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Bench-Top to Clinical Therapies: A Review of Melanocortin Ligands from 1954 to 2016

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Abstract

The discovery of the endogenous melanocortin agonists in the 1950s have resulted in sixty years of melanocortin ligand research. Early efforts involved truncations or select modifications of the naturally occurring agonists leading to the development of many potent and selective ligands. With the identification and cloning of the five known melanocortin receptors, many ligands were improved upon through bench-top *in vitro* assays. Optimization of select properties resulted in ligands adopted as clinical candidates. A summary of every melanocortin ligand is outside the scope of this review. Instead, this review will focus on the following topics: classic melanocortin ligands, selective ligands, small molecule (non-peptide) ligands, ligands with sex-specific effects, bivalent and multivalent ligands, and ligands advanced to clinical trials. Each topic area will be summarized with current references to update the melanocortin field on recent progress.

Keywords

selective ligands; small molecules; sexual dimorphism; bivalent/multivalent ligands; clinical trials

1. Introduction

The melanocortin system consists of five receptor subtypes, discovered to date, that are involved in numerous biological pathways. The melanocortin-1-receptor (MC1R), expressed in the skin, is primarily involved in pigmentation [1, 2]. The melanocortin-2 receptor (MC2R) is involved in steroidogenesis and is expressed in the adrenal cortex [2]. The centrally expressed melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R) are linked to energy homeostasis [3-9]. Additionally, the MC4R has a role in sexual function in humans [10, 11]. While the exact role of the melanocortin-5 receptor (MC5R) has not been elucidated [12, 13], it has been linked to exocrine function [14].

A variety of endogenous ligands interact with the melanocortin receptors (MCRs). The naturally occurring agonists, derived from the proopiomelanocortin (POMC) gene transcript

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[15], stimulate the receptors to increase intracellular cAMP levels. Unique to this GPCR family is the presence of endogenous antagonists, agouti (ASP) and agouti-related protein (AGRP) [16-18]. Additionally, AGRP has been demonstrated to possess inverse agonist activity (directly decreasing cAMP levels within a cell) at the MC4R in mice and humans [19, 20], while agouti has been shown to be an inverse agonist in cells expressing the grey squirrel MC1R [21].

Since changes in pigmentation can be readily visualized, early work on melanocortin ligands focused on the MC1R. The first reports of altered pigmentation dates back to 1916 [22, 23]. Significant advances were achieved with the identification, sequencing, and cloning of the MCRs from 1992 to 1994 [1, 2, 6, 7, 9, 12, 13], coupled with the development of 96-well plate cAMP assays [24]. The genetic information combined with assay platforms generated an experimental paradigm that allowed for the design, synthesis, and investigation of potent, selective compounds for the different receptor subtypes. Many pharmaceutical companies initiated melanocortin ligand programs following the discoveries that the MC4R was linked to food intake, energy homeostasis, obesity, and sexual function in humans [5]. Reports of cardiovascular side effects associated with MC4R ligands [25] coupled with an increase in mergers within the pharmaceutical industry led to diminished industrial interest in melanocortin ligands. However, melanocortin ligands have continued to be advanced to clinical trials.

Over a century of work has been published on the melanocortin receptors, and 60 years of reports focused on melanocortin ligands have resulted in numerous discoveries. As there are too many ligands to summarize succinctly, the scope of this review will focus on the following topics. The first section will review select classic peptide melanocortin ligands followed by a summary of recent advancements in selective ligands. Next, a discussion of small molecule (non-peptide) ligands will focus primarily on the MC4R. Ligands resulting in sex-specific effects will be summarized, followed by an update of bivalent and multivalent ligands. A final section will highlight melanocortin ligands advanced to clinical trials, emphasizing compounds described between 2011 and 2016.

2. Classic Peptide Melanocortin Ligands

Since the first reports of the sequences of adrenocorticotropic hormone (ACTH), β-melanocyte stimulating hormone (β-MSH), and α-MSH in the 1950s [26-28], numerous peptides and small molecule ligands have been developed for the MCRs. This section will focus on some author-perceived classic ligands. In particular, the naturally occurring ligands derived from the POMC gene transcript, the endogenous antagonists ASP and AGRP, and select synthetic derivatives of α-MSH (NDP-MSH, MTII, and SHU9119) will be highlighted (Figure 1).

2.1 Proopiomelanocortin (POMC) Gene Transcript

The endogenous agonists for the melanocortin receptors are all derived from the POMC gene transcript [15]. Cleavage of the pre-proopiomelanocortin polypeptide sequence by prohormone convertases (PC) generates the melanocortin agonist ligands α-MSH, β-MSH, γ-MSH, and ACTH, as well as other peptides including β-endorphin and β-lipotropin

[29-31]. Common to the endogenous melanocortin agonists is a His-Phe-Arg-Trp tetrapeptide sequence which is hypothesized to be the molecular recognition sequence for these ligands (Figure 1A). This sequence is the minimally active truncation product that possesses agonist activity in the classic frog and lizard skin bioassays [32, 33].

Since the endogenous agonists are derived from the POMC gene, the absence of the agonists in POMC-null individual has many effects on pigmentation (MC1R), steroidogenesis (MC2R), and weight gain (MC4R) [34-39]. Following the initial report of POMC-null humans, POMC knock-out (KO) mice were generated by removing the coding region for POMC derived peptides [40, 41]. Similar to the phenotype observed in POMC-null humans, POMC KO mice experienced hyperphagia (MC4R), altered pigmentation (MC1R), and hypocortisolism (MC2R). While it was initially reported that adrenal glands were absent in POMC KO mice [40], it was subsequently observed that POMC mice possess adrenal glands that are significantly smaller than adrenal glands found in wildtype mice [41-43]. An intraperitoneal injection of an exogenous synthetic melanocortin ligand was able to alter the weight gain and pigmentation changes observed in these mice [40]. Untreated, the absence of the POMC gene is fatal in humans [36], underscoring the many critical functions these endogenous ligands perform *in vivo*.

2.1.1 α-MSH—The α-MSH peptide is derived from the N-terminal 13 residues of ACTH (Figure 1A) and is highly conserved across mammalian species. Both termini of α-MSH are modified, with the N-terminal acetylated and the C-terminal carboxyamidated (Figure 1A). Acetylation of the N-terminal has been demonstrated to increase the stability of α-MSH compared to des-acetylated α-MSH [44, 45]. The full length peptide possesses nonselective sub-nanomolar to nanomolar potencies at the MC1R, MC3R, MC4R, and MC5R [46, 47]. Alanine scans of α-MSH have also indicated the importance of the Met^4, Phe^7, Arg^8, and Trp^9 positions for binding and functional activity at the mouse MC1R and rat MC3R [48, 49]. A 2016 report examining the cloned mouse receptors indicated that in addition to positions Met^4, Phe^7, Arg^8, and Trp^9, the Glu^5 and His^6 positions also affect functional activity [47]. Expression of α-MSH in the central nervous system is predominantly in the hypothalamus [50]. Expression of α-MSH is dispersed throughout the arcuate nucleus, however it is found more densely in the lateral regions where it is synthesized [50, 51]. Other locations of α-MSH expression include the dorsomedial nucleus of the hypothalamus (DMH), fibers in the medial preoptic, and the paraventricular, periventricular, and anterior hypothalamic nuclei [50, 52]. The ability of α-MSH to decrease food intake in rodents following intracerebroventricular (icv) administration and alter the skin/hair coloration of humans and small mammals when dosed peripherally demonstrate the importance of this ligand in the regulation of several important pathways [53-59].

2.1.2 β-MSH—The peptide β-MSH consists of 22 amino acids from the POMC gene transcript and is expressed in the hypothalamus [60]. Unlike α-MSH discussed above, β-MSH does not have modifications to either terminal position (Figure 1A). Rodents cannot produce mature β-MSH due to the lack of a di-basic cleavage site [31], although β-MSH has higher affinity at both the human MC4R and the rat MC4R when compared to α-MSH (30- and 4-fold, respectively) [61]. At the human melanocortin receptors, β-MSH has reported to

possess single digit nanomolar binding affinity at the MC1R, approximately 10-, 300-, and 12,000-fold higher affinity when compared to the MC3R, MC4R, and MC5R [62, 63]. It was reported that icv administration of β-MSH decreased spontaneous food intake in rats, and was as potent as α-MSH in rats that were fasted for 24 h [64]. However, a separate study showed that β-MSH had no significant inhibition of food intake after fasting for 48 h [57], perhaps due to the increased fasting time overwhelming the pharmacological response. In humans, β-MSH has also been shown to be important in the regulation of energy homeostasis. A missense mutation of the POMC gene transcript encoding region (Tyr221Cys) producing a nonfunctional β-MSH has been reported [65]. Obese children with the Tyr221Cys mutation experience hyperphagia and increased linear growth, similar phenotypes to those observed in MC4R-deficient individuals [65]. Another mutation in the POMC gene (Arg236Gly) generates a β-MSH/β-endorphin fusion protein which cannot activate the MC4R and results in a similar phenotype [66], underscoring the importance of β-MSH as a physiologically relevant melanocortin ligand.

2.1.3 γ-MSH—The N-terminal domain of POMC encodes for the three γ-MSH peptides, consisting of γ_1-MSH, γ_2-MSH, and γ_3-MSH (Figure 1A). The 23-residue *N*-glycosylated γ_3-MSH [67, 68] can be further cleaved to γ_2-MSH (N-terminal 12 amino acids of γ_3-MSH) and γ_1-MSH (N-terminal 11 amino acids of γ_3-MSH with a C-terminal carboxyamidate). An alanine positional scan of γ_2-MSH indicated residues Met^3, His^5, Phe^6, Arg^7, and Trp^8 were all functionally important for stimulation of the MC3-5R, similar to residues in α-MSH important for activity [69]. Differences in functional receptor selectivity have been observed between species, where γ_2-MSH possessed a 100-fold selectivity for the human (h)MC3R over the hMC5R [69, 70], whereas there was no potency difference between the mouse (m)MC3R and mMC5R [71]. When the activity of γ_2-MSH was compared in parallel between the MC1R, MC3R, MC4R, and MC5R, both the mouse and human MC1R and MC4R possessed similar sub-micromolar potencies [71, 72], although the potency of γ_2-MSH at the hMC3R [72] was approximately 100-fold lower than previous reports [9, 69, 70]. Expression of γ-MSH in the brain is predominantly in the pituitary and hypothalamic arcuate [73-76], but has also been reported in the adrenal medulla [77]. The greater than 10-fold selectivity of γ_2-MSH for the MC3R over the MC4R led to several investigations of the role the MC3R may play on food intake *in vivo* by administering γ-MSH ligands, with mixed results. While icv administration of γ_1-MSH did not inhibit food intake in rats after a 48 h fast [64], icv administration of γ_2-MSH in rats fasted for 48 h caused a significant, yet delayed inhibition of food intake [57]. In another report, icv administration of γ_2-MSH in rats fasted for 24 h yielded no effect on food intake [78], confounding the role γ-MSH peptides may play in regulating food intake. Additional studies of γ-MSH ligands have examined the role of this ligand on cardiovascular, sodium, and blood pressure regulation [79-81].

2.1.4 ACTH—Pro-ACTH is cleaved by PC1 in the anterior pituitary corticotrophs to produce ACTH(1-39) [82, 83], which can be further processed through PC2 to produce ACTH(1-13)NH_2 and α-MSH primarily in the pars intermedia of the anterior lobe of the pituitary [83]. As the only endogenous ligand known to stimulate the MC2R, ACTH is the only endogenous agonist that can stimulate all five melanocortin receptor subtypes [84].

While full length ACTH is 39 residues long, ACTH(1-24) is believed to be the molecular recognition domain while ACTH(25-39) is hypothesized to protect ACTH(1-24) from enzymatic degradation [85]. The N-terminal ACTH(1-24) possessed activity at the central melanocortin receptors, as a 4 μg dose of ACTH(1-24) injected via the lateral ventricle results in 70-80% decreased food intake in rats after a 24 h fast [86]. Direct injection of the same dose into the ventromedial hypothalamus also decreased food intake in free feeding rats during the nocturnal phase [86]. An anorectic effect was reported 4 hours after icv administration of ACTH(1-24) for doses as low as 0.05 μg/animal in mice and 10 μg/animal in rabbits [87]. Exogenous ACTH-mediated feeding inhibition is believed to be controlled solely by the central nervous system and not through peripheral feeding-regulatory pathways [88]. Doses up to 200 μg/kg of ACTH(1-24) administered subcutaneously in rats has no effect on feeding behaviors [88].

2.2 Agouti and AGRP

Unique to the melanocortin system is the presence of two endogenous antagonists, ASP and AGRP, the only naturally occurring GPCR antagonists discovered to date (Figure 1B). Full-length ASP consists of 132 amino acids and expression in the skin has been shown to affect pigmentation through MC1R antagonism [89, 90]. The active form of ASP has been hypothesized to be ASP(23-132), following cleavage of the N-terminal 22 residue signal peptide [91, 92]. The C-terminal domain possesses five disulfide bonds and was found to be equipotent to the full-length peptide [90], with an Arg-Phe-Phe tripeptide sequence shown to be critical for binding to the MC1R [93]. Ectopic expression of agouti due to a mutation at the agouti locus results in the lethal yellow strain of mice (A^y) [89, 94]. The constant antagonism of the MC1R is characterized by overexpression of the skin pigment pheomelanin, resulting in the observed yellow coat color [90, 94]. These mice are also characterized by increased weight gain and increased linear growth [95], characteristic of altered MC4R signaling. Indeed, ASP was found to be a competitive antagonist at the MC4R, but did not interact with the MC3R or MC5R [96]. In a subsequent publication, a synthetic C-terminal agouti fragment with two amino acid substitutions (Q115Y and S124Y, corresponding to the homologous residues in AGRP) was reported to antagonize the MC3R, as well as the MC1R and MC4R [97]. These substitutions to form agouti-YY were required for proper protein folding to generate sufficient quantities of the ligand for *in vitro* assays and NMR characterization [97]. In addition to the skin, ASP has also been found to be expressed in testis, ovary, and adipose tissue [98].

Similar to ASP, AGRP possesses a C-terminal domain with five disulfide bonds that is as active as the full length protein (Figure 1B), as well as an Arg-Phe-Phe tripeptide sequence shown to be critical for antagonist function [18, 99]. The C-terminal domains of agouti-YY, AGRP, and a truncated “mini-AGRP” have all been shown to possess similar structures by solution NMR [97, 100-102]. Despite these structural similarities, these antagonists possess different pharmacological profiles at the melanocortin receptor subtypes. While ASP has been shown to antagonize the MC1R, MC3R and MC4R, AGRP is not an antagonist at the MC1R but does interact with the centrally expressed MC3R and MC4R [17, 18]. Truncated and chimeric ASP-AGRP ligands indicated that the C-terminal loop of ASP was responsible for MC1R selectivity [103]. An additional difference between the antagonists is AGRP has

been demonstrated to be further processed into AGRP(83-132), the proposed functional form *in vivo* [104]. Expression of AGRP is primarily in the arcuate nucleus, the adrenal cortex, posterior hypothalamus, paraventricular nucleus regions of the brain [50, 98, 105]. Similar to the A^y strain in mice, ectopic expression of AGRP results in mice displaying hyperphagia and increased linear growth, purportedly to be due to MC4R antagonism [18, 106]. The orexigenic effect of AGRP(83-132) has been demonstrated to last up to 7 days [107, 108], indicating a long-term mechanism for inducing increased food intake.

2.3 Synthetic Ligands

Since the sequence of α-MSH was reported in 1957 [27], this peptide has been subjected to numerous structure-activity relationship studies, including the classic truncation and alanine-positional scanning experiments [32, 33, 47-49]. Although α-MSH possesses nanomolar to sub-nanomolar potencies at the MCRs, key discoveries led to synthetic compounds with increased potency, length of activity, and altered receptor pharmacological profiles.

2.3.1 NDP-MSH (Melanotan-I)—The [Nle^4,$DPhe^7$]α-MSH (NDP-MSH) ligand was reported to have enhanced potency, increased resistant to proteolysis, and increased duration of action relative to α-MSH in 1980 [109]. Two amino acid residues, Nle^4 and $DPhe^7$, differ between NDP-MSH and α-MSH (Figure 1C). The methionine to norleucine substitution in position 4 was selected since the methionine amino acid was reported to be prone to oxidation when attempting to radiolabel α- or β-MSH [110, 111]. This modification was shown to increase potency relative to α-MSH [111, 112]. The Phe^7 to $DPhe^7$ substitution was explored due to the observation that heat-alkali treatment of α-MSH enhanced activity [113], and the Phe^7 position was a major site of racemization [109]. Incorporation of the two modifications resulted in the NDP-MSH ligand, a sub-nanomolar, nonselective melanocortin receptor agonist. Truncation studies of NDP-MSH indicated an Ac-DPhe-Arg-Trp-NH_2 tripeptide sequence to be the minimally active fragment in both the frog skin bioassay and at the cloned MCRs [46, 114]. An alanine-positional scan of NDP-MSH reported decreased potencies when either the $DPhe^7$ or Trp^9 positions were substituted, indicating the importance of these two aromatic residues for the high potency of NDP-MSH [47]. Thirty-four years after its discovery, NDP-MSH was approved in the European Union as a treatment for adult erythropoietic protoporphria in 2014 [115].

2.3.2 MTII (Melanotan-II)—In 1989, a series of lactam cyclized α-MSH/NDP-MSH analogs were synthesized in order to develop more potent and prolonged-acting melanocortin ligands [116, 117]. Due to a hypothesized salt bridge between the Glu^5 and Lys^{11} of α-MSH/NDP-MSH based upon NMR and computer modeling, truncated ligands were cyclized through a lactam bridge between positions 5 and 10, maintaining the His-DPhe-Arg-Trp active tetrapeptide sequence of NDP-MSH. Truncation of three residues from both the N- and C-termini, in addition to Glu^5 to Asp^5 and Gly^{10} to Lys^{10} substitutions and subsequent lactam bridge formation resulted in MTII, a potent, non-selective melanocortin ligand with agonist activity at the MC1R, MC3R, MC4R, and MC5R [116, 117]. Since its discovery, MTII has been used as an *in vitro* and *in vivo* probe, with central icv administration of MTII inhibiting food intake in mice [5].

2.3.3 SHU9119—The MTII scaffold has been utilized in many structure-activity relationship studies to develop new melanocortin ligands with different selectivity and potency profiles. An early report substituted the DPhe of MTII with a DNal(2′) residue to generate SHU9119 (Figure 1C) [118]. The alteration of one residue changed the pharmacology of the resulting ligand: while SHU9119 maintained potent agonist activity at the MC1R and MC5R, it possessed sub-nanomolar antagonist potency at the MC4R and antagonist/partial agonist activity at the MC3R [118]. As the first peptide ligand discovered with potent antagonist activity at the MC3R and MC4R, icv administration of SHU9119 was shown to significantly increase food intake in mice [5, 108].

3. Selective Compounds

There have been a number of purported selective ligands published for the melanocortin receptor subtypes, with varying degrees of selectivity depending on the definition of the authors. A search of “selective melanocortin ligands” in PubMed (as of October 2016) yields 41 results from 2006 to 2016 alone, with many of these papers reporting a number of ligands. For these reasons, the current review is not meant to provide a comprehensive review of every selective melanocortin ligand, but to highlight selective compounds developed since 2006. For the purposes of this review, a selective compound must possess a 100-fold potency difference between at least two melanocortin receptors. This review will also only discuss ligands with potencies determined at the MC1R, MC3R, MC4R, and MC5R; the MC2R will not be discussed as it is only stimulated by ACTH.

3.1 “Selective” Melanocortin Agonists

In this section, agonists were selected for discussion when functional data (EC_{50} values) at the MC1R, MC3R, MC4R, and MC5R were determined. Ligands for which activity was determined at three or fewer receptors were not included. The search terms “melanocortin” and “melanocortin agonist” were used to search PubMed databases for papers containing agonist melanocortin ligands, focusing on ligands reported since 2006. Selective ligands (ligands with 100-fold differences in potency between at least two receptors) are listed in Table 1 and Table 2. Compounds were separated based upon whether they were assayed at the human (Table 1) or mouse (Table 2) melanocortin receptors. Ligands were additionally divided by selectivity for a particular receptor subtype. The same ligand may be selective for multiple receptors and are listed multiple times in the tables.

There have been a number of selective peptide, peptidomimetic, and small molecule ligands reported for the melanocortin receptors. Of the agonist compounds selective for the hMC1R (Table 1), one was selective for the hMC1R over the hMC4R [119], three were selective for the hMC1R over the hMC3R and hMC4R [119-121], two were selective for the hMC1R over the hMC3R and hMC5R [122], and three possessed at least 100-fold selectivity for the hMC1R over the hMC3R, hMC4R, and hMC5R [121, 123]. For agonist compounds selective for the mMC1R (Table 2), one ligand was selective for the mMC1R over the mMC3R [124], three were selective for the mMC1R over the mMC3R and mMC4R [124, 125], and one compound was at least 100-fold selective for the mMC1R over the three remaining receptors [126]. Of the ligands selective for the hMC1R or mMC1R, three were

based upon the linear structure of α-MSH [119, 123], four were substitutions of the MTII/SHU9119 scaffold [120, 121], five were small molecules [122, 124], and two were cyclic analogues of AGRP possessing a thioether heterocyclic [125, 126].

Unlike the other melanocortin receptors, selectivity for the MC3R has been more difficult to achieve. Two selective agonists have been reported for the hMC3R (Table 1): a partial agonist at the hMC3R was selective over the hMC4R [119] and one compound has been reported selective for the hMC3R over the hMC1R and hMC4R [127]. At the mMC3R (Table 2), one compound has also shown to be selective for the mMC3R over the mMC1R [47]. Of the MC3R selective compounds, two were derivatives of α-MSH [47, 119] and one based upon the MTII/SHU9119 template [127].

Perhaps due to the correlation between the MC4R and obesity, a number of selective ligands have been reported for this receptor subtype. At the hMC4R (Table 1), one compound has been reported for selectivity for the hMC4R over the hMC1R [128], one compound selective for the hMC4R over the hMC5R [128], one ligand for the hMC4R over the hMC3R and hMC5R [128], and two were at least 100-fold selective for the hMC4R over the remaining three receptor subtypes [121, 128]. At the mMC4R (Table 2), two compounds were selective for the mMC4R over the mMC3R [124, 129] and another ligand was reported 100-fold selective for the mMC4R over the mMC1R [130]. Of the selective ligands reported for the MC4R, five were small molecules [124, 128], two were based upon the His-DPhe-Arg-Trp tetrapeptide sequence [129, 130], and one was a derivative of the MTII/SHU9119 scaffold [121].

Investigations into the MC5R have also resulted in several selective ligands. At the hMC5R (Table 1), one compound was selective for the hMC5R over the hMC4R [119], three were selective for the hMC5R over the hMC3R and hMC4R [119-121], and one was selective for the hMC5R over the remaining three receptor subtypes [119]. At the mMC5R (Table 2), two compounds have been reported to be selective for the mMC5R over the mMC3R [124, 129]. Of compounds selective for the MC5R, three were derivatives of α-MSH [119], two were substitutions within the MTII/SHU9119 scaffold [120, 121], one was based upon the tetrapeptide His-DPhe-Arg-Trp [129], and one was a small molecule [124].

3.2 “Selective” Melanocortin Antagonists

Whereas agonists at the melanocortin receptor stimulate the production of cAMP, antagonists inhibit the ability of an agonist to stimulate cAMP production. Antagonists must therefore be assayed in the presence of an agonist, preferably at multiple concentrations of antagonist to generate a Schild analysis [131]. There are no studies which reported functional antagonist pA_2 values at each of the four melanocortin receptors investigated in this review. Therefore, publications were selected when pA_2 values were reported at a minimum of two of the four melanocortin receptors, and functional agonist data at the remaining receptors. For melanocortin receptor antagonists, selectivity was defined as a 100-fold difference in potency between the two melanocortin receptors assayed for antagonist activity. Since pA_2 values represent a log scale, compounds with pA_2 values different by two pA_2 units are 100-fold different. Search terms used for the analysis of melanocortin

antagonists in the PubMed database were “melanocortin” and “melanocortin antagonist,” focusing on ligands reported 2006 - 2016.

From these search parameters, three studies reported selective pA_2 values at two melanocortin receptors (mMC3R and mMC4R) and reported functional agonist EC_{50} values for the other two receptors (mMC1R and mMC5R, Table 3) [132-134]. For the sake of clarity, antagonists in Table 3 were divided into two sections: ligands with antagonist activity at the mMC4R but no agonist or antagonist activity observed at the mMC3R at concentrations up to 10 μM ($pA_2 < 5$) and antagonists that were active at the mMC3R and mMC4R. The antagonists reported by Doering *et al.* were derivatives of the tetrapeptide Ac-Trp-DPhe(*p*-I)-Arg-Trp-NH_2 [132]. Scaffolds reported by Ericson *et al.* were AGRP macrocyclic derivatives [133]. The antagonist reported by Lensing *et al.* possessed the structure Ac-His-DNal(2′)-Arg-Trp-(PEDG20)-NH_2. No ligands within the search parameters were MC1R, MC3R or MC5R selective antagonists. Interestingly, although most reported MC3R antagonists possess partial agonist activity at the MC3R, the peptides developed by Doering *et al.* and Ericson *et al.* did not possess partial agonist activity at the MC3R [132, 133].

4. Small Molecule Ligands

This section will focus on the development of small molecule melanocortin ligands. This topic has been previously reviewed in 2005 [135] and 2007 [136], so the current review will focus primarily on small molecules published since 2008. Numerous non-peptide small molecule ligands have been developed for the melanocortin receptors. In particular, the MC4R was heavily targeted by the pharmaceutical industry due to correlation of the MC4R with obesity [8]. Since MC4R agonists demonstrated an ability to decrease food intake in rodent models [5], small molecule MC4R agonists were investigated as potential therapeutics to promote weight loss. However, a clinical trial in 2009 indicated potent side effects including an increase in blood pressure with the use of the peptide agonist LY2112688 (Ac-DArg-c[Cys-Glu-His-DPhe-Arg-Trp-Cys]-NH_2) [25] and decreased the rate of MC4R-selective small molecule agonist development as anti-obesity treatments. The potential uses of MC4R agonists as pro-erectile agents or MC4R antagonists as weight gain therapeutics for cachexia patients have also been investigated [137-139].

While many different small molecule scaffolds have been reported, they can broadly be divided into two categories. One category originates from the selective small molecule ligand developed by Merck for the MC4R, a tetrahydroisoquinolone ligand THIQ. The other category consists of *de novo* designs or resulted from library screening of ligands not based upon the THIQ scaffold. This review will discuss both sets of molecules, beginning with the THIQ-based ligands. The focus will be on small molecule ligands that have been disclosed and functionally characterized in peer-reviewed publications. Molecules, including SNT207707, SNT209858, and BL-6020/979, that have been described but not pharmacologically characterized or which structures have not been clearly identified outside of the patent literature are not included [140, 141]. When describing the compounds derived from the THIQ ligand, the compounds are described in the approximate order of their publications.

4.1 Historic Overview of Small Molecule Melanocortin Ligands

The first small molecule ligands reported for the melanocortin receptors in 1999 were heterocycles containing a β-turn motif that possessed micromolar agonist potency at the MC1R, but were inactive at the MC3R and MC4R at concentrations up to 100 μM (Figure 2, **EL1** and **EL2**) [142]. In 2002, two groups reported small molecules possessing activity at the MC4R. Bondebjerg *et al*. reported a thioether scaffold that generated one ligand possessing sub-micromolar potencies at the MC1R, MC4R, and MC5R (Figure 2, **JB1n**) [143]. The first small molecule with single-digit nanomolar potency at the MC4R, THIQ, was described by Sebhat *et al*. (Figure 2, **THIQ**) [144]. Following these initial ligands, many additional compounds have been reported as described herein and in previous reviews [135, 136].

The postulated agonist pharmacophore of the endogenous melanocortin ligands is a His-Phe-Arg-Trp tetrapeptide. It has been shown that stereochemical inversion of the Phe to DPhe increased potency, as discussed in section *2.3.1 NDP-MSH (Melanotan-I)*. In efforts to generate a small molecule melanocortin ligand, a group at Merck noted the similarity between the melanocortin His-Phe-Arg-Trp sequence and the active core of the growth hormone secretagogue peptide GHRP-6 (His-DTrp-Ala-Trp) [144]. A clinical candidate was developed by Merck for GHRP-6 with a spiroindanyl piperidine (Figure 3) functioning as an Ala-Trp mimetic. A search of the Merck sample collection for similar compounds resulted in the optimization of the THIQ melanocortin-4 selective agonist (Figure 3), a nanomolar potent ligand with greater than 100-fold selectivity for the hMC4R over the remaining hMCRs [144]. Comparing the structures of His-DPhe-Arg-Trp and **THIQ** (Figure 3), it may be observed that the His is replaced with a constrained tetrahydroisoquinoline moiety in **THIQ**, DPhe by a 4-chlorophenyl ring, and the Trp may be in close proximity to the triazole heterocycle. Later modifications by Merck included replacing the 4-chlorophenyl with a 4-fluorophenyl to improve potency at the hMC4R, and potentially minimizing off-target effects by replacing the triazole with a *t*-butylamide group and substituting the tetrahydroisoquinoline moiety with a piperazine ring (MB243, Figure 3) [145]. Another key contribution from the Merck group was the discovery of the *t*-butylpyrrolidine containing MC4R-selective ligands (including MK0493, Figure 3) [146], a scaffold that is evident in many of the molecules presently reviewed. Through the successive generation of ligands, a piperidine core was maintained along with a halogenated phenyl ring (Figure 3). While many additional structure-activity relation studies and developments around these core structures have been reported, this review updates compounds disclosed since 2008 due to comprehensive reviews published from 2007 and earlier [135, 136].

4.2 THIQ-derived Small Molecule Scaffolds

Guo *et al*. reported a series of N-*tert*-butylpyrrolidine analogues (**1**, Figure 3), maintaining the 4-chloro phenylpiperidine ring of MK0493 with an elongated ethyl group replacing the methyl next to the acetamide [147]. In efforts to improve MC4R binding and functional activity, they investigated aromatic substitutions to replace the 2,4-difluorophenyl group. Both 4-chloro-2-fluorophenyl and 4-bromo-2-fluorophenyl substitutions increased affinity and agonist potency, indicating larger halogens in the 4-position may be beneficial [147]. In examining the affinity and functional effects of the *trans* diastereomers relative to the

pyrrolidine ring (**1**, stereocenters indicated by * and ** in Figure 3) of select compounds, they reported that the (*,** in Figure 3) *S,R* isomers acted as full agonists, while the *R,S* isomers maintained similar affinity but resulted in decreased cAMP production. This work resulted in several nanomolar ligands for the hMC4R [147].

Chen *et al.* from Neurocrine Biosciences, Inc. described a series of pyrrolidine derivatives possessing a piperazine ring in place of the piperidine ring of MK0493 (**2**, Figure 3) [148]. This series optimized a previous reported ligand set combining the pyrrolidine moiety of MK0493 with other work utilizing piperazinbenylamines [149]. Substitutions at the aromatic ring attached to the pyrrolidine, the nitrogen of the pyrrolidine, the 2 and 4 positions of the piperazinephenyl ring, and the benzylamine nitrogen were explored using binding affinity and agonist potency at the hMC4R. Off the pyrrolidine ring, a 4-chlorophenyl group possessed the highest affinity and potency [148]. For substitution at the pyrrolidine nitrogen, a tetrahydropyran derivative exhibited the highest affinity and potency. Similar to the work by Guo *et al.* [147] investigating the two *trans* pyrrolidine diastereomers indicated the (*,** in Figure 3) *S,R* compound possessed high agonist activity while the *R,S* ligand possessed high affinity but was unable to stimulate cAMP production. Relatively small differences in potencies were observed when either a 2-fluoro or 4-methyl phenylpiperazine was used. Further investigation at the benzylamine position indicated that attachment of a *N,N*-dimethylaminopropionic acid through an amide bond resulted in the highest binding affinity, potency, and efficacy (K_i = 1.0 nM, EC_{50} = 3.8 nM, 122 % max signal for α-MSH) and possessed 200-fold selective binding for the hMC4R over other receptor subtypes [148]. This compound was shown to dose dependently decrease food intake in diet-induced obese (DIO) mice [148].

Maintaining the 4-chlorophenyl group, the Neurocrine Biosciences group next investigated the pyrrolidine ring by substituting in tetrahydrothiophenes and tetrahydrofurans in efforts to develop more potent hMC4R antagonists for cachexia treatment (**3**, Figure 3) [138]. Additionally, they explored 4-chloro, 4-trifluoromethyl, 4-methyl, or 2-fluoro phenylpiperazine substitutions. As desired, none of the compounds significantly increased cAMP production. Select compounds possessed nanomolar binding affinity and were able to block α-MSH stimulated cAMP release (with IC_{50} values of 590 nM or higher) [138], indicating functional antagonism of the MC4R. Notably, none of the compounds significantly increased cAMP production.

A similar study by the Neurocrine Biosciences group modified the location of the pyrrolidine nitrogen, explored various substitutions in the 2 and 4 positions of both phenyl ring systems, varied the benzylamide substitution, and attached different carboxylic acids to the pyrrolidine nitrogen (**4**, Figure 3) [139]. Similar to the tetrahydrothiophenes and tetrahydrofurans (**3**) [138], this compound series was unable to stimulate hMC4R induced cAMP production even though several compounds possessed nanomolar binding affinities [139]. The most potent compound in this series possessed a 4-chlorophenyl group, a 4-methyl substitution at the phenylpyrrolinidine ring, and an urea off of the pyrrolidine nitrogen. This compound blocked α-MSH stimulated cAMP production with a IC_{50} value of 93 nM. Interestingly, the *cis*-isomers of the pyrrolidine substitution were found to impart

higher binding affinity, in contrast to the *trans*-isomers previously reported when the pyrrolidine nitrogen is in a different location (**4** versus **1** and **2**, Figure 3) [147, 148].

The group at Neurocrine also investigated a series of piperazinebenzylalcohols and related ketones and amine analogs without the pyrrolidine ring (**5**, Figure 3) [150]. An isopropyl group off of the benzyl position increased binding affinity, similar to the increased affinity for a benzylamine or cyclohexyl-carboxylate at a similar position [151, 152]. A trend for increased binding affinities for benzylamines over the benzylketones or benzylalcohols was observed. The benzylalcohols were all found to possess significantly more binding affinity for the hMC4R over the other hMCRs, though none of the compounds possessed agonist activity. A pharmacokinetic comparison between the most potent alcohol with the corresponding amine indicated similar trends, although the charged amine had slightly higher brain penetration than the uncharged alcohol [150].

With this same general scaffold, the group from Neurocrine also reported a series of analogues with an amine one methylene further removed from the phenylpiperazine rings (**6**, Figure 3) [153]. Due to previous enhancements in potency by incorporating a basic nitrogen from the phenylpiperazine ring, the group wanted to further remove the nitrogen further removed from this ring system. Several compounds were shown to possess sub-nanomolar binding affinities at the hMC4R, although their reported functional activity was greater than 100 nM [153].

Another non-pyrrolidine scaffold from the Neurocrine group probed the benzylamine position and substitutions on both aromatic rings (**7**, Figure 3) [154]. Ligands with 6-fluoro, 6-chloro, or 4-methyl substitutions in the phenylpiperazine ring were synthesized, all of which possessed nanomolar binding affinities. Insertion of 4-H, 4-methoxy, and 4-chloro in the halogenated ring resulted in similar affinities. Substitutions at the benzylamine position were also found to be beneficial. This series possessed minimal affinity at the hMC1R and was >40-fold selective for the hMC4R over the hMC3R. One compound evaluated in a mouse cachexia model was able to increase body weight in treated mice versus vehicle [154].

A group from Merck group also utilized a piperazine scaffold possessing a 2,4-difluorophenyl aromatic ring attached to the *N*-*tert*-butyl pyrrolidine (**8**, Figure 3) [128]. Bulkier aliphatic groups (*iso*-propyl, *tert*-butyl, or cyclohexyl) were incorporated off of the piperazine ring in a similar orientation to the cyclohexyl group of MB243, in addition to examining a variety of amine substitutions. Both cyclohexyl and *iso*-propyl substitutions increased potency relative to a *tert*-butyl group. Select amide substitutions resulted in nanomolar potent MC4R agonists with 10-fold selectivity for this receptor over other melanocortin receptor subtypes [128].

The Merck group also generated a series of analogs using a spiroindane motif attached to the piperidine ring (**9**-**12**, Figure 3) [155-158]. The spiroindanyl piperidine motif had previously been utilized in a growth hormone secretagoue project at Merck. It was developed to mimic an Ala-Trp functionality with improved pharmacokinetics parameters for the growth hormone program [144, 157, 159]. They hypothesized this motif may have similar beneficial

results in the MC4R ligand project. This scaffold initially maintained the 2,4 difluorophenyl group and *N-tert*-butylpyrrolidine elements of MK0493 (**9**, Figure 3). The stereochemistry of the two pyrrolidine ring substitutions was explored [157]. Similar to the pyrrolidine scaffolds with a piperazine (**2**) or piperidine (**1**) core [147, 148], a (*,** in Figure 3) *S,R* substitution possessed more potent binding and agonist potency at the hMC4R, although the *R,S* substitution in this scaffold still possessed agonist pharmacology with 10-fold decreased potency [157].

As a follow-up study, the Merck group utilized the spiroindanyl piperinde scaffold, but reversed the amide bond off of spiroindane (**10**, Figure 3) [158]. This modification allowed the rapid screening of many amines through amide bond formation with the carboxylic acid. The stereochemistry of the carboxylic acid attached to the spiroindanyl ring was demonstrated to be important (*** in Figure 3), as an S-conformation increased agonist potency and binding at the hMC4R. From this series, a number of ligands were generated which possessed sub-nanomolar agonist potencies at the hMC4R and were greater than 100-fold selective for the hMC4R over the hMC1R [158].

The Merck group continued to utilize the spiroindanyl piperidine core and examined positions 3 and 4 off the spiroindane phenyl group with additional substitutions off of the spiroinanyl piperidine, and pyrrolidine amine substitutions (**11**, Figure 3). The 3-chloro, 4-methyl substitutions were found to possess the highest affinity and agonist potency compared to a 3-fluoro, 4-fluoro or 3-fluoro, 4-chloro substitutions. Primarily a nitrile was used off of the spiroindanyl core, although morpholine or pyrrolinol substitutions further increased agonist potency to sub-nanomolar values. While the *N-tert*-butyl substitution at the pyrrolidine amine was the most potent and possessed the highest affinity, a 2-fold decrease in binding affinity and agonist potency was observed when the *N-tert*-butyl group was replaced with a tetrahydrofuran analog. The tetrahydrofuran substitution decreased lipophilicity and addressed an unpublished metabolic issue observed for the *N-tert*-butyl substitution [155]. Combining these data resulted in the 3-chloro, 4-methyl spiroindanyl substitution pattern with a nitrile group off of the spiroindanyl moiety coupled with a tetrahydropyranal amine substitution ligand that possess nanomolar potency at the hMC4R and was greater than 620-fold selective for the hMC4R over the hMC1R [155]. This ligand decreased food intake and bodyweight at 10 mg/kg orally dosed in DIO wt mice and had no significant effect in MC3R/MC4R KO mice [155].

An additional series from the spiroindanyl piperidine scaffold by the Merck group investigated the substitution of a heterocycle ring motif off of the spiroindanyl core, similar to the ring system observed in THIQ (**12** and **THIQ**, Figure 3) [156]. Additionally, they probed substitutions off of the pyrrolidine. Incorporating a cyclopentyl ring with a tertiary nitrogen attached to a methyl group and tetrahydropyran ring resulted in the compound with the highest affinity (0.43 nM) and potency (0.11 nM), and decreased food intake in a rat DIO model when orally dosed at 1 and 3 mg/kg [156].

A series of analogs possessing the *N-tert*-butylpyrrolidine core with an attached phenylpiperidine ring system were studied by Pfizer for potentially treating male sexual dysfunction. (**13**, Figure 3) [137]. A previous MC4R agonist developed by this group

possessing a 4-alcohol on the phenylpiperidine ring suggested small flanking substitutions on the C3 and C5 position of the piperidine ring had a beneficial effect, and was the basis for their structure-activity relation study. Probing substitutions on the aryl portion of the phenylpiperidine ring and substitution on the pyrrolidine nitrogen led to two compounds, a phenyl/*tert*-butyl or 4-fluorophenyl/*tert*-butyl substitution pattern possessing 12 and 18 nM agonist potency at the hMC4R and approximately 100-fold selectivity for this receptor [137]. The phenyl/*tert*-butyl compound was advanced into a human trial for male erectile dysfunction. A 200 mg dose demonstrated similar efficacy to sildenafil and demonstrated a significant effect compared to the placebo control [137].

As a final small molecule for the MC4R derived from the MB243 scaffold, the Merck group incorporated a substituted piperazine attached through a urea linkage while preserving the halogenated aromatic ring and *tert*-buyl/cyclohexyl substituted piperidine of **MB243** (**14**, Figure 3).[160] This series was designed to partially activate the receptor with the aims of decreasing food intake while not inducing an erectile response. The 4-chlorophenyl ring substitution did not possess activity at the hMC4R, and so a 4-fluorophenyl motif was used. A *cis*-methyl substitution at the C3 and C5 positions along with an unsubstituted nitrogen in the piperazine ring resulted in a 4.9 nM binding affinity compound with 22 nM agonist potency at the hMC4R with an efficacy of 59% relative to α-MSH. This compound was able to reduce body weight in an DIO rat model without causing an erectile response, indicating that it was possible to modulate weight without pro-erectile activities in rodents [160].

4.3 De novo designed/library screened small molecule scaffolds

In addition to small molecule melanocortin scaffolds with origins from the THIQ ligand, other novel scaffolds have been described. These *de novo* scaffolds were the result of serendipitous discoveries, screening GPCR privileged structures, or by adopting peptide structures into small molecule designs (Figure 4)

In a melanocortin program at Novo Nordisk for the treatment of obesity, a scaffold presenting four pharmacophores was synthesized to contain 384 random compounds from 41 building blocks [122]. While possessing a piperazine ring and aromatic substitutions (**15** and **16**, Figure 4), a cyclophane scaffold was discovered through a random screening approach. The structures of the lead ligands were also initially incorrectly assigned, as the active compounds were side reactions that occurred during syntheses, demonstrating the importance of resynthesizing and screening lead ligands compounds. Small molecules **15** and **16** possessed 12 and 16 nM agonist potency at the hMC1R, 700 and 470 nM agonist potency at the hMC4R, and were inactive at the hMC3R and hMC5R at concentrations up to 10 μM [122]. Compound **16** was also shown to dose-dependently decrease food intake in schedule fed male rats [122].

Another scaffold reported by Singh *et al.* incorporated an urea motif with an additional aromatic moiety and primary amine (**17** and **18** as examples, Figure 4) [161]. This was a follow-up to a previously synthesized library from the same group which reported a micromolar MC3R agonist that was unable to stimulate the MC4R [162]. A series of 27 analogues were synthesized which resulted in five compounds with micromolar agonist potencies at the MCRs [161]. A comparison of **15** and **16** to **17** and **18** shows both possess a

primary amine and aromatic moieties in approximately the same orientation, resulting in some similarities between these scaffolds.

Two groups have also reported small molecule melanocortin ligands based upon diazepine scaffolds, a privileged structure for other GPCR systems. Joseph *et al.* generated a series of 1,4-benzodiazepine-2,5-dione ligands [163], a scaffold previously reviewed to possess activity at the cholecystokinin receptor, opiate receptor, and antitumor properties [164]. The most potent of this compound set (**19**, Figure 4) resulted in sub-micromolar agonist activity at the MCRs [163]. Another diazepine scaffold was developed from a 1-4-dihydro-[1,4]diazepine-5,7-dione core, reported by Szewczyk *et al.*, after screening compounds from this series against numerous GPCRs [165]. Their analogs (**20**, Figure 4) possessed partial agonist activity (relative to α-MSH) at the MC1R and MC4R, with sub-nanomolar potencies at these receptors.

5. Sex-Specific Effects of Melanocortin Ligands

Various studies have reported a sexual dimorphism within the melanocortin system. An example is the differences in body weight gained between MC4R knockout male and female mice that are similar to the trends in human genetic epidemiology studies [8, 166-168]. Based on physiological observations of sex differences, it has been hypothesized that melanocortin ligands with sex-specific effects may be developed. This section will review published reports that directly compare the effects of melanocortin ligands in males and females on various physiological properties, summarized in Table 4. Non-pharmacological data is beyond the scope of this review, but has previously been examined [169].

Melanocortin ligands have utilized in basic and clinical research in both males and females for treating sexual function disorders [170]. Although the MTII derivative bremelanotide has shown efficacy in both males and females, bremelanotide is currently only in clinical trials for female sexual desire disorder (For structure, see Figure 6 below) [170-173]. To the authors knowledge, the decision to focus on females for therapeutic development is the first clinical attempt in utilizing sex differences of melanocortin ligands. The differential effects of melanocortin ligands on sexual function in males and females has been extensively reviewed [170, 171, 174], and will not be discussed in further detail herein.

In 1977, Beckwith and coworkers reported melanocortin ligands had differential effects on male and female rats [176]. Neonatal administration of α-MSH in male rats resulted in better performance on learning tasks as adults than control animals, an effect not observed in female rats [176]. This group also demonstrated sex differences in response to neonatal administration of α-MSH in an open-field test. Male rats treated with α-MSH demonstrated enhanced effects at 45 days old and 120 days old, but significant effects were observed only at 45 days old in female rats [175]. In more recent studies, neonatal administration of melanocortin agonists to prairie voles affects social behaviors in a sex-dependent manner [177]. Daily neonatal peripheral injection of MTII reduced juvenile play behaviors in males, but not females. In contrast females receiving daily neonatal injection of MTII displayed enhancements in partner preference after non-mated cohabitation with males, which was not observed in males after cohabitation with females. Classical melanocortin agonist responses

were observed in both prairie vole sexes after MTII administration, including darkened pigmentation (MC1R activation) and reduced body weight (MC3R/MC4R activation) [177].

Another established line of melanocortin sexual dimorphism has focused on the role of the MC1R in meditating a female-specific mechanism of κ-opioid analgesia [178-180, 184, 185]. Agonist κ-opioid analgesia could be blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonism in males, but not females [186]. It has been postulated that female mice use a MC1R-mediated pathway instead of the NMDA receptor [178, 180, 184, 187]. It was demonstrated that icv administration of the melanocortin ligands Ac-Nle-Asp-Trp-DPhe-Nle-Trp-Lys-NH_2 (pA_2=8.4 in the frog skin assay, IC_{50} = 260, 60, and 910 nM at the hMC3R, hMC4R, and hMC5R) respectively or c[Gly-Cpg-DNal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (K_i = 53 nM at the hMC1R by Schild analysis, IC_{50} = 12, 44, and 1300 nM at the hMC3R, hMC4R, and hMC5R, respectively) potentiated κ-opioid analgesia in female but not male mice [178, 188, 189]. The presumed melanocortin antagonism rendered females sensitized to blockage of κ-opioid analgesia by a NMDA receptor antagonist similar to male mice [178]. These melanocortin pharmacological studies were supported by studies in mice and humans that lack functional MC1R, implicating the role of the MC1R over the other melanocortin receptor subtypes in the analgesic response [178, 185, 187]. Similar to rodent studies, women with mutations in the MC1R displayed greater analgesic responses to pentazocine on thermal and ischemic pain stimuli compared to women or men with no variant MC1R [178]. Ovariectomized (OVX) female mice possessed NMDA antagonist sensitivity while treatment with estrogen or progesterone to OVX mice reinstates NMDA resistance, implicating sex hormones for this effect [184, 186, 190, 191].

Further studies on the icv administration of a presumed MC1R antagonist MSG606 (c[$(CH_2)_3$CO-Gly-His-DPhe-Arg-DTrp-Cys(S-)]-Asp-Arg-Phe-Gly-NH_2) demonstrated this ligand reversed morphine hyperalgesia in female mice, but had no effect in male mice in two different strains (CD-1 and C57BL/6J) [180]. The MSG606 ligand was reported to be have IC_{50} = 17, 3900, >10000, and 1100 nM, and EC_{50} = >10000, 59, >10000, and 1300 nM at the hMC1R, hMC3R, hMC4R, and hMC5R, respectively [192]. However, MSG606 was not fully functionally characterized as a competitive MC1R antagonist by a traditional Schild analysis. In 2015, it was observed that both icv and intrathecal (i.t.) administration of MSG606 or a NMDA receptor antagonist in mice followed the same sex-specific pattern as previously observed. Administration of MSG606 reversed morphine-induced hyperalgesia in females, but not males. Antagonism of the NMDA receptor reversed morphine-induced hyperalgesia in males, but not females. Ovariectomized female mice were sensitized to NMDA receptor antagonism, but not to MSG606 administration. Progesterone treatment administered to OVX females re-sensitized them to both icv and i.t. administration of MSG606 to reverse morphine-induced hyperalgesia [179]. Male mice treated with progesterone were sensitized to icv administration of MSG606 to reverse morphine-induced hyperalgesia, but not to i.t. administration [179]. While these studies suggest mMC1R activation may be responsible for the algetic effects, all of the ligands utilized bind or activate multiple melanocortin receptor subtypes. Studies with selective functionally confirmed MC1R antagonists will be necessary to fully understand the pharmacological effects in relation to the other melanocortin receptors, especially as the MC4R has also been implicated in analgesia [193-195]. To date, pharmacological and genetic data support the

hypothesis that males primarily use the NDMA receptor pathway and females use a melanocortin pathway, although both sexes can compensate with the other pathway depending on sex hormone levels [184].

Melanocortin ligands have been suggested to differentially affect energy homeostasis in males and females. For example, AGRP was reported to reduce energy expenditure (measured by vO_2) in female rats more than in male rats [181]. Removal of the gonads attenuated the observed differences in energy expenditure in females, suggesting sex hormones mediated the effect. Following icv administration, AGRP induced significantly elevated feeding for 5 days in males compared to only 3 days in female mice [181]. Lensing *et al*. reported a tetrapeptide, Ac-Trp-DPhe(*p*-I)-Arg-Trp-NH_2, that displayed sex-specific metabolic responses in mice [182]. The Ac-Trp-DPhe(*p*-I)-Arg-Trp-NH_2 peptide possesses micromolar antagonist potency at the mMC3R (pA_2 = 5.4) and nanomolar antagonist potency at the mMC4R (pA_2 = 7.8) [132]. This ligand is also a micromolar agonist at the mMC1R (EC_{50} = 2000 nM) and mMC5R (EC_{50} = 2800 nM) [132]. A 7.5 nmol icv administered dose increased food intake, increased respiratory exchange ratio (RER), and trended towards decreasing energy expenditure in male mice, but had minimal effects in female mice. A 2.5 nmol dose significantly increased food intake, RER, and energy expenditure in female mice, but had minimal effects in males at this dose. These data suggest that Ac-Trp-DPhe(*p*-I)-Arg-Trp-NH_2 could be used as a sex-specific probe *in vivo* to study the underlying mechanisms of the melanocortin sexual dimorphism as it related to energy homeostasis [182]. From these results, it may be hypothesized that melanocortin ligands have different therapeutic windows in males and females, with clinically relevant implications. However, there have been several reports indicating no ligand differences on energy homeostasis between male and female rodents [196-199].

A surprising sex-specific effect was observed in 2014, when icv administration of SHU9119 resulted in no significant effect on blood pressure in young or old female rats, but reduced blood pressure in male rats [183]. Food intake increased in all mice after SHU9119 administration, signifying MC3R/MC4R antagonism in both sexes [183]. This suggests ligands may be designed with ideal pharmacological profiles for therapeutic use in only one sex. For example, melanocortin anti-obesity therapeutics may be developed that decrease food intake but lack cardiovascular side effects in females.

Overall, it appears melanocortin ligands may differentially affect males and females (Table 4). However, more research will be necessary to exploit the sexual dimorphism for sex-specific therapeutics. Establishing whether the sex-specific effects are ligand dependent or present with all ligands with similar *in vitro* pharmacologies and the underlying causes (including melanocortin receptor, POMC, AGRP or other gene expression levels, changes in neuron connectivity, sex hormones differences, or other system wide changes) of the sexual dimorphisms will aid in the development of sex-specific ligands.

One current difficulty discovering these ligands is that no current *in vitro* technology is capable of detecting melanocortin sex-specific properties. Therefore, detection of ligands with sex difference relies on *in vivo* experimental paradigms, presenting no opportunity to optimize ligands prior to more extensive animal studies. The development of *in vitro* assays

that can detect sex differences may represent a major advancement in the design of melanocortin ligands with sex-specific effects. Melanocortin ligands with pre-established sex-specific effects will be necessary to validate that the *in vitro* assays developed are physiological relevant and correlate with the *in vivo* pharmacology. Important considerations include: 1) reporting the sex of the cell lines used *in vitro* [200], 2) studying pharmacological effects in both males and females, and reporting null effects if observed only in a single sex, 3) clearly reporting the sex of animals used in individual experiments, especially when multiple paradigms are performed in a single manuscript, and 4) when combining data that is not significantly different between males and females, reporting the sexes separately in the supplemental materials or the number of females and males used within a data set. These suggestions may allow the development of ligands with sex-specific effects that possess reduced side effects and potential therapeutic applications in one sex.

6. Bivalent and Multivalent Melanocortin Ligands

Bivalent and multivalent ligand design strategies targeting the melanocortin receptors have often been utilized in order to achieve high affinity ligands. These strategies lower the entropic cost of binding by allowing multiple binding interactions per ligand resulting in cooperative binding affinity (Figure 5 A-C) [201-206]. These probes may take advantage of the aggregation or “clumping” of multiple receptors together on the cell membrane (Figure 5 D-E). Recent studies have suggested the presence of melanocortin receptors dimers (or higher-order oligomers) for every known melanocortin subtype [207-214]. Furthermore, radiolabeled ligand binding studies suggest that there are two tandem binding sites with different binding properties on cells expressing melanocortin receptors, indicating targetable dimers [215, 216]. Bivalent and multivalent ligands may therefore preferentially interact with melanocortin receptor dimers or higher-order oligomers.

One difficulty in understanding the pharmacology of bivalent and multivalent ligands is discerning whether the effects are from a cooperative synergistic binding mode or due to the effect of increasing the pharmacophore concentration without synergy effects. Large increases in binding affinity compared to the monovalent controls (>10-fold) are hypothesized to be due to a cooperative synergistic bivalent or multivalent binding mode (Figure 5). While bivalent ligands have been shown to enhance binding affinity, rarely do additional pharmacophores beyond two result in further potency gains [205, 217].

Melanocortin multivalent ligands were first reported in 1977 [218]. Eberle *et al.* coupled α-MSH peptides (six to several hundred) to albumin, thyroglobulin, and tobacco mosaic virus which resulted in 1500-fold higher potency than α-MSH alone [218, 219]. Bivalent ligands were first reported in the clinic by Barb *et al.* as diagnostic tools [220-223]. Since melanoma cells often express elevated levels of the MC1R, it was hypothesized that ligands with high MC1R affinity could be conjugated to dyes or other labels and utilized as imagining or diagnostic tools [224-228]. These high affinity ligands might also be used to deliver therapeutics selectively to melanoma cells. However, this targeting strategy has been criticized because stimulation of the MC1R has been shown to increase melanocyte proliferation, and potentially could lead to melanoma growth [227, 229]. The use of multivalent melanocortin ligands as diagnostic tools has been reviewed previously

[224-228]. Therefore, this review will focus on updating the use of bivalent and multivalent ligands as imagining tools. Bivalent ligands containing two pharmacophores will be reviewed first, followed by multivalent ligands possessing more than two pharmacophores.

6.1 Melanocortin Bivalent Ligands

Bivalent ligands are the simplest form of multivalent ligands featuring two pharmacophores separated by a linker or spacer. The two pharmacophores are intended to target two different binding sites. A subclass of bivalent ligands is bitopic ligands, which target both an orthosteric and an allosteric binding site on the same receptor. To the authors' knowledge, there are no known bitopic melanocortin ligands. Therefore, in this review the term bivalent ligand will be used exclusively for ligands which target two orthosteric binding sites on two different receptors.

Bivalent ligands for various GPCRs have been demonstrated to have unique effects compared to their monovalent counterparts. In particular, bivalents ligands are uniquely poised to study GPCR dimerization [202, 230-232]. Carrithers and Lerner developed a series of homobivalent (containing two of the same pharmacophores) melanocortin ligands in 1996 to target two separate melanocortin receptors by crosslinking them. They utilized either an agonist pharmacophore based on α-MSH or the pharmacophore Met-Pro-DPhe-Arg-DTrp-Phe-Lys-Pro-Val tethered by a poly-lysine linker [233, 234]. They demonstrated that the agonist bivalent ligand increased functional activity 5- to 7-fold. In the frog melanocyte dispersion assay, the Met-Pro-DPhe-Arg-DTrp-Phe-Lys-Pro-Val based monovalent ligand was reported to antagonize α-MSH mediated dispersion in a dose-dependent manner. At high concentrations, the bivalent based on Met-Pro-DPhe-Arg-DTrp-Phe-Lys-Pro Val resulted in an agonist functional response [233]. This was the first report demonstrating improvement in functional activity with melanocortin bivalent ligands targeting putative dimers.

After the above report, homobivalent ligand development primarily focused on increasing the binding affinity at the hMC4R through various bivalent ligand design strategies [134, 201, 205, 217, 235-242], with some reports focusing on the MC1R and *in vivo* imaging [134, 243-245]. While high affinity ligands are desirable for biological responses, the use of low affinity pharmacophores in bivalent ligand design allow the greatest detection of synergistic binding [201, 204, 206, 217, 235, 236, 239, 243]. Therefore, analogs with lower initial monovalent binding affinities result in the greatest observable fold enhancements via a bivalent design strategy. Research programs primarily utilized different analogs of the agonist NDP-MSH. These included the tetrapeptide His-DPhe-Arg-Trp [134, 201, 205, 217, 235, 236, 238, 240-242], six residue analogs[235, 244, 245], seven residue analogs [237, 239, 243], and full length NDP-MSH [201, 236, 238]. Antagonist analogs in which the DPhe was replaced with DNal(2′) have been utilized to produce antagonist analogs with increased binding affinity [134, 239]. There has also been one report in which a melanocortin agonist pharmacophore was attached to an antagonist pharmacophore via a linker that also resulted in increased binding affinity [239].

Linker optimization has been a primary goal of melanocortin homobivalent ligand design. The optimal linker length must be long enough to bridge or crosslink two receptors, but not

too long to eliminate entropic gains. Various linker systems have been incorporated, including poly-lysine [233, 237], polyethylene glycol [134, 235, 237-239, 241], Ala-Gly [235], Pro-Gly [134, 235, 237, 239, 240], rigid amino acids [236], squalene [201], glycerol [241], D-mannitol [241], phloroglucinol [242], tripropargylamine [242], 1,4,7-triazacyclononane [242], others [205, 217, 243], and mixtures of these different linker systems together. Improper linker design may result in some increased binding affinity (<10-fold) that can be attributed to simply doubling the pharmacophore concentration [235-237]. Greater fold enhancements (>10-fold) are observed with linkers that appear to bridge two receptors resulting in cooperative synergistic binding (Figure 5A-C). An optimal linker length of approximately 23 ± 5 Å has been suggested by multiple studies at the hMC4R [217, 237, 239, 241, 242].

An assumption in the field was that a bivalent ligand optimized for the MC4R would be effective as a diagnostic tool for melanoma (that highly expresses the MC1R), as long as a non-selective pharmacophore was incorporated. As a result, the majority of studies only investigated *in vitro* pharmacology using HEK293 cells overexpressing the hMC4R, which resulted in ligands optimized for the hMC4R. In contrast, Lensing *et al*. reported the linker systems, independent of the binding pharmacophore, displayed preferential patterns for different melanocortin receptor subtypes [134]. A 36 atom $(Pro\text{-}Gly)_6$ linker system was optimal for mMC1R binding (14-fold enhancement compared to monovalent counterpart), suggesting a cooperative bivalent binding mode (Figure 1 A-C). However, a 20 atom polyethylene glycol-based linker was less effective at the mMC1R. The lower-fold enhancement was mostly likely an effect of increasing the pharmacophore concentration and is not indicative of a synergistic bivalent binding mode. The 20 atom polyethylene glycol-based linker was optimal at the mMC4R (22-fold enhancement compared to monovalent counterpart). The 36 atom $(Pro\text{-}Gly)_6$ linker system resulted in only a 6-fold enhancement at the mMC4R. Both linker systems had binding affinity increases consistent with bivalent binding at the mMC3R (23- to 25-fold enhancement). Currently, this is the only parallel structure activity relationship (SAR) study of various linker systems between different melanocortin receptor subtypes [134]. Similar trends for other GPCR systems have been reported [230, 246, 247], highlighting the importance of optimizing the linker and pharmacophore for a specific receptor subtype if high affinity is desired.

Binding affinity is usually the optimized parameter in melanocortin bivalent designs, but there are a few reports discussing bivalent ligand effects on functional potency. As described above, Carrithers and Lerner observed a 5- to 7-fold enhancement relative to the monovalent ligand in a functional frog-melanocyte dispersion assay (presumably through the MC1R) [233]. Another study by Brabez *et al*. compared the effects of a monovalent, bivalent, and trivalent ligands on cAMP signaling in HEK293 cells expressing the hMC4R and observed increased cAMP signaling corresponding to increased valency [217]. Lensing *et al*. reported that although bivalent ligands increased binding affinity (14- to 25-fold), more moderate increases in cAMP signaling potency were observed (3- to 5-fold). Considering that at least a doubling in functional potency would be expected due to doubling the pharmacophore concentration, these increases suggested minimal synergy in function due to bivalent ligand design [134]. Several hypotheses have been proposed for the possible divergences between binding affinity and functional potency, including unique βγ subunit signaling, potency

masking, auxiliary binding sites, and asymmetric dimer signaling [134, 217, 233]. Given the limited reports of bivalent functional effects, further studies may be warranted to investigate the mechanism of bivalent ligands' functional signaling and how they may differ from monovalent ligands.

In vivo studies on melanocortin bivalent ligands have focused on their utility as diagnostic tools [220, 221, 224, 225, 243-245]. As noted above, the use of high affinity ligands for the MC1R coupled to dyes or radiolabeled have been identified as possible imagining tools, diagnostic tools, or targeting molecules for melanoma. In 1992, a bivalent chelating derivative of α-MSH was examined in humans, but showed high non-specific uptake in the liver and other organs [220-223, 245]. In 2012, Morais and coworkers demonstrated that ^{99m}Tc(I)-labeled bivalent analogs of NDP-MSH resulted in approximately 20-fold enhancements in binding affinity to B16F1 murine melanoma cells (presumably expressing the MC1R) compared to a monovalent counterpart [243]. These ligands displayed negligible degradation when incubated *in vitro* with human blood serum [243]. The biodistribution of ^{99m}Tc(I)-labeled bivalent analogs were studied in melanoma-bearing mice. The lead bivalent analog displayed increased tumor retention in which 98% of the signal from a tumor at 1 h was still detected at 4 h compared to 72% with the monovalent analog [243]. The enhanced tumor retention, hypothesized to be due to increased kinetics of binding, partially accounted for the better tumor-to-blood and tumor-to-muscle ratios 4 h after injection compared to the monovalent ligand. However, despite improved *in vitro* MC1R binding affinity and high cellular internalization/retention, the bivalent ligand did not increase tumor uptake or improve the pharmacokinetic profiles relative to the monovalent counterpart [243]. Previous reports demonstrated a similar result that bivalent ligands increased *in vitro* binding affinities at the MC1R but not enhanced *in vivo* properties relative to monovalent ligands, possibly due to reduced tissue penetration [220-223, 244, 245].

The first report of *in vivo* functional effects of a melanocortin homobivalent ligand was of CJL-1-87 (Ac-His-DPhe-Arg-Trp-PEDG20-His-DPhe-Arg-Trp-NH_2) [134]. This ligand consists of two His-DPhe-Arg-Trp pharmacophores connected by a 20 atom polyethylene glycol-based linker. Administration of CJL-1-87 icv resulted in dose-dependent decreased food intake [134]. Comparison to the monovalent ligand Ac-His-DPhe-Arg-Trp-NH_2 suggested little improvement in a nocturnal feeding paradigm [108, 134, 214]. However, a direct comparison study utilizing a fast-refeeding paradigm showed significant differences between CJL-1-87 and Ac-His-DPhe-Arg-Trp-NH_2 after ICV administration [214]. Administration of the bivalent CJL-1-87 icv resulted in 50% less food intake than the monovalent ligand 2 to 8 h post-treatment [214]. Treatment also resulted in significantly lowered respiratory exchange ratio (RER) as well as significantly decreased insulin, C-peptide, leptin, and resistin plasma levels compared to the monovalent ligand Ac-His-DPhe-Arg-Trp-NH_2 [214].

6.2 Heterobivalent Ligands

Another promising tool for targeting melanoma or other cancers has been the utilization of heterobivalent ligands featuring pharmacophores for two different GPCR types [248-254]. These ligands feature a pharmacophore for the melanocortin receptors and a pharmacophore

for a different receptor system (including opioid receptors or cholecystokinin). Monovalent binding occurs to cells expressing one receptor subtype. However, synergistic bivalent binding occurs only on cells expressing both receptor types [248-254]. If appropriate receptors pairs are selected that are co-expressed in cancer cells but not in normal cells, the synergistic bivalent binding will selectively occur on cancer cells. A fluorescent label can be conjugated in the inert linker region, resulting in high affinity ligands that can be used for cancer imagining and diagnostics. Replacement of the fluorescent label with a chemotherapeutic can result in a highly selective drug targeting strategy [248-254]. This heterobivalent ligand targeting strategy has been validated both *in vitro* and *in vivo* [248-253]. In 2012, Xu *et al.* synthesized heterobivalent ligands containing a melanocortin pharmacophore and a cholecystokinin pharmacophore separated by a fluorescently-labeled synthetic linker. They observed the ligands had up to a 12-fold higher specificity for tumors co-expressing both of these receptors than for tumors expressing one receptor, providing a proof-of-principle for future studies [253].

6.3 Melanocortin Multivalent Ligands

Multivalent ligands are defined as ligands that feature more than two pharmacophores for the melanocortin receptors, ranging from three to several hundred. Conjugates of multiple copies α-MSH derivatives to larger biomolecules were reported starting in 1977 [218, 255]. Eberle *et al.* reported conjugating α-MSH to human serum albumin at a ratio of four and six α-MSH hormones to one molecule of albumin and showed approximately equal activity to non-conjugated α-MSH analogs in testing on *Rana pipiens*. Schwyzer *et al.* reported loading the tobacco mosaic virus (TMV) with approximately 400-600 molecules of α-MSH analogs, resulting in enhanced potency, affinity, resistance towards enzymatic degradation, and prolonged activity at target cells [219, 256-260]. Sharma *et al.* developed a class of multivalent fluorescent melanotropin-macromolecule conjugates [261, 262]. They used a polyvinyl alcohol (PVA) scaffold that had an approximate molecular weight of 110,000 and 2500 hydroxyl groups available for derivation. The hydroxyl groups were conjugated to introduce 10-16 molecules of a melanocortin pharmacophore (based on NDP-MSH) and 10-16 molecules of a fluorophore (fluorescein isothiocyanate or FTIC) to create macromolecular conjugates (MSH-PVA-FITC). These conjugates possessed increased binding affinity and increased levels of melanocortin receptor detection in labeling experiments comparing different cells that did or did not express the melanocortin receptors [261, 262]. Sharma *et al.* also developed both latex bead and polyamide bead conjugates to NDP-MSH analogs and achieved similar results as Schwyzer *et al.* [263-265]. The latex beads were considered microspheres (~1 μm in diameter) and the polyamide beads were classified as macrospheres (40 to 100 μm in diameter). Electron and light microscopy imaging indicated that mutiple latex microspheres were bound to B16/F10 mouse melanoma cells (~10-15 μm in diameter). The larger polyamide macrospheres were bound to multiple cells, a difference presumed to be due to the relative sizes of the conjugated beads and the cells.

In 2007, Newton *et al.* engineered and fused α-MSH analogs to phages and used these multivalent phage constructs to image B16-F1 mouse melanoma *in vitro* and *in vivo* [266]. In 2011 and 2013, Barkey *et al.* attached hMC1R selective α-MSH analogs to stabilized

triblock polymer micelles through Cu-catalyzed click chemistry [267, 268]. Though the ligand decreased binding affinity of the polymer micelles after attachment, it increased specificity for the hMC1R over the hMC4R and hMC5R [268]. Further cross-linking the targeted polymer micelles generated constructs that were used as delivery systems for contrast-enhancing gadolinium complexes of texaphyrin (Gd-Tx) [267]. These agents were efficacious at penetrating and delivering the contrast agent into xenografted tumors in mice with minimal accumulation in healthy tissues, including the kidney and liver [267].

Besides conjugated multivalent ligands, additional research has focused on the design of smaller, synthetic ligands. Trivalent ligands have been synthesized by incorporating a lysine in the linker of bivalent ligands, providing an additional chemical handle to add another pharmacophore. Trivalent ligands possessed increased binding affinity over bivalent and monovalent ligands at the hMC4R, albeit the result was not indicative of trivalent binding [235].

Additional trivalent ligands were shown to increase binding affinity and supported a cooperative binding mode. In 2011, Brabez *et al.* reported a series of trivalent melanocortin ligands with increased binding affinity. They found the optimal distance between His-DPhe-Arg-Trp tetrapeptide pharmacophores to be 24 ± 5 Å when targeting the hMC4R [217]. In competitive binding experiments with HEK293 cells expressing the hMC4R, the monovalent, bivalent, and trivalent analogs had IC_{50} values of 4900, 310, and 14 nM, respectively. The increased affinity with each valency suggested that three receptors were involved in a trivalent cooperative binding mechanism with the trivalent ligand [205, 217]. The authors noted that although cAMP signaling potency increased with each valency, the levels of cAMP signaling corresponded to receptor occupancy independent of valency. This suggested that the ligands activated cAMP signaling in a monovalent fashion and no allosterism or synergy in function was detected [217]. In a follow-up study, the authors combined the trivalent ligands onto additional scaffolds, resulting in ligands with 6 and 9 pharmacophores [269]. The 6- and 9-valent compounds decreased binding affinity 3-fold at the hMC4R compared to the trivalent analog, but were approximately 100-fold more potent than the monovalent compound. These data suggest the 6- and 9- pharmacophore ligands achieved cooperative binding but three or fewer receptors were involved. The cAMP signaling was also independent of the number of pharmacophores present, as previously described. They reported that all compounds were internalized within 90 minutes, suggesting these constructs could potentially be used for drug delivery purposes [269].

Solanesol-derived and sucrose-derived scaffolds were utilized to make both bivalent and tetravalent ligands attached to the His-DPhe-Arg-Trp tetrapeptide sequence [270, 271]. Moderate improvement in binding affinity at the hMC4R was observed, likely due to proximity effects and increasing the moles of pharmacophore present, but not indicative of cooperative or multivalent binding. The authors hypothesized their ligands may not possess the correct linker length or improperly presented the pharmacophores for cooperative binding [270, 271].

An unique strategy to synthesize multivalent ligands featuring the His-DPhe-Arg-Trp tetrapeptide was reported by Dehigaspitiya *et al.* in 2015. Linear ligands that had up to eight

His-DPhe-Arg-Trp units were synthesized and separated by a $(Pro\text{-}Gly)_3$ linker. The binding affinities for the hMC4R were slightly enhanced in competitive binding experiments when adjusted for pharmacophore concentrations, suggesting the observed enhancement were not from cooperativity or multivalent binding [240].

In 2015, Elshan *et al*. presented trigonal scaffolds and compared monovalent, bivalent and trivalent ligands featuring the His-DPhe-Arg-Trp tetrapeptide pharmacophore. The bivalent ligands increased binding affinity 10- to 30-fold, indicating synergistic bivalent binding affinity at the hMC4R. The trivalent ligands were reported to be marginally better binders (~2-fold) than the bivalent ligands, supporting a model where trivalent ligands bind in a synergistic bivalent binding mode, but not a trivalent mode [242]. Their results suggested the optimal linker length to bridge two receptors is between 17 and 23 Å. However, considering a third receptor did not appear to be utilized, it is likely that the third pharmacophore linker is not optimized to achieve trivalent binding [242]. Dehigaspitiya *et al*. reported similar results when comparing monovalent, bivalent, trivalent and tetravalent ligands featuring the tetrapeptide His-DPhe-Arg-Trp in different scaffolds [241]. All multivalent compounds possessed 30- to 40-fold higher binding affinities at the hMC4R compared to monovalent controls, although valencies beyond two did not result in further affinity gains. This finding is consistent with bivalent binding to putative melanocortin receptor dimers, without evidence for trivalent or tetravalent binding. They also reported the optimal distance between pharmacophores was between 17 and 23 Å, as previously reported [241, 242].

In summarizing the reported bivalent and multivalent ligand designs for the melanocortin receptors, two key observations can be made. First, in order to observe synergistic effects, the proper pharmacophore must be used. In almost all cases, the tetrapeptide His-DPhe-Arg-Trp was observed to result in the greatest-fold affinity enhancements, presumably due to synergistic binding. This is likely due to the lower initial binding affinity of the tetrapeptide compared to longer analogs that allows easier detection and observation of the synergistic binding mode. The second key is the design of proper linker length to bridge putative melanocortin dimers. The greatest-fold enhancements were with linkers of approximately 23 ± 5 Å. One difficulty in estimating the exact length is that the linkers are flexible and therefore nearly impossible to measure precisely. The estimated range for the optimal melanocortin linker is similar to that of other GPCR systems including the oxytocin (~25 Å) [272], opioid (~22 Å) [246, 273, 274], and dopamine receptors (~25 Å) [247]. This provides strong evidence for a common design of bivalent ligands targeting GPCR systems and suggests this length may be the result of a common GPCR phenomenon (dimerization or high-order oligomerization).

Although the increases in binding affinity support the use of bivalent and multivalent melanocortin ligands as diagnostic tools, imaging probes, and drug delivery vehicles for melanoma, their functional effects require further investigation. In the limited studies evaluating functional effects, some increases in potency were observed. However, these enhanced *in vitro* potencies observed could be due to increased concentration and increased binding affinity, and not due to allosterism or synergistic functional effects [134, 217]. Future studies on bivalent and multivalent ligands may need to focus on evaluating the functional effects of this ligand class both *in vitro* and *in vivo*, on developing SAR at

different receptor subtypes (and heterodimers), and optimizing the linker length to a third pharmacophore to extend beyond bivalent binding.

7. Clinical Candidates

There has been a concerted effort to translate melanocortin ligands into clinical therapies. Both α-MSH and β-MSH were injected in humans in 1961 [55]. However, many unexpected challenges such as receptor-mediated pressor effects limit the translation of these pharmacologically active compounds into viable therapies [25]. The clinical study involving Eli-Lilly compound LY2112688, which had unexpected cardiovascular effects, is the archetype of unexpected challenges targeting the melanocortin receptors [25].

This section will focus on clinical studies from 2011 to 2016, in peer-reviewed publications on melanocortin compounds in preclinical and clinical studies to complement previous reviews [171, 275]. A survey was conducted on the databases ClinicalTrials.gov, PubMed, and the Cochrane Central Register of Controlled Trials (CENTRAL). Search criteria included “melanocortin” and “pharmacotherapy OR drug therapy OR pharmaceutic OR drug.” Studies were excluded if they were not peer-reviewed or did not possess patient data. In addition, studies focusing on polymorphisms of the melanocortin receptors and the resulting effects on different pathways were excluded. These criteria generated more than 70 articles that were evaluated. Compounds currently in clinical trial (Figure 6) include bremelanotide, afamelanotide, setmelanotide, MC4-NN2-0453, and MSH/ACTH(4-10).

7.1 Bremelanotide

The cyclic heptapeptide, sequence Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-OH (bremelanotide, Figure 6), is a derivative of the potent melanotan-II (MTII, Figure 6) with a C-terminal carboxylic acid [116, 117]. Bremelanotide is in clinical development by Palatin Technologies for the treatment of hypoactive sexual desire disorder in women. The initial development of these cyclic α-MSH analogues targeted sunless tanning. It was discovered by Dr. Hadley *et al.* that a single 10 mg injection of MTII was able to induce an instantaneous and unrelenting erection lasting approximately 8 hours in duration [174], indicating sexual effects in males with other sexual effects also observed in females.

A phase 2 clinical study consisting of 394 women experiencing sexual dysfunction reported clinical efficacy was achieved with 1.25 and 1.75 mg subcutaneous doses. These doses induced a maximal 3.0 mm Hg pressure increase in both systolic and diastolic blood pressures between 0 and 4 h post injection. Importantly, the increases in blood pressure were transient, approximately 15 minutes in duration, and quickly reduced back to pretreatment levels [276].

Bremelanotide was taken into a phase 2 trial in postmenopausal women with self-reported sexual dysfunction [277]. A total of 327 women with female sexual arousal disorder and/or hypoactive sexual desire disorder were randomized in a double-blind placebo-controlled study. Treatment consisted of placebo, 0.75, 1.25, or 1.75 mg subcutaneous doses of bremelanotide. Two week base-line measurements were followed by a 12 week at-home study where the compound was administered as desired once daily (up to 16 doses in a 4-

week period) 45 minutes prior to anticipated sexual activity. Efficacy was determined as changes in score using the self-reported female sexual distress scale-desire/arousal/orgasm via an electronic diary for all sexual encounters for the duration of the study. Results indicated a significant increase in the number of sexual encounters per month (+0.75 for the 1.25/1.75 mg pooled data) in addition to a decrease in the distressed scores produced from the self-reported questionnaire (indicating greater satisfaction) [277]. Positive results were also reported from a phase 3 clinical study in August 2016 in premenopausal women, and may perhaps be the first US approved melanocortin ligand drug.

7.2 Afamelanotide

The linear 13-residue afamelanotide is another name for the synthetic melanocortin analog NDP-MSH (Figure 6) that was initially reported in 1980 [109, 278]. Afamelanotide has been used in phase 2 trials for the treatment of skin conditions associated with inflammation. These studies have focused on rare skin conditions such as Hailey-Hailey disease in addition to diseases with a broader scope such as acne vulgaris, although these studies are limited due to small patient size [279, 280].

In clinical development and evaluation sponsored partially by Clinuvel Pharmaceuticals, afamelanotide has produced successful results in phase 3 clinical trials for erythropoietic protoporphyria, a photosensitivity skin condition (ClinicalTrials.gov identifiers NCT01605136 and NCT00979745), summarized in 2015 [281]. Presumably signaling through the MC1R, afamelanotide induces the production of eumelanin in the skin, serving as a chemical tanning agent that would allow increased exposure to sunlight for individuals afflicted with erythropoietic protoporphyria. These studies were conducted in both the United States (94 patients) and the European Union (74 patients) using similar methodology. In a randomized, placebo-controlled, double-blind study, patients were treated with 16 mg injections of afamelanotide (proprietary name Scenesse) formulated in a biodegradable, implantable polymer matrix [282]. In the European study, the patients received five doses over 9-months, while in the United States study patients received three doses over 6-months. The results from both locations indicated less-severe phototoxic reactions with shorter recovery times. In a self-reporting questionnaire, patients indicated the treatment produced a positive impact on their daily lives [281]. Patients also experienced a significant improvement to light tolerance [281]. Afamelanotide was approved by European regulators to treat erythropoietic protoporphyria in 2014 [115] and is the first approved melanocortin ligand for therapeutic use.

7.3 Setmelanotide

The disulfide cyclized octapeptide setmelanotide (RM-493, formerly BIM-22493, IRC-022493, Figure 6), Ac-Arg-c[Cys-DAla-His-DPhe-Arg-Trp-Cys]-NH_2, is in clinical evaluation for weight-loss by Rhythm Pharmaceuticals. Preclinical studies in obese rhesus macaques indicated chronic subcutaneous administration of setmelanotide reduced overall food intake, decreased body weight, improved glucose tolerance, and did not induce negative cardiac effects [283]. Phase 1 and 2 studies have successfully evaluated the safety, efficacy, tolerability, pharmacokinetics, and pharmacodynamics of the octapeptide in obese volunteers (ClinicalTrials.gov identifiers NCT02431442 and NCT02041195). A phase 2

clinical trial (ClinicalTrials.gov identifier NCT01867437) evaluated the efficacy and safety of setmelanotide, and consisted of 12 obese, mean body mass index (BMI) 35.7 kg/m^2, otherwise healthy individuals. In a randomized crossover experimental paradigm, individuals received a 1 mg/24 hour subcutaneous infusion of drug or placebo for 72 hours. A significant increase in resting energy expenditure was observed, without corresponding increases in blood pressure or heart rate [284]. An active study of volunteers with Prader-Willi syndrome to evaluate the compound efficacy is ongoing in 2016 (ClinicalTrials.gov identifier NCT02311673). In addition, there are results from an investigator initiated study consisting of two obese patients with proopiomelanocortin deficiencies [285]. Daily subcutaneous administration of setmelanotide in these patients resulted in substantial weight loss (patient 1 = 51.0 kg in 42 weeks, patient 2 = 20.5 kg in 12 weeks) and significantly decreased blood pressure in 1 patient [285].

7.4 MC4-NN2-0453

In development by Novo Nordisk, MC4-NN2-0453 is a α-MSH analog with an N-terminal fatty acid extension [286]. The modified peptide, 16-(tetrazol-5-yl)hexadecanoyl-Oeg-Gly-Ser-Gln-His-Dap[bis(carboxymethyl)amino]acetyl-Nle-c[Glu-Hyp-DPhe-Arg-Trp-Lys]-NH_2 (Figure 6), has K_i of 2700, 71, 0.58, and 13 nM at the hMC1R, hMC3R, hMC4R, hMC5R, respectively [286]. These *in vitro* results did not translate into clinically relevant effects on body weight in obese, otherwise healthy, patients [287]. A four day multi-dose, randomized, double-blind, placebo-controlled trial tested multiple concentrations administered subcutaneously up to 3.0 mg/day [287]. Analysis of the pharmacokinetic parameters indicated the compound possessed a long (>200 hour) $t_{1/2}$ half-life, but did not alter body weight. Side effects of this compound included non-serious skin adverse events, primarily hyperpigmentation. Further development of this compound as an anti-obesity therapy appears to be halted, perhaps due to lack of efficacy.

7.5 MSH/ACTH(4-10)

The linear heptapeptide H-Met-Glu-His-Phe-Arg-Trp-Gly-OH (Figure 6), also referred to as MSH/ACTH(4-10), has been subject to at least one human study between 2011 and 2016. In this study, 10 healthy, BMI between 20 and 25 kg/m^2, male volunteers participated in a double-blind randomized crossover study. The participants received a 10 mg dose via intranasal administration. Interstitial glycerol, an indicator of lipid hydrolysis, was monitored via microdialysis in abdominal white adipose tissue and in skeletal muscle of the forearm. Results indicated a significant increase in glycerol in the white adipose tissue 45 minutes after dosing compared to no change in the glycerol levels in the skeletal muscle tissue. These results indicate the peptide alters lipid metabolism in humans [288].

8. Conclusions

Numerous melanocortin ligands have been developed in the 60 years since the sequences of the first endogenous ligands were elucidated. While much of the early focus was on the development of compounds that alter pigmentation, the cloning of the receptors and identification of other biological pathways controlled by these receptors (including obesity and sexual function) led to the development of potent and selective ligands. Many classical

medicinal chemistry structure-activity relationship study techniques have led to a greater understanding of ligand/receptor interactions. Although many unique ligands have been reported, the expanding signaling pathways associated with the melanocortin receptors provides opportunities for continued investigations. With new biological functions and pathways associated with the melanocortin receptors, it may be unwise to “believe that we have reached the limits of insight that can be reasonably provided by structure-activity studies” for melanocortin ligands [85].

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Highlights

- An updated melanocortin ligand review.
- Classic, selective, small-molecule, sex-specific, bivalent, and clinical ligands.
- Focuses on melanocortin ligand publications from 2006-2016.

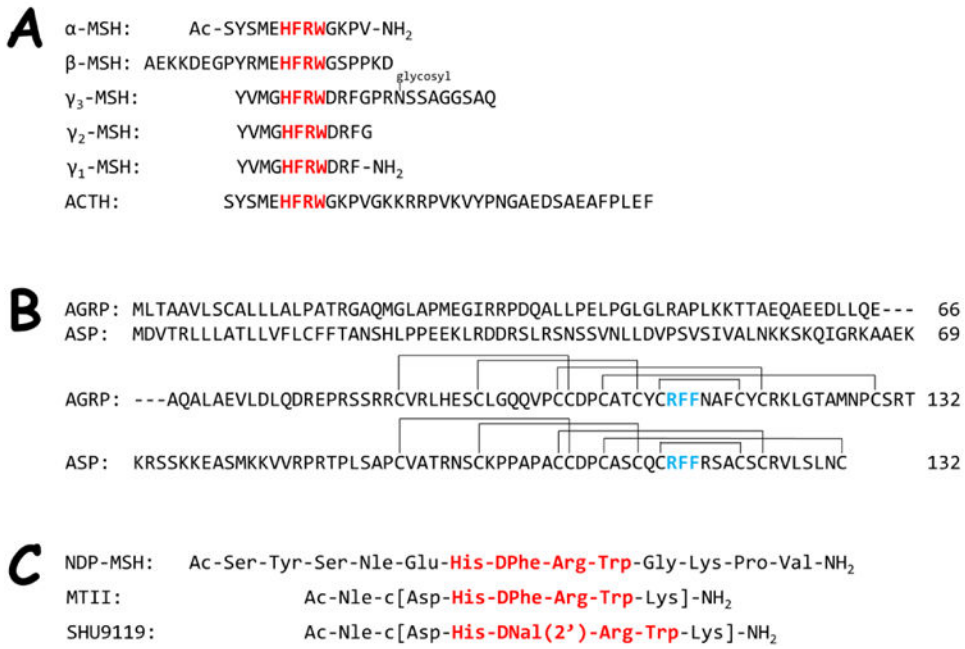

Figure 1.

Structures of classical melanocortin ligands. (A) POMC-derived naturally occurring agonists (the common His-Phe-Arg-Trp tetrapeptide is highlighted in red). (B) Sequences of the endogenous antagonists AGRP and ASP (the active Arg-Phe-Phe tripeptide is highlighted in blue). (C) NDP-MSH, MTII and SHU9119 (hypothesized pharmacophore region highlighted in red).

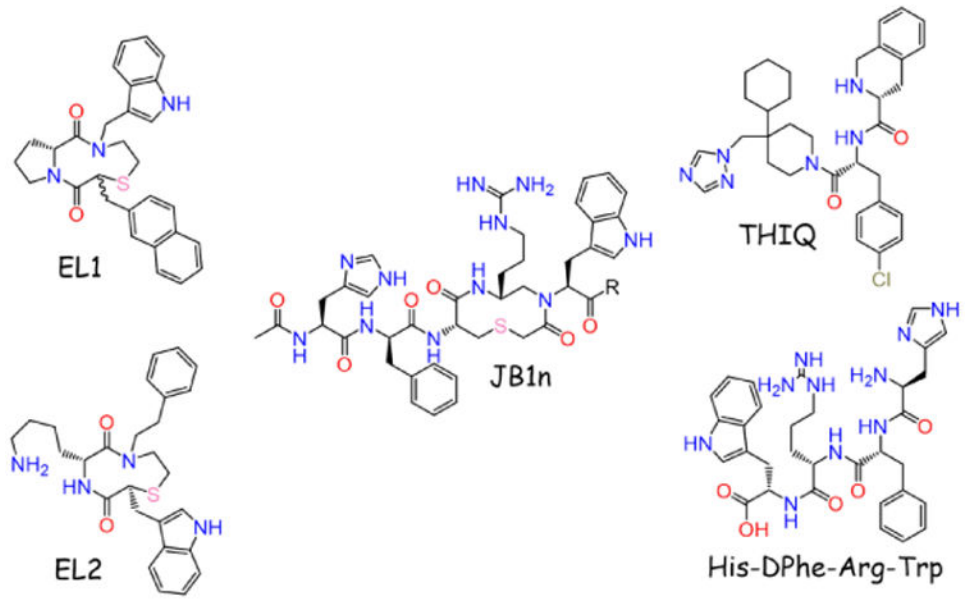

Figure 2.

First reported melanocortin small molecule ligands, along with the hypothesized Ac-His-DPhe-Arg-Trp-NH_2 melanocortin pharmacophore. The C-terminus of JB1n was reported as a mixture of an amide and carboxylic acid.

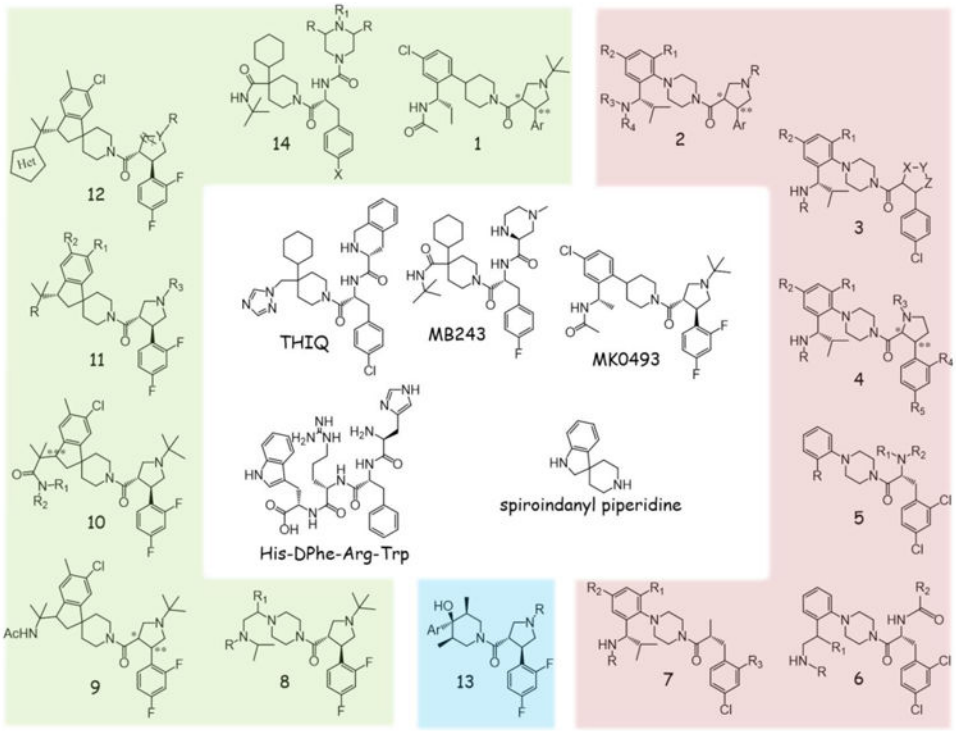

Figure 3.

Small-molecule melanocortin scaffolds derived from the Merck compound THIQ. Scaffolds are grouped by company disclosing the structures: Merck (green), Neurocrine Biosciences, Inc. (red), or Pfizer (blue). In the center, THIQ, MB243, and MK0493 are select Merck compounds disclosed before 2007, the His-DPhe-Arg-Trp hypothesized melanocortin pharmacophore, and an illustrative spiroinanyl piperidine core structure.

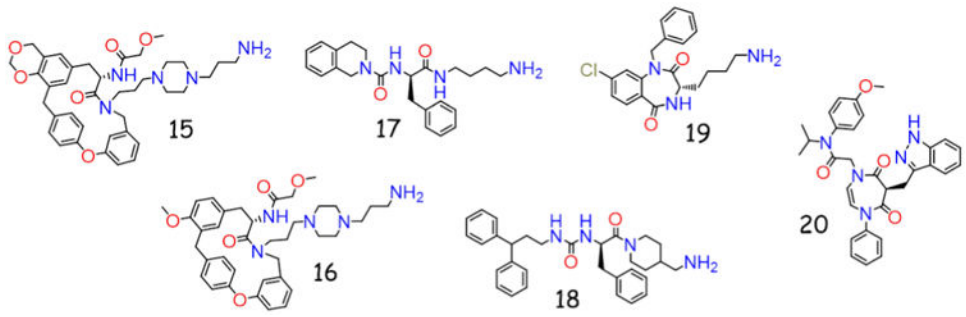

Figure 4. De novo
melanocortin ligand small molecule scaffolds.

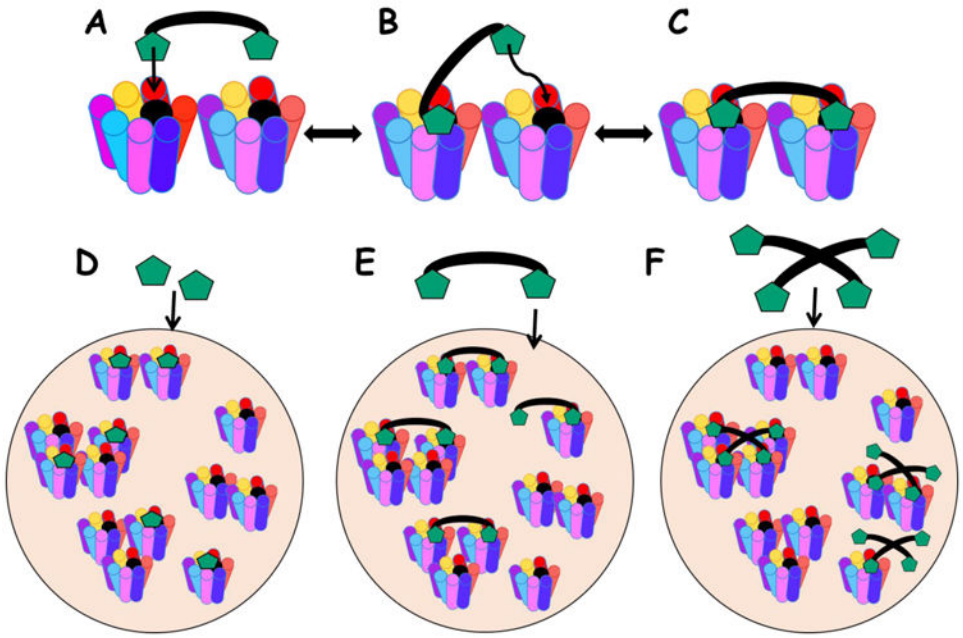

Figure 5.

Bivalent and multivalent ligand binding modes. **(A)** The bivalent ligand first binds a receptor with one pharmacophore in a monovalent fashion. **(B)** The second pharmacophore is tethered in close proximity to the second binding site. **(C)** The second pharmacophore can bind the second receptor with reduced entropic cost. Similar binding mode may exist for multivalent ligands with more than two pharmacophores. **(D)** Monovalent ligands bind monomers, dimers, and higher-order oligomers equally. **(E)** Bivalent ligands bind dimers and higher-order oligomers in a cooperative synergistic fashion. **(F)** Multivalent ligands bind GPCR clusters in a cooperative synergistic fashion. Image modified from Lensing, *et. al.* [134].

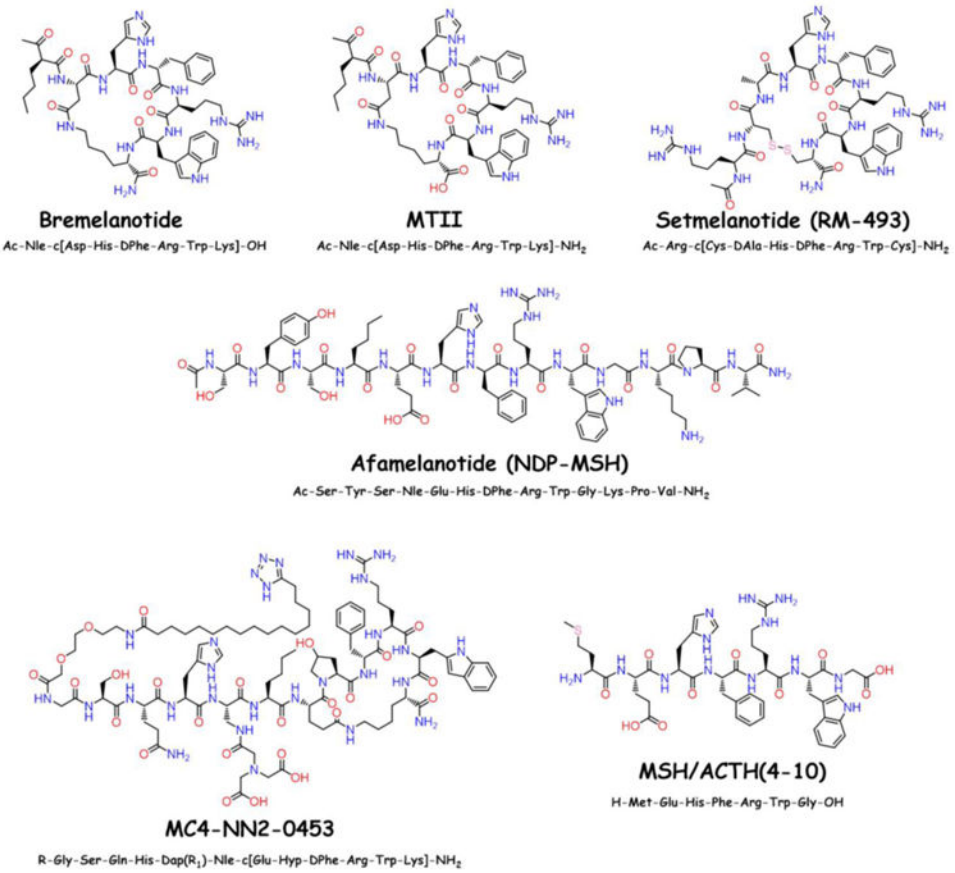

Figure 6.

Chemical structures of the melanocortin ligands used in clinical trials from 2011 to 2016. The ligand MTII is included as a comparison to bremelanotide.

Table 1

Table of selective melanocortin agonists for the human MCRs. Selectivity was defined to be at least 100-fold more potent between at least two receptor subtypes. Ligands are grouped by receptor selectivity. In some publications, numerous ligands were reported with the same selectivity profiles. In these instances, only the most potent ligands were chosen.

Selective For:	First Author	Year	Ref	Compound ID	Compound EC_{50} (nM)			
					hMC1R	hMC3R	hMC4R	hMC5R
hMC1R	Bednarek, Maria A	2007	[119]	11	***1.4 (59%)***	0.54 (43%)	4% @ 2.5μM	0.11
	Cai, Minying	2015	[121]	1	***15***	>10000	>10000	7
	Bednarek, Maria A	2007	[119]	5	***0.23 (67%)***	3% @ 2.5μM	0% @ 2.5μM	0.088
	Bednarek, Maria A	2007	[120]	2	***90***	0% @ 10μM	0% @ 10μM	50
	Conde-Frieboes, K	2011	[122]	6a	***12***	>10000	700	>10000
	Conde-Frieboes, K	2011	[122]	7	***16***	>10000	470	>10000
	Cai, Minying	2015	[121]	15	***3.7***	>10000	>10000	>10000
	Cai, Minying	2015	[121]	8	***2.1***	>10000	>10000	450
	Bednarek, Maria A	2007	[123]	2	***2.4***	34% @ 1μM	22% @ 2.5μM	26% @ 1.5μM
hMC3R	Bednarek, Maria A	2007	[119]	11	1.4 (59%)	***0.54 (43%)***	4% @ 2.5μM	0.11
	Carotenuto, Alfonso	2015	[127]	2	940	***1.9***	>1000	10
hMC4R	Hong, Qingmei	2010	[128]	21A	1200	90	***5.4***	490
	Hong, Qingmei	2010	[128]	23A	520	79	***6.4***	1000
	Hong, Qingmei	2010	[128]	20B	540	1800	***13***	>5000
	Hong, Qingmei	2010	[128]	25B	>5000	2500	***19***	>5000
	Cai, Minying	2015	[121]	17	>10000	>10000	***5 (67%)***	>10000
hMC5R	Bednarek, Maria A	2007	[119]	11	1.4 (59%)	0.54 (43%)	4% @ 2.5μM	***0.11***
	Cai, Minying	2015	[121]	1	15	>10000	>10000	***7***
	Bednarek, Maria A	2007	[119]	5	0.23 (67%)	3% @ 2.5μM	0% @ 2.5μM	***0.088***
	Bednarek, Maria A	2007	[120]	23	3.8 (46%)	8% @ 5μM	3% @ 5μm	***0.15***
	Bednarek, Maria A	2007	[119]	7	20% @ 5μM	54% @ 5μM	36% @ 5μM	***0.41***

Table 2

Table of selective melanocortin agonists for the mouse MCRs. Selectivity was defined to be at least 100-fold more potent between at least two receptor subtypes. Ligands are grouped by receptor selectivity. In some publications, numerous ligands were reported with the same selectivity profiles. In these instances, only the most potent ligands were chosen.

Selective For:	First Author	Year	Ref	Compound ID	Compound EC_{50} (nM)			
					mMC1R	mMC3R	mMC4R	mMC5R
mMC1R	Todorovic, Aleksandar	2007	[124]	10	***5.9***	>100000	41	30
	Todorovic, Aleksandar	2007	[124]	11	***14***	>100000	>100000	25
	Todorovic, Aleksandar	2007	[124]	12	***5.1***	10% @ 100μM	63% @ 100μM	19
	Singh, Anamika	2014	[125]	AMW6103	***0.81***	5300	440	31
	Singh, Anamika	2013	[126]	4	***1.9***	4500	290	415
mMC3R	Todorovic, Aleksandar	2016	[47]	[Ala^4]α-MSH	12000	***17***	120	60
mMC4R	Todorovic, Aleksandar	2007	[124]	10	5.9	>100000	***41***	30
	Hess, Shmuel	2008	[129]	4	28	1300	***4***	2
	Proneth, Bettina	2008	[130]	11	280	240	***2.8***	2.9
mMC5R	Todorovic, Aleksandar	2007	[124]	10	5.9	>100000	41	***30***
	Hess, Shmuel	2008	[129]	4	28	1300	4	***2***

Table 3

Table of selective mouse MC4R antagonists. Selectivity was defined to be greater than 100-fold more potent between at least two receptors (two pA_2 units). Ligands are grouped by possessing or not possessing agonist or antagonist activity at the mMC3R at concentrations up to 10 μM ($pA_2 < 5$). In some publications, numerous ligands were reported with the same selectivity profiles. In these instances, only the most potent ligands were chosen.

First Author	Year	Ref	Compound ID	Receptor Activity			
				mMC1R EC_{50} (nM)	mMC3R pA_2	mMC4R pA_2	mMC5R EC_{50} (nM)
Ericson, Mark D	2015	[133]	17	20000 nM	< 5	***8.2***	>100000
Doering, Skye R	2015	[132]	3	8300 nM	< 5	***7.1***	24000 nM
Ericson, Mark D	2015	[133]	10	1500 nM	< 5	***7***	>100000
Doering, Skye R	2015	[132]	2	21000 nM	< 5	***6.3***	7800 nM
Ericson, Mark D	2015	[133]	22	75% @ 100μM	*6.9*	***9.1***	>100000
Lensing, Cody J	2016	[134]	10	110 nM	*6.1*	***8.4***	70% @ 100μM
Doering, Skye R	2015	[132]	6	5500 nM	*5.9*	***8.3***	20000 nM
Doering, Skye R	2015	[132]	7	5800 nM	*5.7*	***8.3***	20000 nM
Doering, Skye R	2015	[132]	1	2000 nM	*5.4*	***7.8***	2800 nM
Ericson, Mark D	2015	[133]	19	13000 nM	*5.6*	***7.8***	>100000

Table 4

Examples of melanocortin ligands affecting males and females differently.

Ligand	Effect	Species	Strain	Ref
α-MSH	Improved learning tasks in males, but not females.	Rats	Holtzman albino	[175, 176]
MTII	Reduced juvenile play behaviors in males, but not females after neonatal administration	Voles	Prairie Voles	[177]
Ac-Nle-Asp-Trp-DPhe-Nle-Trp-Lys-NH_2	Effected κ-opioid analgesia in females, but not in males	Mice	Crl:CD-1	[178]
C[Gly-Cpg-DNal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	Effected κ-opioid analgesia in females, but not in males	Mice	Crl:CD-1	[178]
C[$(CH_2)_3$CO-Gly-His-DPhe-Arg-DTrp-Cys(S-)]-Asp-Arg-Phe-Gly-NH_2	Reversed morphine hyperalgesia in female mice, but had no effect in males	Mice	C57BL/6J; CD-1	[179, 180]
AGRP	Reduced energy expenditure (measured by vO_2) in female rats more than in male rats.	Rats	Long-Evans	[181]
Ac-Trp-DPhe(*p*-I)-Arg-Trp-NH_2	Different doses necessary to affect energy homeostasis in males and females	Mice	Mixed C57BL/6J and 129/Sv background	[182]
SHU9119	Increased blood pressure in males, but not in females	Rats	Spontaneously hypertensive rats (SRH)	[183]