Systematic Backbone Conformational Constraints on a Cyclic Melanotropin Ligand Leads to Highly Selective Ligands for Multiple Melanocortin Receptors

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Abstract

Human melanocortin receptors (hMCRs) have been challenging targets to develop ligands that are explicitly selective for each of their subtypes. To modulate the conformational preferences of the melanocortin ligands and improve the biofunctional agonist/antagonist activities and selectivities, we have applied a backbone N-methylation approach on Ac-Nle-c[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH$_2$ (Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$), a nonselective cyclic peptide antagonist at hMC3R and hMC4R and an agonist at hMC1R and hMC5R. Systematic N-methylated derivatives of Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$, with all possible backbone N-methylation combinations, have been synthesized and examined for their binding and functional activities toward melanocortin receptor subtypes 1, 3, 4, and 5 (hMCRs). Several N-methylated analogues are selective and potent agonists or antagonists for hMC1R or hMC5R or have selective antagonist activity for hMC3R. The selective hMC1R ligands show strong binding for human melanoma cells. We have also discovered the first universal antagonist (compound **19**) for all subtypes of hMCRs.

Graphical abstract

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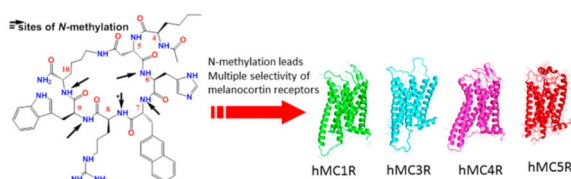
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The authors declare no competing financial interest.

INTRODUCTION

The melanocortin system[1–3] remains a challenging target for rational peptide and peptidomimetic design because the 3D topographical requirements for specific melanocortin receptor subtype recognition have not been fully elucidated.[4–7] Nevertheless, the numerous multifaceted physiological functions of the five known subtypes of human melanocortin receptors (hMC1–5R), including skin pigmentation,[8,9] control of the immune system,[10,11] erectile function,[12,13] blood pressure and heart rate,[14,15] control of feeding behavior and energy homeostasis,[3,6,16–21] modulation of aggressive/defensive behavior,[22,23] and mediation of pain,[24–26] continue to provide a strong stimulus for further development of potent and selective melanocortin agonists and antagonists. Until recently, much of this work was focused on hMC4R due to its direct involvement in the regulation of feeding behavior and energy homeostasis[3,6,16–21] as well as sexual behavior.[1,6,12,13,27] The hMC3 receptor has been suggested to play a complementary role in weight control.[20,21,28] Although the full scope of physiological functions of this receptor is yet to be unraveled, the current understanding based on the observed stimulation of food intake by peripheral administration of an MC3R-selective agonist[29] and MC3R agonist-induced inhibition of spontaneous action of pro-opiomelanocortin (POMC) neurons[30] suggests that hMC3R is an inhibitory autoreceptor on POMC neurons.[3] In addition, development of selective ligands for the hMC1 and hMC5 receptors is receiving increasing attention lately due to the roles of these receptors in regulating pain and skin pigmentation,[6,7] controlling the immune system (hMC1R),[8,11] regulating exocrine gland function,[31] and coordinating central and peripheral signals for aggression (hMC5R).[22,23]

Another consideration in the development of selective ligands for the melanocortin system bears intrinsic challenges due to conserved amino acid sequences and their structural similarity contained in the seven transmembrane GPCR fold of the melanocortin receptors.[1–3,32,33] Unlike many other G protein coupled receptor (GPCR) targets, the hMCRs, known to have the shortest N-terminus among GPCRs, have separate natural agonist and antagonist molecules for functional regulation.[1–3,32,33] This aspect imposes a second dimension for the development of melanocortin ligands that achieve selectivity for receptor subtype as well as the desired agonist, antagonist, and bioavailability properties.

To accomplish this, we have envisioned the application of an N-methylation strategy[34–36] to the melanocortin ligands. Recently, N-methylation of backbone amide NHs of peptides has been shown to substantially improve the physicochemical, structural, and biological properties of peptides.[34–40] N-Methylation is one of the simplest ways to include conformational restraints into the peptide backbone, as it introduces steric restrictions, allows for cis-peptide bonds, and also prevents hydrogen-bond formation. In addition, N-methylated amino acids often act as turn-promoting moieties (akin to prolines)[34,40] and thus this strategy helps to generate secondary structure features in peptides without changing the constituent peptide sequence. The specific impact of N-methylation on melanocortin peptide ligands had not been investigated systematically until we used this strategy on Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH$_2$ (MT-II) to develop completely selective hMC1R agonists.[41,42] Interestingly, we have also observed that some of the N-methylated Ac-Nle^4-c[Asp^5,DPhe^7, Lys^{10}]-NH$_2$ (MT-II) peptides possessed antagonist activity, which is a switch

in functional selectivity, although the amino acid sequence remained unchanged. Here, we have modulated the peptide conformation and the functional side chain disposition of Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ (peptide 1, SHU9119),[43] a peptide with antagonist activities for hMC3R and hMC4R and with agonist activities for hMC1R and hMC5R, by systematic N-methylation of the backbone amide NHs to gain further insight into the intricate correlations among amino acid sequence, conformation, and activity–selectivity. A combinatorial library consisting of N-methylated Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ (peptide 1, SHU9119) and analogues with single and multiple N-methylation in the core cyclic sequence of Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ has been designed, synthesized (Figure 1 and Table 1), and characterized chemically, and *in vitro* studies have been performed for binding affinities and efficacies at the hMCRs (Figure 2b–e and Tables 1–3). Syntheses were performed in analogy to the synthesis of the library of N-methylated Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-NH$_2$ (MT-II) peptides.[41]

RESULTS AND DISCUSSION

SAR Studies of N-Methylated Ac-Nle^4-c[Asp^5,D-Nal-(2′)^7,Lys^{10}]-NH$_2$ Analogues

Effects of N-methylation on Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ were evaluated on stably cloned hMCR (hMC1R, hMC3R, hMC4R, and hMC5R) cell lines (HEK 293 cells) via competition binding assays and functional activity assays (cAMP assay). Competitive binding assays with [^{125}I]-[Nle^4,D-Phe^7]-α-MSH (NDP-α-MSH) on whole cells and [^{3}H]-adenylate cyclase assays for cAMP were examined along with the application of standard Ac-Nlec[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) and Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ (SHU9119). The results are shown in Figure 2 and Tables 2 and 3. For the pharmacological characterization of the ligands in this study, a binding efficiency >70% is defined as competitive binding and binding efficiency <70% is considered to be noncompetitive binding. For the characterization of the potency of the ligand, we used Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) as the standard full agonist (Act% = 100%). Therefore, the cAMP functional activities (cAMP level) induced by ligands are expressed as a percent of the activity that is generated by the standard Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) and are defined as Act%. Act% < 30% is defined as no activity, Act% between 50 and 80%, partial agonist, and Act % > 80, agonist.

The first group of peptides are mono-N-methylated derivatives of Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ (peptides 2–6; Figures 1 and 2a). The competitive binding assays show that mono-N-methylation at each position of the pharmacophore does not have a remarkable influence on the binding affinity toward hMCRs except for the hMC4R for N-methylation at position 7 (*N*Me-DNal(2′)^7, peptide 5), implying that *N*Me-DNal(2′)^7 causes a drastic loss of binding affinity at hMC4R. cAMP assays show that peptide 3, with *N*Me-Trp^9, leads to a greater loss of functional activity (cAMP activity) at hMC1R compared to that with the *N*Me-DNal(2′)^7 analogue 5, except at hMC5R. Therefore, the single N-methylated derivatives of Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ at the Trp^9 position (peptide 3) lead to a selective agonist for hMC5R.

The second group of peptides are di-N-methylated derivatives in the core sequence of Ac-Nle^4-c[Asp^5,D-Nal-(2′)^7,Lys^{10}]-NH$_2$ (peptides 7–16; Figure 1 and 2a). The first subgroup is

NMe-Lys^{10} + NMeXaa (peptides **7–10**; Xaa refers to the rest of the amino acids in the cyclic structure of Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ considered for N-methylation). The results show that any site of N-methylation combined with NMe-Lys^{10} (peptides **7–10**, Figure 2) retains the same binding behavior as that of the first group for hMC1R, hMC3R, and hMC5R and that a loss of binding affinity by 1 to 2 orders of magnitude occurs at hMC4R. The exception is peptide **9**, which lost binding affinity for all subtypes of melanocortin receptors, possibly due to NMe-DNal(2′)^7. It is interesting to note that when both of the positively charged amino acids (Arg^8 and Lys^{10}) are N-methylated (peptide **8**) the binding affinity for hMC4R decreases by 3 orders of magnitude but increases at hMC3R. Therefore, peptide **8** becomes a potent selective antagonist of hMC3R (with pA$_2$ value 9.1) and a potent selective agonist for hMC1R. This observation agrees with the previous studies using chimeric human melanocortin 4 receptor and truncation studies demonstrating that Arg^8 is critical for binding at hMC4R.[42] The N-methylation of Arg8 changes Φ and Ψ space and reduces the flexibility of the charged side chain group, which, in turn, reduces the binding affinity toward the receptor. When NMe-DNal(2′)^7 is included (peptide **9**), binding affinity is drastically lost at hMC1R, hMC3R, and hMC4R. Hence, peptide **9** is a very selective potential antagonist for hMC5R. The second subgroup of di-N-methylated Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ analogues is NMe-Trp^9 + NMeXaa (peptides **7** and **11–13**). Peptide **7** retains the same binding affinities and functional activities as those of the mono-N-methylated derivatives of Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$. Peptides **11–13** all show potential antagonist activity at hMC1R, hM3R, and hMC4R based on the EC$_{50}$ and Act% values. In the third subgroup, consisting of NMe-Arg^8 + NMeXaa (peptides **8**, **11**, **14**, and **15**), the combination with NMe-Arg^8 shows no significant changes in the binding behavior compared to that of the mono-N-methylated compounds, except for hMC4R, as mentioned above. cAMP agonist activity at hMC5R is lost when it is combined with NMe-His. Interestingly, when NMe-Arg^8 is combined with NMe-DNal(2′)^7 (peptide **14**), partial agonist activity is observed at hMC1R, hMC3R, and hMC4R. Among the fourth subgroup of NMe-DNal(2′)^7 + NMeXaa peptides (peptides **9**, **12**, **14**, and **16**), the combination with NMe-DNal(2′)^7 showed a significant loss of binding affinity at all receptor subtypes. The fifth and last of the di-N-methylated Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ compounds is NMe-His^6 + NMeXaa (peptides **10**, **13**, **15**, and **16**). When NMe-His^6 is involved in di-N-methylated analogues, the agonist activity at hMC5R is diminished compared to that of the first subgroup. When the di-N-methylated combination of NMe-His^6 and NMe-Trp^9 is used, the binding affinity is often reduced by 1 order of magnitude and the functional cAMP activities are lost at the hMC1R, as was observed with the mono-N-methylated compounds (peptides **7**, **12**, and **13**). When two N-methylated aromatic residues (NMe-His^6 and NMe-DNal(2′)^7, peptide **16**) are used in combination, all of the agonist activities are lost and most of the binding affinities and cAMP activities are greatly diminished. However, the combination of NMe-His^6 with positive charged residues NMe-Lys^{10} and NMe-Arg^6 (peptides **10** and **15**) shows increased, selective agonist activities at hMC1R. Peptide **15** is a selective agonist for hMC1R, with possible antagonist activities for the rest of the hMCRs. It has a 6-fold selective, potent antagonist activity at hMC3R with a pA$_2$ value of 8.2 compared to that at hMC4R. Peptide **16**, with binding affinity and binding efficiency toward all hMCRs, has possible universal antagonist activity for all subtypes of melanocortin receptors. To our knowledge, this is the first melanotropin peptide that has possible

universal antagonist activity for all hMCRs. The fifth subgroup also presented moderate, selective antagonist for hMC5R (peptide **13**). Generally, when the N-methylated aromatic amino acids are involved in dimethylated Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ analogues (peptide **16**), most of the binding affinities and cAMP activities are greatly diminished.

The third group of peptides consists of tri-N-methylated derivatives in the core sequence of Ac-Nle^4-c[Asp^5,$_D$-Nal-(2′)^7,Lys^{10}]-NH$_2$ (peptides **17–26**; Figure 1 and 2a). Except for a few cases of dramatically increased selectivity, this group of peptides in general shows significantly reduced binding affinities, which might be due to hindrance caused by the multiple N-methylations that are involved. However, in a few cases, there was a dramatic increase in selectivity. Peptide **17**, with consecutive N-methylations at Arg^8-Trp^9-Lys^{10}, leads to a selective partial agonist ligand at hMC4R. Compared to hMC4R, its binding affinity toward hMC3R is more potent than toward hMC4R, with binding affinities of 9.7 and 203 nM, respectively. The pA$_2$ value for peptide **17** at hMC3R is 8.5. The combination of NMe-DNal(2′)^7, NMe-Trp^9, and NMe-Lys^{10} (peptide **18**) shows full agonist activity at hMC1R and partial agonist activity at hMC4R. The combination of NMe-His^6, NMe-Trp^9, and NMe-Lys^{10} (peptide **19**) shows good binding to all hMCRs but no activity to any hMCR. The pA$_2$ values for peptide **19** at hMC1R, hMC3R, hMC4R, and hMC5R are 7.5, 8, 8, 7.4, respectively. Hence, in peptide **19**, a potent, universal antagonist is discovered. Peptide **20** also shows potential antagonist activity at all 4 hMCRS (with a very weak activity at hMC3R). For the combination of NMe-DNal(2′)^7 with positively charged residues NMe-His^6 and NMe-Lys^{10} (peptide **22**) or NMe-Arg^8 and NMe-Trp^9 (peptide **23**), all functional activities are lost. The combination of NMe-His^6, NMe-Trp^9, and NMe-Arg^8 (peptide **24**) shows selective partial agonist activity at hMC1R and hMC5R. This observation is consistent with our earlier Ac-Nle^4-c[Asp^5,$_D$Phe^7, Lys^{10}]-α-MSH(4–10)-NH$_2$ N-methylation studies.[41] In this group, peptide **19** is an antagonist toward all hMCRs with reduced binding affinities compared to those of the mono-N-methylated group of peptides (peptide **2–6**), whereas peptide **21** retains agonist activity at hMC5R. It is noteworthy that when NMe-DNal(2′)^7 is involved nearly all of the binding affinities are reduced (peptides **20**, **22**, **23**, **25**, and **26**).

The fourth group of peptides has five different tetra-N-methylated Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ derivatives (peptides **27–31**; Figure 1 and 2a). The studies show that the sites of N-methylation when combined with NMe-DNal(2′)^7 reduce the binding affinity at all hMCRs. Only compound **28** retains nanomolar binding at all hMCRs, whereas cAMP activity for this peptide shows agonist activity at hMC1R and hMC5R and potential antagonist activity at hMC3R and hMC4R, much like Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$.

The final penta-N-methylated compound **32** shows potent binding at hMC1R, with an IC$_{50}$ of 68 nM (91% binding efficiency), poor binding efficiency at hMC5R (37%), and micromolar binding at hMC3R and hMC4R. Therefore, on the basis of its binding affinity, it has potent and selective binding to hMC1R, with a pA$_2$ value of 8.8, consistent with its binding affinity. In the cAMP assay, the analogue shows much lower activation on peptide stimulation (lower Act% shown in Table 3); it was inactive at all hMCRs.

Comparison of N-Methylation on Ac-Nle^4-c[Asp^5,$_D$Phe^7, Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) and Ac-Nle^4-c-[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ (SHU9119)

Our earlier N-methylation studies on Ac-Nle^4-c[Asp^5,$_D$-Phe^7, Lys^{10}]-α-MSH(4–10)-NH$_2$ demonstrated that N-methylation of cyclized melanotropins increased the selectivities for the hMCRs. For example, N-methylation of Ac-Nle^4-c[Asp^5,$_D$Phe^7, Lys^{10}]-α-MSH(4–10)-NH$_2$ led to the most selective agonist for hMC1R, which is also a specific biomarker for melanoma cells.[41] The present results showed that N-methylation of Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ also leads to selective agonists and antagonists toward various hMCRs, but it does so with a much broader spectrum of selectivity compared to that for N-methylated Ac-Nle^4-c[Asp^5,$_D$-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$. In Figure 2, the red spots demonstrate increases and decreases in agonist activity at all subtypes of melanocortin receptors. In this study, we have found selective hMC1R agonists (peptide **8**, **10**, **15**) and the most selective highly potent hMC1R antagonist (peptide **32**), with a pA$_2$ value of 8.8. In addition to possible selective antagonists for hMC3R (peptides **8**, **15**, **17**), with pA$_2$ values of 9.1, 8.2, and 8.5, respectively, a highly selective hMC5R agonist (peptide **3**) and antagonists (peptides **9** and **13**) were found. Finally, through this study, we discovered a unique universal antagonist (peptide **19**) for all four hMCRs, which is an important advancement for examining the biological roles of the endogenous agonist MSH system. Direct comparison of binding and activity values for the same methylation pattern shows that N-methylation influences structural changes of Ac-Nle^4-c[Asp^5,$_D$-Phe^7,Lys^{10}]-α-MSH-(4–10)-NH$_2$ (MT-II) and Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ (SHU9119) in different ways. It should not be expected that influencing both compounds in a parallel manner will lead to similar changes in binding and activity properties for each methylation pattern because the difference between Ac-Nle^4-c[Asp^5,$_D$-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) and Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ (SHU9119) involves substitution of $_D$-Phe^7 by the bulky DNal(2′)^7. Since this amino acid is presented in Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ but not in Ac-Nle^4-c[Asp^5,$_D$-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$, any N-methylation on these aromatic amino acids and combination with other N-methylated amino acids might induce different conformational changes that can dramatically change binding and functional selectivity properties. Introducing N-methylation on amide NHs of aromatic amino acids can be expected to reorient the backbone conformation and side chain stacking (aromatic groups), all of which can be important factors for melanocortin receptor subtype selectivity.

Binding Assays of Selective hMC1R Ligands at Human Melanoma Cells

The above-discussed SAR studies revealed that peptide **15** is a quite good, selective agonist of hMC1R. We have therefore further investigated binding and cAMP activities in human melanoma cells (A375). As shown in Table 4, these peptides have nanomolar binding affinities for A375 melanoma cells. The cAMP activities of these two peptides in melanoma cells are different from those for HEK 293 cells expressing hMC1R. This is due to the different expression level of hMC1R between these two cell lines. Another possible reason could be the different signaling system in these two cell lines. Nevertheless, these two peptides can be used to target melanoma cells.

CONCLUSIONS

A lack of selectivity and potency in the agonist or antagonist properties of melantroptin ligands for the melanocortin receptor subtypes is still the most difficult hurdle for their application in medicine. To ameliorate the biological properties of melanocortin ligands and to achieve selective agonists and antagonists for melanocortin receptors via the conformational modulation of the peptide backbone that is imparted by N-methyl steric constraints, mono-and multiply N-methylated analogues of the Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ peptide were investigated. It was found that the activity and selectivity profile induced by N-methylation is very different for Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ compared to those resulting from a similar approach using Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$. This is striking as both stem peptides differ only in the exchange of one amino acid (DNal(2′)^7 instead of D-Phe^7 in Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$).

The systematic N-methylation of Ac-Nle^4-c[Asp^5,D-Nal-(2′)^7,Lys^{10}]-NH$_2$ leads to a highly selective antagonist of hMC1R (peptide **32**) based on binding affinity. This strategy still retains several hMC1R agonists (peptides **8**, **10**, and **15**) as previously described.[41] As anticipated, we have found a selective agonist for hMC5R (peptide **3**). The selective antagonist for hMC3R (peptide **17**) discovered in this study can be potentially used to treat obesity and diabetes. Apart from this, the newly discovered peptide **19** is a universal antagonist of the hMCR system, which could be important to modulate the endogenous agonist MSH in the melanocortin system. These results strengthen the role of our earlier discovered D-Trp^8-γ-MSH as a selective agonist of hMC3R. Further conformational and topographical characterization of all of the important peptides discussed here to obtain a more detailed correlation between melanocortin ligand conformation and the observed receptor discrimination is in progress.

The results obtained from multiply the N-methylated Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$ peptide along with those from multiply N-methylated Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) will help to understand the finer details of the functional properties of melanocortin receptors and the bioactive conformational preferences of the ligands required by them. The studies further highlight the synthetic simplicity and impact of the N-methylation strategy on finetuning the conformational preferences of the ligands to achieve the desired biological effects. The studies, in general, are an encouragement toward a future for peptide chemistry for the development of peptide therapeutic agents.

EXPERIMENTAL SECTION

Materials

N^α-Fmoc-amino acids, peptide coupling reagents, Rink amide MBHA resin, and solvents were reagent grade and used without further purification unless otherwise specified. These chemicals were obtained from Aldrich, Novabiochem, Iris Biotech GmbH, Merck, NeoMPS, ORPEGEN Pharma, and GLS. The following amino acids were used: Fmoc-Lys(Alloc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg-(Pbf)-OH, Fmoc-DNal(2′)-OH, Fmoc-His(Trt)-OH, Fmoc-Asp-(OAllyl)-OH, and Fmoc-Nle-OH. The polypropylene reaction vessels (syringes with

frits) were purchased from B. Braun Melsungen AG. The purity of the peptides was checked by analytical reverse-phase HPLC using an Amersham Pharmacia Biotech Äkta Basic 10F with a ODS-A C18 (120 Å, 5 μm, 250 mm × 4.6 mm) column (Omnicrom YMC Europe GmbH) monitored at 220 and 254 nm and by high-resolution mass spectral analysis

Synthesis

The Ac-Nle[4]-c[Asp[5],D-Nal(2′)[7],Lys[10]]-NH$_2$ analogues were synthesized manually by Fmoc-SPPS and characterized using methods described in Doedens et al.[41] All physicochemical data for each new peptide is available in Table 1. All peptides were HPLC-purified using a ODS-A C18 (120 A, 5 μm, 250 mm × 20 mm) column (Omnicrom YMC Europe GmbH). For purification, a linear gradient of acetonitrile and water containing 0.1% TFA was used. All peptides when purified exhibited 95% purity, as analyzed by analytical HPLC and HPLC-MS.

Biological Activity Assays

Receptor Binding Assay—Competition binding experiments were carried out using both HEK 293 and A375 melanoma (ATCC) cell lines. HEK 293 cells stably expressed human MC1, MC3, MC4, and MC5 receptors as described before. HEK 293 cells transfected with hMCRs were seeded on 96-well plates 48 h before the assay was performed (50 000 cells/well). For the assay, the cell culture medium was aspirated and the cells were washed once with freshly prepared minimum essential medium (MEM) containing 100% minimum essential medium with Earle's salt (MEM, GIBCO) and 25 mM sodium bicarbonate. Next, the cells were incubated for 40 min at 37 °C with different concentrations of unlabeled peptide and labeled [^{125}I]-[Nle[4],D-Phe[7]]-α-MSH (PerkinElmer Life Science, 20 000 cpm/well, 33.06 pM) diluted in 125 μL of freshly prepared binding buffer containing 100% MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. The assay medium was subsequently removed, and the cells were washed once with basic medium and then lysed by the addition of 100 μL of 0.1 M NaOH and 100 μL of 1% Triton X-100. The total labeled [^{125}I]-[Nle[4],D-Phe[7]]-α-MSH detected in lysed cells was measured by a Wallac Micro β-TriLux 1450 LSC and luminescence counter (PerkinElmer Life Science, Boston, MA) (Table 2).

The binding studies with melanoma cells were done using the same methodology, and the results are presented in Table 4.

Adenylate Cyclase Assay—HEK 293 cells transfected with human melanocortin receptors were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 h before the assay was performed (50 000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 100 μL of MEM buffer (GIBCO). An aliquot (100 μL) of Earle's balanced salt solution with 5 nM isobutylmethylxanthine (IBMX) was placed in each well for 1 min at 37 °C. Next, aliquots (25 μL) of melanotropin peptides of varying concentration were added, and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the assay buffer and adding 60 μL of ice-cold Tris/EDTA buffer to each well followed by placing the plates in a

boiling water bath for 7 min. The cell lysates were then centrifuged for 10 min at 2300*g*. A 50 μL aliquot of the supernatant was transferred to another 96-well plate, and placed, along with 50 μL of [^{3}H] cAMP and 100 μL of protein kinase A (PKA) buffer, in an ice bath for 2–3 h. The PKA buffer consisted of Tris/EDTA buffer with 60 μg/mL PKA and 0.1% bovine serum albumin by weight. The incubation mixture was filtered through 1.0 μm glass fiber filters in MultiScreen-FB 96-well plates (Millipore, Billerica, MA). The total [^{3}H] cAMP was measured by a Wallac Micro β-TriLux 1450 LSC and luminescence counter (PerkinElmer Life Science, Boston, MA). The cAMP accumulation data for each peptide analogue was determined with the help of a cAMP standard curve generated by the same method as described above. The maximal cAMP produced at 10 μM of each ligand was compared to the amount of cAMP produced at 10 μM of the standard agonist, Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II), and is expressed as a percentage (% max effect) in Table 3. The antagonist properties of the lead compounds were evaluated by their ability to competitively displace the Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II) agonist in a dose-dependent manner at concentrations up to 10 μM.

The cAMP assays for the melanoma cell line were done using the same methods as above, and the results are given in Table 4.

Data Analysis—IC$_{50}$ and EC$_{50}$ values represent the mean of two experiments performed in triplicate. IC$_{50}$ and EC$_{50}$ estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis with GraphPad Prism 5 (GraphPad Software, San Diego, CA). pA$_2$ analysis was done with a Schild plot followed by the cAMP assay.

ACKNOWLEDGMENTS

ABBREVIATIONS USED

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature, *J. Biol. Chem.*, 1972, 247, 977–983;

Boc	*tert*-butyloxycarbonyl
Fmoc	fluorenylmethoxycarbonyl
CH$_3$CN	acetonitrile
Cl-HOBt	1-hydroxy-6-chlorobenzotriazole
DCM	dichloromethane
DIPEA	diisopropylethylamine

DMF	*N,N*-dimethylformamide
DIC	diisopropyl carbodiimide
HOBt	*N*-hydroxybenzotriazole
_h_MCR	human melanocortin receptor
MSH	melanocyte-stimulating hormone
Nal(2')	2′-naphthylalanine
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
TFA	trifluoroacetic acid
SPPS	solid-phase peptide synthesis
RP-HPLC	reverse-phase high-performance liquid chromatography
Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]-NH$_2$	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH$_2$ (cyclic analogue of α-MSH)
Ac-Nle^4-c[Asp^5,D-Nal(2′)^7,Lys^{10}]-NH$_2$	Ac-Nle-c[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH$_2$ (cyclic analogue of α-MSH)
GPCR	G protein coupled receptor

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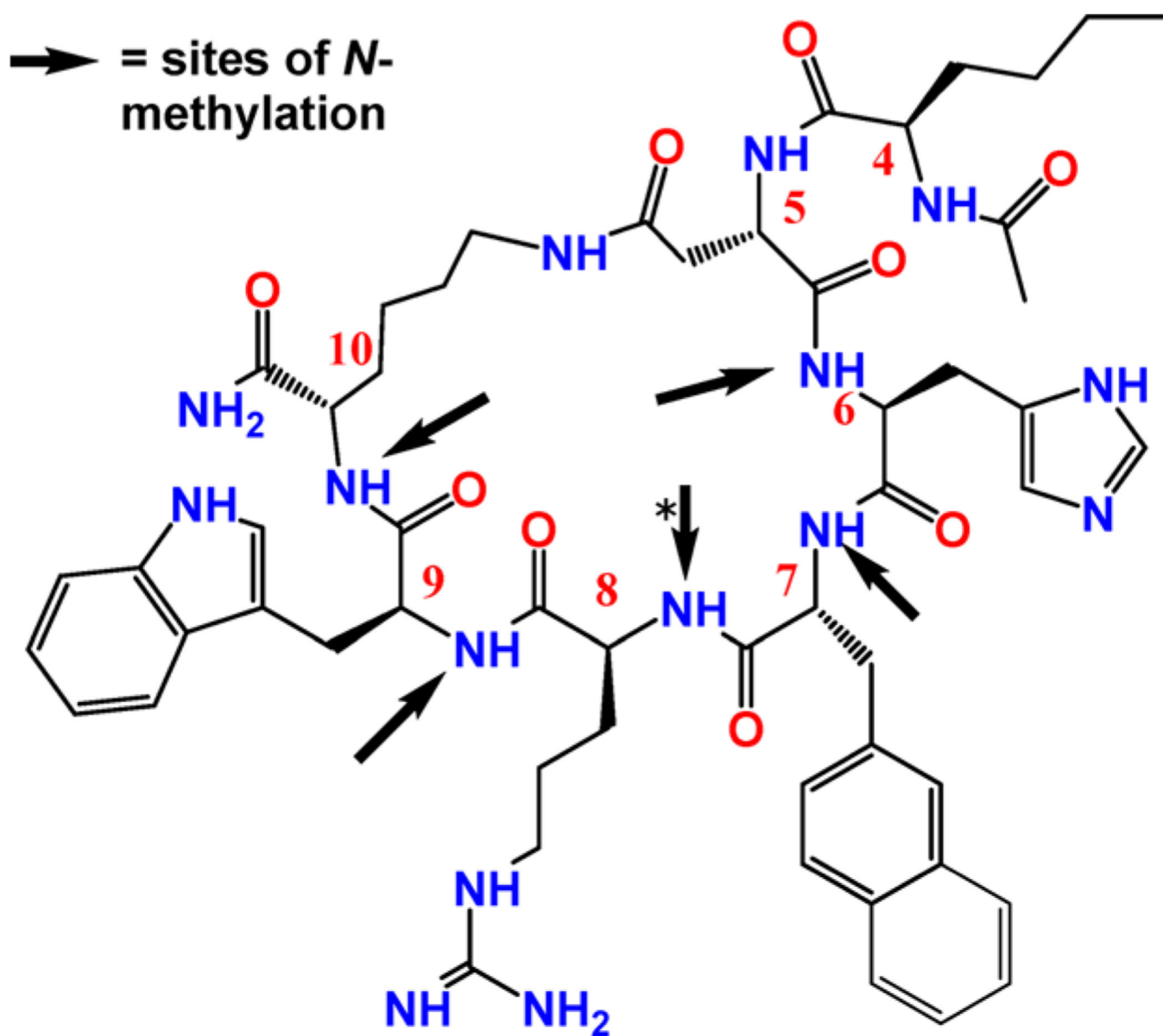
Figure 1.
Structure of Ac-Nle[4]-c[Asp[5],D-Nal(2′)[7],Lys[10]]-NH$_2$ peptide. The arrows indicate the sites of N-methylation in analogues synthesized, with all combinations examined systematically (see text).
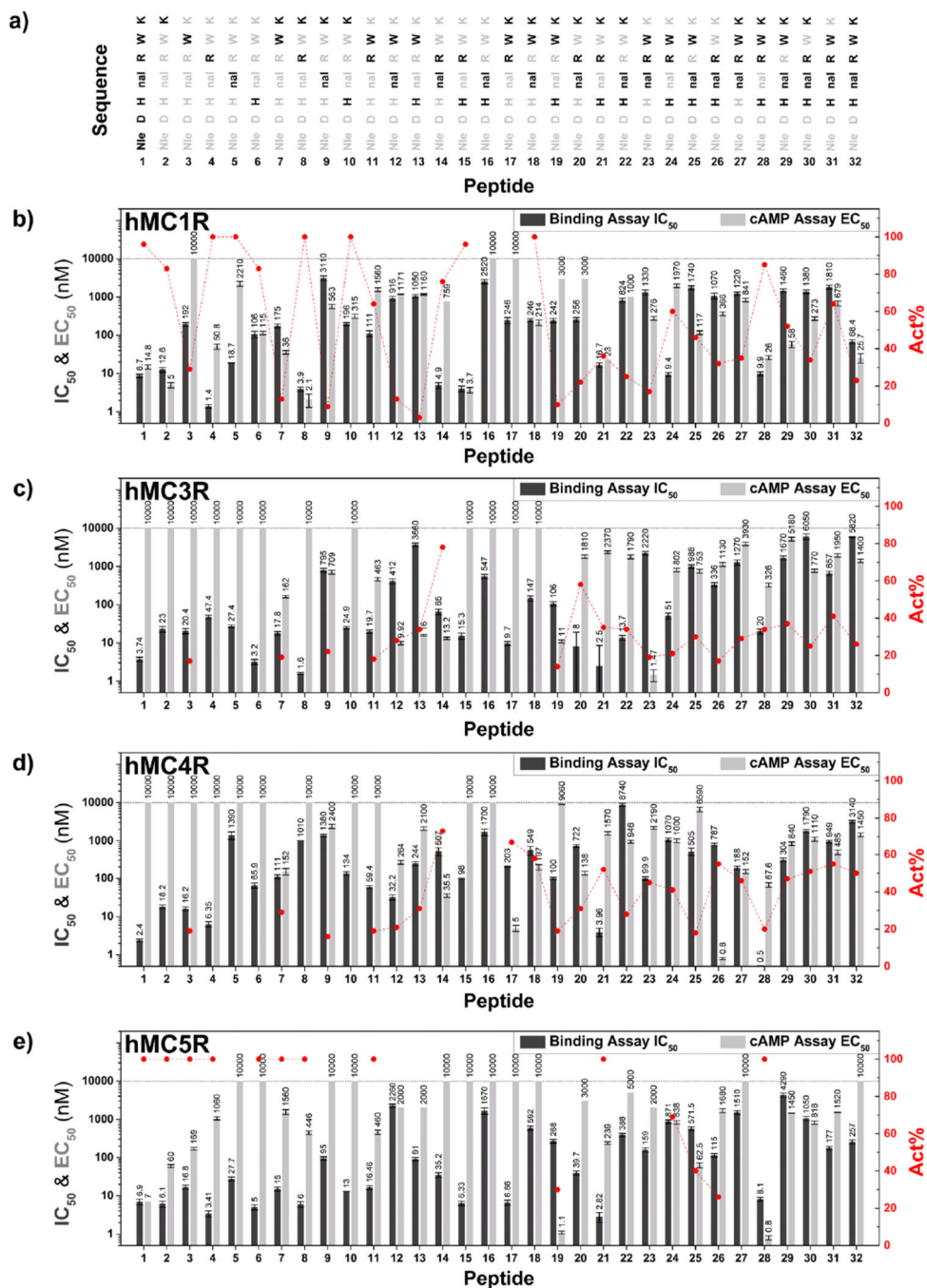
Figure 2.
(a) Amino acid sequences for Ac-Nle[4]-c[Asp[5],D-Nal(2′)[7],Lys[10]]-NH$_2$ (peptide **1**) and N-methylated Ac-Nle[4]-c[Asp[5],D-Nal(2′)[7],Lys[10]]-NH$_2$ analogues (peptides **2–32**; the site of N-methylation is highlighted in bold. (b–e) Histograms showing the results for IC$_{50}$ from binding assays (black bars) and EC$_{50}$ from cAMP assays (light gray bars) at hMC1R, hMC3R, hMC4R, and hMC5R, respectively. Although IC$_{50}$ and EC$_{50}$ values on the *y* axis are in real number units, for convenient representation and comparison, log$_{10}$ scaling is used on the *y* axis. The specific IC$_{50}$ and EC$_{50}$ values for each peptide are indicated above its

corresponding histogram bar. Act% values (>0%) of the ligands for the cAMP assay are plotted in red with a second y dimension on the right. The location of the red spots demonstrates increased and decreased agonist activity. For more details, including the binding efficiency, refer to Tables 2 and 3. IC_{50}, concentration of peptide at 50% specific binding ($N = 4$); EC_{50}, effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$).

Table 1

HPLC and Mass Data for N-Methylated Ac-Nle[4]-c[Asp[5],$_D$-Nal(2')[7],Lys[10]]-NH$_2$ Peptides[a]

#	Sequence							Calculated Mass (M+H)+	ESI-MS (M+H)+	RP-HPLC 10–100% in 30 min [tR] min	RP-HPLC 10–50% in 30 min [tR] min
1	Nle	D	H	DNal(2')	R	W	K	1074.6	1074.7	15.58	26.70
2	Nle	D	H	DNal(2')	R	W	K	1088.6	1088.8	16.16	28.13
3	Nle	D	H	DNal(2')	R	W	K	1088.6	1088.8	14.80	24.52
4	Nle	D	H	DNal(2')	R	W	K	1088.6	1088.8	13.71	22.73
5	Nle	D	H	DNal(2')	R	W	K	1088.6	1088.8	14.07	23.80
6	Nle	D	H	DNal(2')	R	W	K	1088.6	1089.1	15.82	27.57
7	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	15.86	27.28
8	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	13.89	23.08
9	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	14.43	24.21
10	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.6	15.71	27.28
11	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.6	14.02	22.90
12	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	14.09	23.52
13	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.6	15.52	26.33
14	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	13.93	23.28
15	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	13.93	23.12
16	Nle	D	H	DNal(2')	R	W	K	1102.6	1102.7	15.25	25.94
17	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	13.80	22.75
18	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	14.55	24.51
19	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	15.98	27.65
20	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	13.98	23.27
21	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	14.17	23.77
22	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	14.49	24.48
23	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	13.91	23.05
24	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	13.69	22.57
25	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	14.87	25.08
26	Nle	D	H	DNal(2')	R	W	K	1116.6	1116.7	14.00	23.24

#	Sequence						Calculated Mass $(M+H)^+$	ESI-MS $(M+H)^+$	RP-HPLC 10–100% in 30 min [t_R] min	RP-HPLC 10–50% in 30 min [t_R] min
27	Nle	D	H	DNal(2′)	R	W	K	1130.6	1130.7	14.35	24.57
28	Nle	D	H	DNal(2′)	R	W	K	1130.6	1130.7	13.97	22.95
29	Nle	D	H	DNal(2′)	R	W	K	1130.6	1130.7	15.05	25.65
30	Nle	D	H	DNal(2′)	R	W	K	1130.6	1130.8	14.45	24.09
31	Nle	D	H	DNal(2′)	R	W	K	1130.6	1130.7	13.40	22.05
32	Nle	D	H	DNal(2′)	R	W	K	1144.6	1144.7	14.17	23.73

[a] Amino acids with N-methyl groups are highlighted in gray. The calculated mass $(M + H)^+$ for each peptide provides the theoretically calculated mass based on light isotope composition; ESI-MS $(M + H)^+$ represents the experimentally determined mass. For all peptides, purity was characterized based on RP-HPLC measurements using a C18 column and employing a linear gradient of acetonitrile (MeCN) and water. All peptides showed a purity 95%. The gradients 10–100% and 10–50% indicate solvent gradients of MeCN in water from 10 to 100% in 30 min and from 10 to 50% in 30 min, respectively. Last two columns give RP-HPLC retention times [t_R] (in min) for each peptide.

Table 2

Binding Assay Results of N-Methylated Ac-Nle^4-c[Asp^5,$_D$-Nal(2')^7,Lys^{10}]-NH$_2$ Analogues at hMCRs^a

#	sequence							hMC1R IC$_{50}$, nM	Bind-Effic	hMC3R IC$_{50}$, nM	Bind-Effic	hMC4R IC$_{50}$, nM	Bind-Effic	hMC5R IC$_{50}$, nM	Bind-Effic
1	Nle	D	H	DNal(2')	R	W	K	8.7±0.9	100	3.74±0.5	97	2.4±0.2	98	6.9±1.2	98
2	Nle	D	H	DNal(2')	R	W	K	12.6±1.8	100	23±4	97	18.2±2.1	97	6.1±1.1	98
3	Nle	D	H	DNal(2')	R	W	K	192±20	77	20.4±3	77	16.2±2	70	16.8±2.3	77
4	Nle	D	H	DNal(2')	R	W	K	1.4±0.15	90	47.4±5.2	95	6.35±1.0	90	3.41±0.6	96
5	Nle	D	H	DNal(2')	R	W	K	18.7±0.30	80	27.4±2.1	90	1390±310	90	27.7±3.4	90
6	Nle	D	H	DNal(2')	R	W	K	106±21	90	3.2±0.5	90	65.9±10	90	5±0.8	90
7	Nle	D	H	DNal(2')	R	W	K	175±20	86	17.8±2	95	111±13	89	15±1.8	94
8	Nle	D	H	DNal(2')	R	W	K	3.9±0.5	80	1.6±0.1	90	1010±1.1	90	6±1.0	90
9	Nle	D	H	DNal(2')	R	W	K	3110±456	82	795±90	90	1380±110	101	95±10	87
10	Nle	D	H	DNal(2')	R	W	K	196±16	90	24.9±2.1	99	134±15	90	13±0.12	90
11	Nle	D	H	DNal(2')	R	W	K	111±20	89	19.7±2	93	59.4±6	89	16.46±2.1	93
12	Nle	D	H	DNal(2')	R	W	K	916±120	92	412±60	92	32.2±5	88	2260±230	72
13	Nle	D	H	DNal(2')	R	W	K	1050±116	88	3660±410	95	244±31	90	91±9	86
14	Nle	D	H	DNal(2')	R	W	K	4.9±0.9	90	65±11	90	507±120	90	35.2±5	90
15	Nle	D	H	DNal(2')	R	W	K	4.0±0.7	90	15.3±2.8	80	98±4.1	90	6.33±0.9	90
16	Nle	D	H	DNal(2')	R	W	K	2520±320	86	547±70	80	1700±340	90	1670±300	90
17	Nle	D	H	DNal(2')	R	W	K	246±45	80	9.7±1.2	90	203±7	80	6.66±1	90
18	Nle	D	H	DNal(2')	R	W	K	246±24	80	147±23	90	549±150	90	592±70	80
19	Nle	D	H	DNal(2')	R	W	K	242±32	74	106±13	90	100±8	79	268±31	91
20	Nle	D	H	DNal(2')	R	W	K	256±35	84	8±11	86	722±81	81	39.7±5	90
21	Nle	D	H	DNal(2')	R	W	K	16.7±2.2	9	2.5±06	94	3.96±1	76	2.82±0.8	90
22	Nle	D	H	DNal(2')	R	W	K	824±100	68	13.7±2	38	8740±841	59	388±41	70
23	Nle	D	H	DNal(2')	R	W	K	1330±200	70	2220±242	61	99.9±10	36	159±19	67
24	Nle	D	H	DNal(2')	R	W	K	9.4±1.2	86	51±9	78	1070±110	76	871±91	84
25	Nle	D	H	DNal(2')	R	W	K	1740±210	68	988±110	90	505±111	67	571.5±61	93
26	Nle	D	H	DNal(2')	R	W	K	1070±189	93	336±42	82	787±82	68	115±13	92
27	Nle	D	H	DNal(2')	R	W	K	1220±123	94	1270±210	92	188±21	37	1510±159	32

#	sequence							hMC1R IC$_{50}$, nM	Bind-Effic	hMC3R IC$_{50}$, nM	Bind-Effic	hMC4R IC$_{50}$, nM	Bind-Effic	hMC5R IC$_{50}$, nM	Bind-Effic
28	Nle	D	H	DNal(2′)	R	W	K	9.9±1.2	93	20±3	88	0.5±0.02	64	8.1±1	89
29	Nle	D	H	DNal(2′)	R	W	K	1460±163	42	1670±193	97	304±35	27	4290±521	64
30	Nle	D	H	DNal(2′)	R	W	K	1380±150	87	6050±985	93	1790±190	24	1050±120	24
31	Nle	D	H	DNal(2′)	R	W	K	1810±210	89	657±76	94	949±85	8	177±21	75
32	Nle	D	H	DNal(2′)	R	W	K	68.4±8	91	5820±200	84	3140±270	19	257±34	37

[a] Amino acids with N-methyl groups are highlighted in gray. IC50, concentration of peptide at 50% specific binding (N = 4), NB, 0% 125I-NDP-α- MSH displacement observed at 10 µM. Percent binding efficiency, maximal % of 125I-NDP-α-MSH displacement observed at 10 µM.

Table 3

cAMP Assay Results of N-Methylated Ac-Nle^4-c[Asp^5,$_D$-Nal(2′)^7,Lys^{10}]-NH$_2$ Analogues at hMCRs^a

# sequence								hMC1R EC$_{50}$, nM	%max	hMC3R EC$_{50}$, nM	%max	hMC4R EC$_{50}$, nM	%max	hMC5R EC$_{50}$, nM	%max
1	Nle	D	H	DNal(2′)	R	W	K	14.8±2.0	96	>10000	NA	>10000	NA	7	100
2	Nle	D	H	DNal(2′)	R	W	K	5.0±0.8	83	>10000	NA	>10000	NA	60±5	100
3	Nle	D	H	DNal(2′)	R	W	K	>10000	29	>10000	17	>10000	19	169±14	100
4	Nle	D	H	DNal(2′)	R	W	K	50.8±8	100	>10000	NA	>10000	NA	1060±100	100
5	Nle	D	H	DNal(2′)	R	W	K	2210±310	100	>10000	NA	>10000	NA	>10000	NA
6	Nle	D	H	DNal(2′)	R	W	K	115±12	83	>10000	NA	>10000	NA	>10000	100
7	Nle	D	H	DNal(2′)	R	W	K	36±4	13	162±12	19	152±30	29	1560±210	100
8	Nle	D	H	DNal(2′)	R	W	K	2.1±0.8	100	>10000	NA*	>10000	NA	446±50	100
9	Nle	D	H	DNal(2′)	R	W	K	563±70	9	709±87	22	2400±290	16	>10000	NA
10	Nle	D	H	DNal(2′)	R	W	K	315±40	100	>10000	NA	>10000	NA	>10000	NA
11	Nle	D	H	DNal(2′)	R	W	K	1560±170	64	463±52	18	>10000	19	460±61	100
12	Nle	D	H	DNal(2′)	R	W	K	1171±21	13	9.92±1	28	264±31	21	>2000	NA
13	Nle	D	H	DNal(2′)	R	W	K	1160±52	3	16±0.8	34	2100±231	31	>2000	NA
14	Nle	D	H	DNal(2′)	R	W	K	759	76	13.2±0.9	78	35.5±3.9	73	>10000	NA
15	Nle	D	H	DNal(2′)	R	W	K	3.7±0.6	96	>10000	NA*	>10000	NA	>10000	NA
16	Nle	D	H	DNal(2′)	R	W	K	>10000	NA	>10000	NA	>10000	NA	>10000	NA
17	Nle	D	H	DNal(2′)	R	W	K	>10000	NA	>10000	NA*	5±0.9	67	>10000	NA
18	Nle	D	H	DNal(2′)	R	W	K	214±35	100	>10000	NA	197±29	58	>10000	NA
19	Nle	D	H	DNal(2′)	R	W	K	>3000	10*	11.±1	14*	9060±110	19*	1.1±0.1	30*
20	Nle	D	H	DNal(2′)	R	W	K	>3000	22	1810±190	58	138±15	31	>3000	NA
21	Nle	D	H	DNal(2′)	R	W	K	23	36	2370±180	35	1570±170	52	239±20	100
22	Nle	D	H	DNal(2′)	R	W	K	>1000	25	1790±213	34	946±78	28	>5000	NA
23	Nle	D	H	DNal(2′)	R	W	K	276±32	17	1.47±0.5	19	2190±178	45	>2000	NA
24	Nle	D	H	DNal(2′)	R	W	K	1970±213	60	802±91	21	1000±120	41	838±90	69
25	Nle	D	H	DNal(2′)	R	W	K	117±13	46	753±89	30	6590±912	18	62.5±9	40
26	Nle	D	H	DNal(2′)	R	W	K	366±41	32	1130±134	17	0.8±0.05	55	1680±192	26

#	sequence							hMC1R EC$_{50}$, nM	%max	hMC3R EC$_{50}$, nM	%max	hMC4R EC$_{50}$, nM	%max	hMC5R EC$_{50}$, nM	%max
27	Nle	D	H	DNal(2')	R	W	K	841±91	35	3930±410	29	152±19	46	>10000	NA
28	Nle	D	H	DNal(2')	R	W	K	26±3	85	326±41	34	67.6±9	20	0.8±0.1	100
29	Nle	D	H	DNal(2')	R	W	K	58±11	52	5180±589	37	840±93	47	1450±14	NA
30	Nle	D	H	DNal(2')	R	W	K	273±31	34	770±77	25	1110±141	51	818±83	NA
31	Nle	D	H	DNal(2')	R	W	K	679±98	64	1950±200	41	485±67	55	1520±19	NA
32	Nle	D	H	DNal(2')	R	W	K	25.7±7	23*	1400±150	26	1450±1 62	50	>10000	NA

[a] Amino acids with N-methyl groups are highlighted in gray. EC50, effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$). Percent activity, percent of cAMP produced at 10 µM ligand concentration in relation to Ac-Nle^4-c[Asp^5,$_D$-Phe^7,Lys^{10}]-α-MSH(4–10)-NH$_2$ (MT-II). NA, 0% cAMP accumulation observed at 10 µM. The peptides were tested at a range of concentrations from 10^{-10} to 10^{-5} M.

* Asterisks show the ligands and receptors for which we have determined pA2 values. The pA2 values for the peptide 8, 15, and 17 at the hMC3R are 9.1, 8.2, and 8.5, respectively; The pA2 values for the peptide 19 at the hMC1R, hMC3R, hMC4R, hMC5R are 7.5, 8, 8, and 7.4, respectively. The pA2 value for peptide 32 at the hMC1R is 8.8.

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Table 4

Binding and cAMP Activities of Selected Peptides at Human Melanoma Cells (A375)

Peptide	Sequence	IC$_{50}$ (nM)	Binding Efficiency	EC$_{50}$ (nM)	Act%
15	Ac-Nle-c[Asp-(NMe)His-DNal(2′)-(NMe)Arg-Trp-Lys]-NH$_2$	14.8 ± 2	100 ± 10	22 ± 2.5	NA
28	Ac-Nle-c[Asp-(NMe)His-DNal(2′)-(NMe)Arg-(NMe)Trp-(NMe)Lys]-NH$_2$	8.7 ± 1	100 ± 10	20 ±2	NA
MT-II	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH$_2$	6.7 ± 2.9	100 ± 10	110 ± 0.6	100
1	Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH$_2$	100 ± 96	80 ± 34	3.3 ± 2.6	NA