest affinity for the MC3R. Significant activation of the MC3R occurs only with the more common *D*-Tic-*D*-*pCl*-Phe dipeptide, found to be optimal for MC4R activity. The most potent ligand (2) is a partial agonist with an *N*-methyl substitution to the heterocycle within the privileged structure portion of the molecule.

A number of other putatively GPCR-biased privileged structures have been appended to the *D*-Tic-*D*-*pCl*-Phe sequence. Three examples of substituted arylpiperazines are shown in Fig. 7, but their affinities for the hMC3R are in the micromolar range [74–76].

Compounds with modifications to both the privileged structure and the dipeptide consensus sequence have been reported. Two examples are shown in Fig. 8 [77]. Interestingly, in this series only the analogue *N*-methylated at the II nitrogen of the imidazole shows no activity at the MC3R. This substitution is likely to change the orientation and hydrogen-bonding properties of this heterocycle, and illustrates the importance of the histidine or histidine-mimicking side chain in MC3R binding and activation.

The compounds in Fig. 9 provide further support for this assertion [78]. Varying the position of *N*-methylation in the His-mimicking piperazine produces the only analogue which significantly activates the MC3R.

Clearly the privileged structure approach has had a major impact on research efforts aimed at developing melanocortin-targeted small molecules. Molecular modeling provides a rationale for the activity of this molecular template, as the pharmacophore elements found in the small molecules appear to overlap with the side-chain functionality found in the key residues of bioactive peptide ligands [79].

A logical alternative approach to creating small molecule ligands for these receptors, then, is the use of the experimentally-derived structures of known active ligands to rationally design new molecules. In the first report of work employing this strategy [80], the solution structure of an active cyclic peptide ligand related to MT-II was first determined using NMR-derived constraints. Models of a variety of ring systems appended with the appropriate side chain functionality were overlapped with the peptide NMR structure, and compounds demonstrating the best overlap were synthesized. This study seems to indicate that in some compounds, changing or removing any of the pharmacophore elements will cause the complete loss of MC3R activity (Fig. 10). Generally, it illustrates the potential to generate active compounds by a ligand-based design approach.

Our own rational design of small molecules for the melanocortin receptors relied on a similar approach [81]. Instrumental to achieving this goal was the determination of an NMR solution structure for MT-II [67], against which potential new structures could be superimposed. Modeling indicated that our amino acid-derived scaffold could mimic the β-turn backbone of

the peptide, and facilitated the prediction of which positional and stereochemical isomers were most likely to be active. All members of a test set of eight compounds were found to be high-affinity antagonists, and most subtype-selective. In this series, there appears to be a significant similarity in the binding requirements for the MC1R and MC3R, as typified by the nanomolar binding of 13 to both subtypes (Fig. 11). Demonstrating the crucial nature of the stereochemistry of these ligands, inversion of the *C6* center to produce the diastereomeric 12 yields complete MC5R selectivity. This work further demonstrates that a ligand-based rational design approach may be a highly efficient means of producing hMC3R-active molecules.

More traditional approaches, such as the optimization of activity for a compound series originally derived from a screening hit, have also led to the discovery of novel ligands for the MCRs. An example of a pyridazinone-based molecule [82] derived in this way is shown in Fig. (12).

DATA BASE SEARCH FOR DRUG DESIGN

Computational tools to search chemical structure databases are useful for finding leads early in a drug discovery program. Similarity searches are among the most diverse and most useful, and have become a standard tool for target specific library design [83,84]. The underlying theory is that, given a compound with desired biological activities, compounds that are similar to it in structure are likely to have similar activity. In a common practice of focused library design, the investigator provides a set of chemical structures as a "probe" for searches of a database of compounds, and finds those that are most similar, and then submits them for testing. Similarity searches can be done on the basis of either 2D or 3D structure. 2D similarity searches are computationally very inexpensive and rapid; their popularity is related to the trend of applying high-throughput medicinal chemistry methodologies.

Successful approaches to the design of focused compound libraries have recently been reported for the melanocortin 4 receptor and other GPCRs [73,85–88]. They range from 2D simulation algorithms, to the analysis of ligand-receptor spatial arrangements and to neural network learning QSAR systems. With this variety of tools, it now appears possible to design libraries that are enriched in compounds possessing the desired target-specific properties.

THE hMC3R STRUCTURE AND RELATED ASPECTS OF LIGAND-RECEPTOR INTERACTION

Comparison of the human melanocortin receptor sequences revealed that most of the conserved amino acids are located within the putative transmembrane helices, with the most sequence deviations occurring within the C-terminus, first extracellular and third intracellular loops, whereas the other extra- and intracellular loops as well as the amino-terminus show regions of substantial similarity [89].

Chen *et al*. examined the significance of 19 transmembrane domain amino acids for molecular recognition and receptor signaling (Fig. 13) [90]. The hMC3R residues E131, D154, D158, and H298 were found to be homologous to the hMC1R residues E94, D117, D121, and H260, and to the hMC4R residues E100, D122, D126, and H264, respectively [91–93]. The existence of an ionic pocket formed by amino acid residues E131 in transmembrane domain 2 [TM2] and D154, D158 in TM3 of hMC3R and a second hydrophobic binding pocket consisting of F295, F296, and H298 in TM6 of hMC3R was hypothesized, as replacement of these amino acids with alanine resulted in dramatically reduced NDP-α-MSH binding affinity and agonist potency (Fig. 14). Furthermore, substitutions of D121 in TM2 and D332 in TM7 with alanine resulted in the complete loss of ligand binding, ligand induced receptor activation, and cell surface protein expression. These findings suggest that TM3 and TM6 are important for NDP-

α-MSH binding, while D121 in TM2 and D332 in TM7 are crucial for receptor signaling. Interestingly, unlike MC1R and MC4R, only TM6 of hMC3R seems to be involved in a hydrophobic binding pocket, as mutations of F216A, F233A, F234A, F288A, F290A, and F318A did not significantly alter NDP-α-MSH binding affinity and receptor activity. Tao and Segaloff also described the hMC3R mutation 1183N, found in patients with high fat contents, which completely abolishes agonist-mediated receptor activation [93].

POTENTIAL THERAPEUTIC USES FOR THE MC3R

Until the discovery and cloning of the MC3R, MC4R and MC5R receptors in the 1990s most of the work with POMC related peptides (β-endorphin not included) centered around the possible roles of α-MSH in pigmentation, pigmentary disorder, and melanoma cancer, and the role(s) of ACTH in adrenal function. Though much critical research still is ongoing in these areas, since then a large number of academic, pharmaceutical and biotechnology groups have been examining the role(s) primarily of α-MSH and related peptides, peptidomimetics and non-peptide ligands on the MC3R, MC4R and MC5R. It is not our intention here to review this rapidly expanding literature (see e.g. refs. [5,6] for examples of many areas under investigation). In this review we will concentrate on those aspects related to the MC3R.

In the last 15 years, major advances in our understanding of the regulation of body weight and energy homeostasis have been reported [59,94]. It is now widely understood that the disorders of leptin feedback or hypothalamic melanocortin signaling can lead to pathological weight gain and diabetes in humans [e.g. 95, 96]. Downstream of leptin signaling, the interplay between melanocortin peptides and neuropeptide Y (NPY) has been examined [e.g. 97] and it has been demonstrated that melanocortin peptide administration within the PVH leads to the regulation of feeding behavior as well as energy expenditure. The central melanocortin system modulates energy homeostasis through the actions of the agonist α-MSH, a POMC cleavage product, and the endogenous antagonist AGRP on the MC3R and MC4R [98,99]. The MC3R is primarily expressed in the brain in the regions of the hypothalamus and limbic system, as well as in the placenta and gut [100,101]. On the other hand, MC4R is widely expressed throughout the brain [15,102]. Additionally, the control of food intake has been suggested to be modulated through the MC4R [103], whereas the MC3R and MC5R have not been linked to any specific actions on feeding behavior. Several investigators [12,13,104] have attempted to solve this puzzle by studying MC3R-deficient mice and to comparing these mice with mice deficient for both the MC3R and MC4R genes. It was suggested that inactivation of the mouse MC3R leads to increased fat mass, reduced lean mass and higher feed efficiency than their wild type littermates. Furthermore, MC3R−/− mice also exhibited a unique metabolic syndrome leading to ≈50% increase in adipose mass, an unusual increase in respiratory quotient and reduced energy expenditure [13].

The role of MC3R also has been proposed for anti-inflammation as well as cardiovascular regulation. Getting *et al.* [105] have suggested that activation of MC3R by ACTH(4–10) suppresses cytokine release from microphages which then leads to the inhibition of inflammation. Furthermore, the MC3R is the only melanocortin receptor subtype for which γ_2-MSH is selective [106] which makes this receptor an obvious leading candidate for mediation of cardiovascular regulation [107,108] and inhibition of TRH release [109]. Recently, Marks *et al.* [110] have shown that peripheral injections of D-Trp^8-γ-MSH, a potent and selective MC3R agonist can stimulate food intake in mice which suggests that individuals affected with involuntary weight loss due to chronic diseases can be helped [111]. This is the first time such a physiological role for MC3R has been suggested where this receptor acts as an inhibitory autoreceptor of POMC neurons. Table 1 describes a number of potent and selective MC3R agonists and antagonists that might prove useful for the treatment of obesity, cachexia and cardiovascular, inflammatory, and other diseases.

CONCLUDING REMARKS

POMC is clearly a primordial gene whose presence is found in virtually all multicellular animals that have a central nervous system. From this perspective it perhaps is not surprising that the processed ligands from this gene and their target G-protein coupled receptor are involved in most aspects of human behavior, endocrine and central nervous system function. Though α-MSH, ACTH and some of the other processed peptides of POMC, especially from the pituitary gland, were among the first peptide hormones/neurotransmitters whose biological functions were evaluated in considerable detail at least with respect to melanopigmentary and adrenal function, it was the cloning of the receptors for these functions [i.e. MC1R and MC2R] and the additional three receptors (i.e. MC3R, MC4R, MC5R) that led to recognition of the central role of this system of ligands and receptors in numerous endocrine and CNS functions in normal health and disease. Though much of this research has focused on the MC4R and feeding behavior it has become increasingly clear that the MC3R is often as crucial as the MC4R in a variety of biological functions, and it seems clear that future research will have to evaluate the MC3R receptor system in much more detail, especially its relationships in various physiological and pharmacological situations where they and other neurotransmitter and hormonal systems are involved in health and disease. Clearly highly potent and selective ligands, both agonists and antagonists, will be needed to sort out the complex biology involved. Though we have developed some reasonably selective hMC3R ligands, there is clearly a great need for further development, and we plan to pursue this vigorously into the future.

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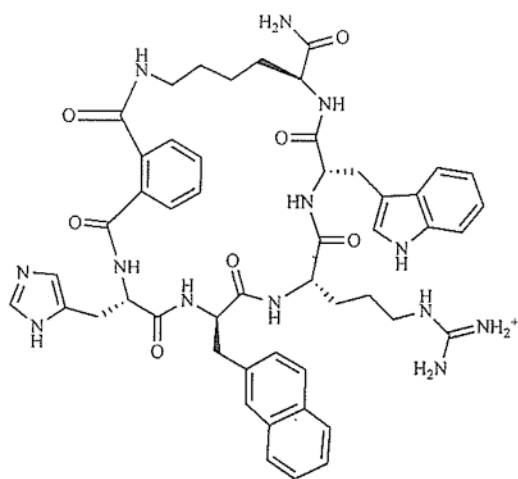
Peptide (7)
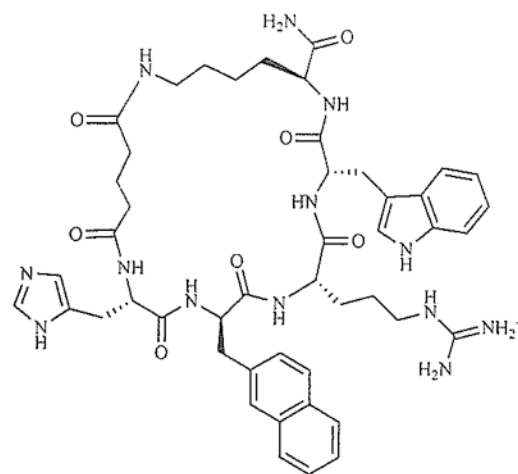
Peptide (9)

Fig. (1).
Key analogues of 1, Peptide 7 synthesized by introducing an *o*-phthalic acid in the cyclization linker of 1; Peptide 9 synthesized by replacing *D*-Phe[7] with *D*-Nal(2′)[7], and by replacing the cyclization linker of 1 by a glutaric acid linker [60].
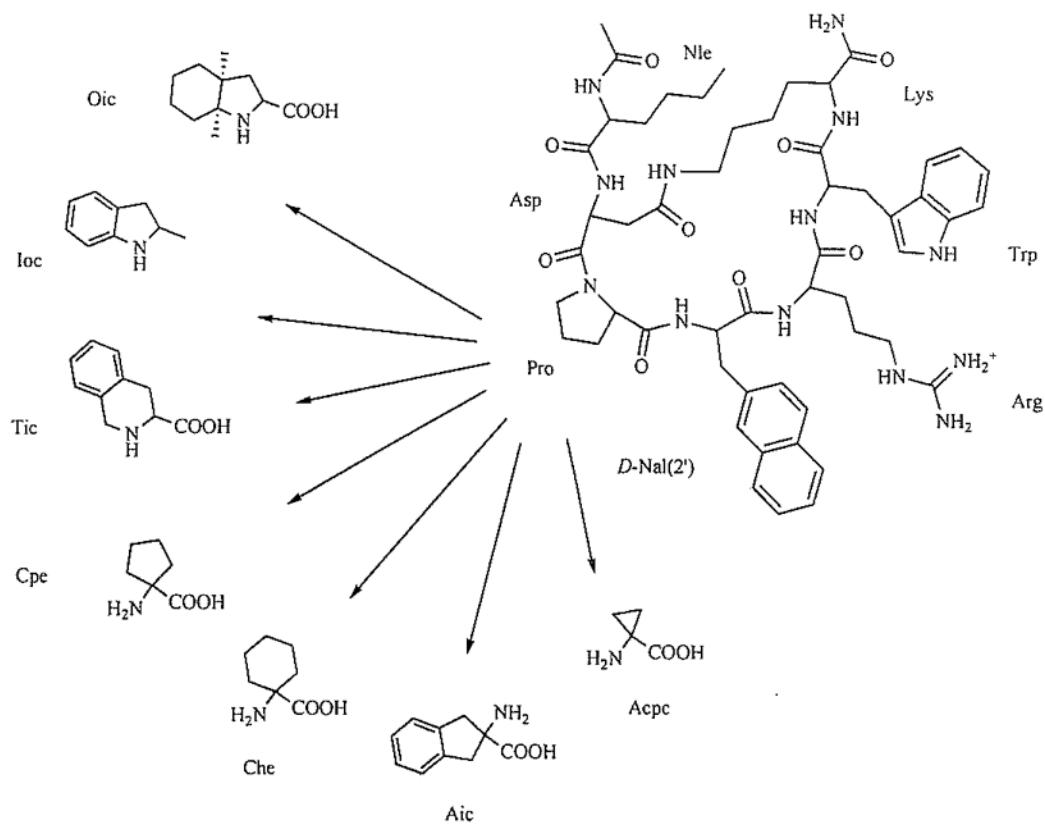
Fig. (2).
Structure of the potent monocyclic peptide Ac-Nle-c[Asp-Pro-*D*-Nal(2′)-Arg-Trp-Lys]NH$_2$, referred to as PG-901, and the conformationally constrained amino acids [61].
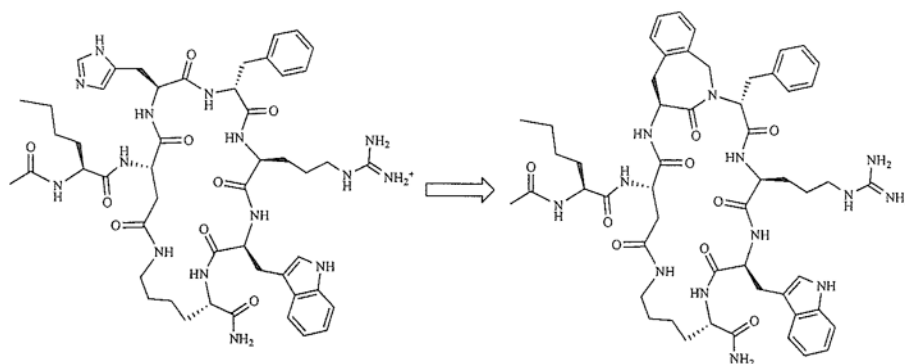
Fig. (3).
Design of the γ-MSH-derived cyclic lactam scaffold [69].

Fig. (4).
Design of the MT-II-derived Aba analogues [71].
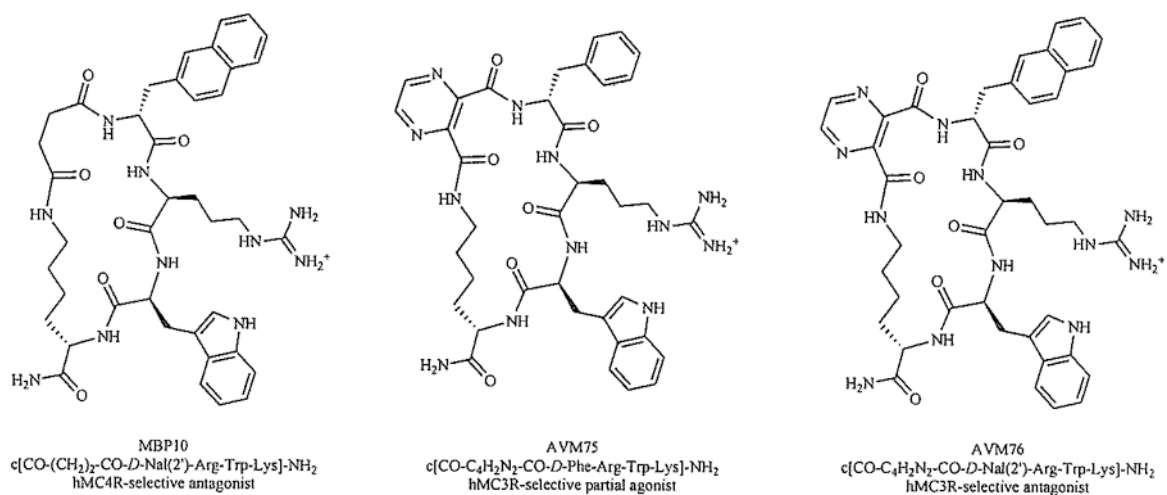
Fig. (5).
Development of highly selective 'tripeptide-pharmacophore' analogues *via* modulation of the linker arm [70,72].

IC_{50} (MC3R) / IC_{50} (MC4R) = 0.3

EC_{50} (MC3R) = 59 nM
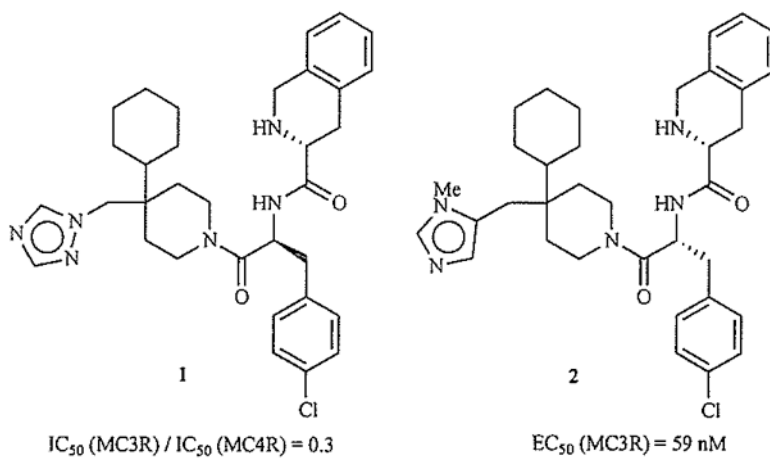
Fig. (6).
Most MC3R-selective (1) and potent (2) ligands from ref. [73].

Fig. (7).
Selected Arylpiperazines from refs. [74–76].

6

EC$_{50}$ (MC3R) = 1.7 µM

7

No activity at MC3R

Fig. (8).
N-Methylation changes functional activity [77].

No dose-response in range tested;
11% activation (cAMP) relative to a-MSH

EC_{50} (MC3R) = 2.9 μM;
19% activation (cAMP) relative to a-MSH
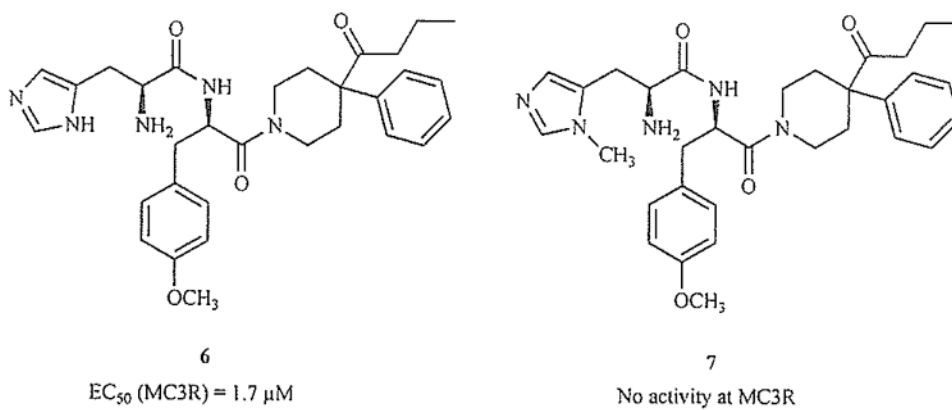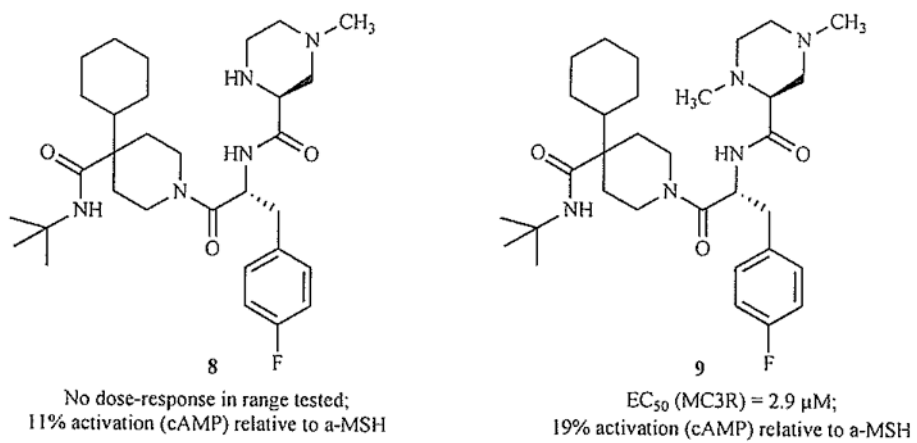
Fig. (9).
N-Methylation changes functional activity [78].
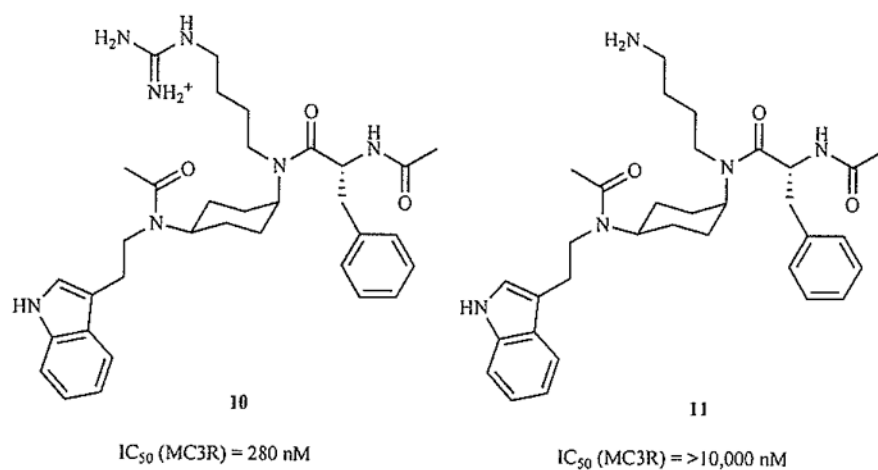
Fig. (10).
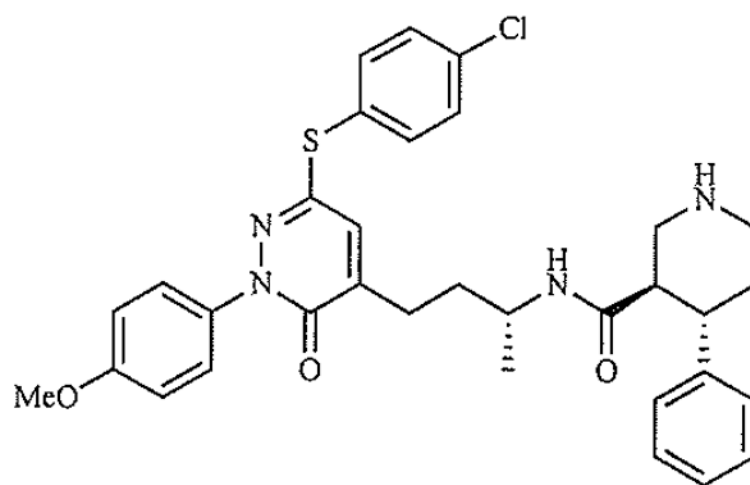Removing functional groups eliminates MC3R binding of rationally designed molecules [80].

IC$_{50}$ (MC3R) = >10,000 nM

IC$_{50}$ (MC3R) = 1.2 nM
IC$_{50}$ (MC1R) = 4.0 nM
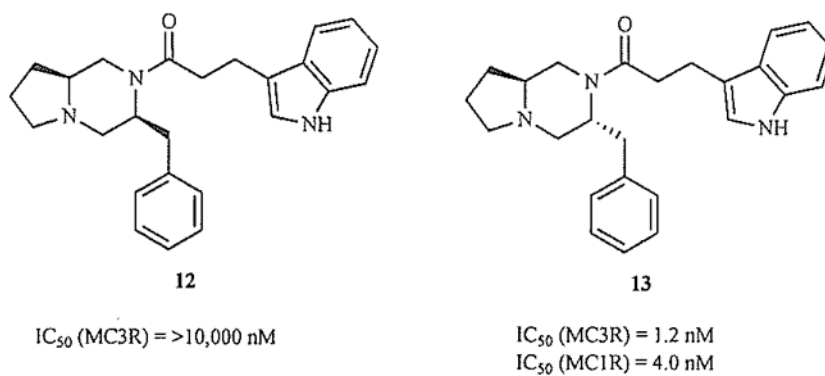
Fig. (11).
Stereochemistry crucial for MC3R activity in rationally designed molecules [81].

$$IC_{50} (MC3R) = 2.172 \ \mu M$$

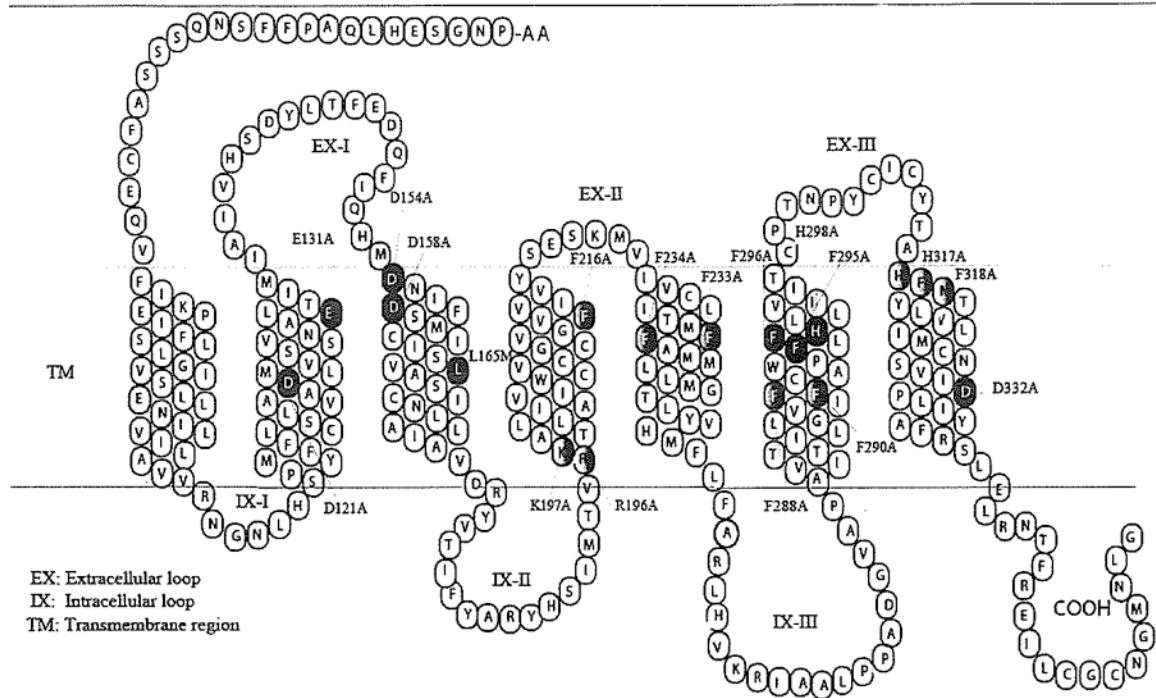**Fig. (12).**
Ligand optimized from screening hit [82].

Fig. (13).
Two-dimensional representation of the hMC3R sequence and secondary strucmre [90].
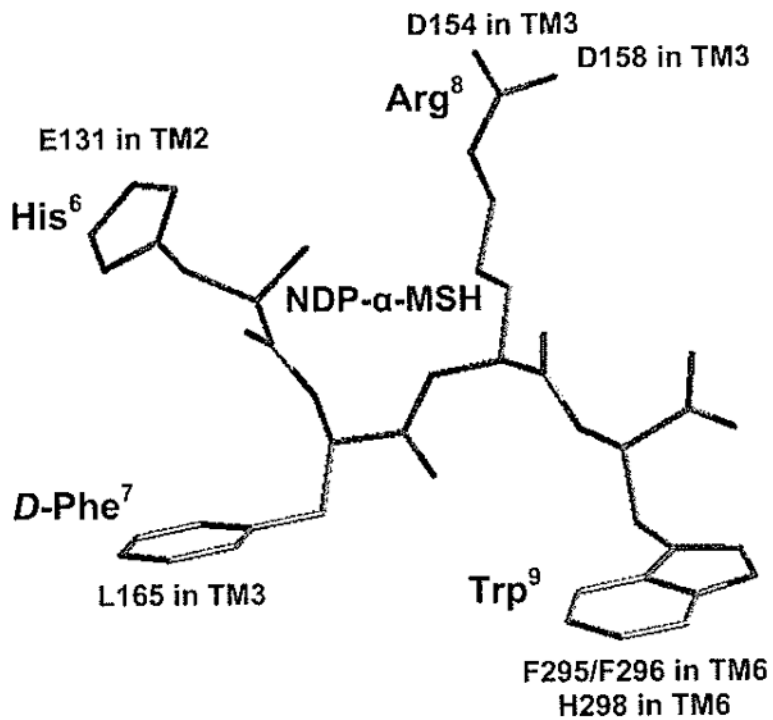
Fig. (14).
Two-dimensional representation of a proposed three-dimensional model illustrating the synthetic melanocortin NDP-α-MSH docked inside the hMC3R. Two receptor binding pockets are hypothesized, the first being a predominantly ionic pocket formed by D154 and D158, and the second is a hydrophobic pocket formed by aromatic residues in TM6 [90].

Table 1

Melanotropin Peptide Analogues Selective for Human Melanocortin-3 Receptor

a) Agonists/Partial Agonists

Ref.	Sequence	hMC1R			hMC3R			hMC4R			hMC5R		
		IC$_{50}$,nM	EC$_{50}$,nM	% max effect	IC$_{50}$,nM	EC$_{50}$,nM	% max effect	IC$_{50}$,nM	EC$_{50}$,nM	% max effect	IC$_{50}$,nM	EC$_{50}$,nM	% max effect
[43]	H-Tyr-Val-Met-Gly-His-Phe-Arg-D-Trp-Asp-Arg-Phe-Gly-OH	ND	ND	ND	6.7±1	0.33±0.01	100	600±70	100±11	99	340±40	82±10	97
[69]	c[Nle-Arg-D-Phe-Arg-Trp-Glu]-NH$_2$	1,400±100	>10,000	0	140±10	1.2±0.2	112	270±26	174±18	63	68.0±2	>10,000	0
[62]	H-D-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Lys)-NH$_2$	ND	ND	ND	3.7±0.05	4.87±0.5	61	700±100	>10,000	0	1,100±180	>10,000	0

b) Antagonists

Ref.	Sequence	hMC1R			hMC3R			hMC4R			hMC5R		
		IC$_{50}$,nM	EC$_{50}$,nM	% max effect	IC$_{50}$,nM	EC$_{50}$,nM	% max effect	IC$_{50}$,nM	EC$_{50}$,nM	% max effect	IC$_{50}$,nM	EC$_{50}$,nM	% max effect
[60]	c[(O)C-(CH$_2$)$_3$-C(O)-His-D-Nal(2')-Arg-Trp-Lys]-NH$_2$	ND	ND	ND	5.9±0.19	>10,000	0	220±13	1,200±200	22.8	26±1.4	1.01±0.09	81
[61]	Ac-Nle-c[Asp-Tic-D-Nal(2')-Arg-Trp-Lys]-NH$_2$	ND	ND	ND	6.7±0.6	>10,000	0	3.7±0.3	>10,000	0	1.24±0.13	2.05±0.2	102
[61]	Ac-Nle-c[Asp-Acpc-D-Nal(2')-Arg-Trp-Lys]-NH$_2$	ND	ND	ND	2.5±0.2	>10,000	0	270±30	>10,000	0	4.1±0.5	390±50	50
[63]	Ac-c[Cys-Glu-Pro-D-Nal(2')-Arg-Trp-Cy5]-Pro-Pro-Lys-Asp-NH$_2$	ND	ND	ND	31±10	>10,000	0	17,000	>10,000	0	97,000	>10,000	0
[63]	Ac-c[Pen-Glu-His-D-Nal(2')-Arg-Trp-	ND	ND	ND	3.0±0.5	>10,000	0	750±100	>10,000	0	94±11	>10,000	0

a) Agonists/Partial Agonists

Ref.	Sequence	hMC1R			hMC3R			hMC4R			hMC5R		
		IC_{50},nM	EC_{50},nM	% max effect	IC_{50},nM	EC_{50},nM	% max effect	IC_{50},nM	EC_{50},nM	% max effect	IC_{50},nM	EC_{50},nM	% max effect
	Cys]-Pro-Pro-Lys-Asp-NH$_2$												
[52]	H-Tyr-Val-Nle-Gly-His-D-Phe-Arg-D-Nal(2')-Asp-Arg-Phe-Gly-OH	0.7±0.1	2.0±0.2	87	6.0±0.5	>10,000	0	6.0±1	36±4	100	5.0±1	200±20	67
[69]	c[Nle-Val-D-Nal(2')-Arg-Trp-Glu]-NH$_2$	4,000	>10,000	0	1.7±0.1	>10,000	0	180±20	16±0.3	13	2.2±0.5	>10,000	0
[71]	Ac-Nle-c[Asp-Aba-D-Phe-Arg-Trp-Lys]-NH$_2$	>10,000	>10,000	0	50±6	>10,000	0	>10,000	>10,000	0	2,900 ±300	>10,000	0