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Deletion of Ac-NMePhe¹ from [NMePhe¹]arodyn under Acidic Conditions: 2. Effects of Substitutions on Pharmacological Activity

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Abstract

Arodyn (Ac[Phe^{1,2,3},Arg⁴,D-Ala⁸]Dyn A(1-11)-NH₂) is an acetylated dynorphin A (Dyn A) analog that is a potent and selective κ opioid receptor antagonist (Bennett *et al. J. Med. Chem.* **2002**, *45*, 5617), and its analog [NMePhe¹]arodyn shows even higher affinity and selectivity for κ opioid receptors (Bennett *et al., J. Pep. Res.* **2005**, *65*, 322). However, the latter compound is prone to deletion of the Ac-NMePhe moiety from the N-terminus of the peptide during acidic cleavage as described in the accompanying paper. Several stable analogs of [NMePhe¹]arodyn and [NMePhe¹,Trp³]arodyn where the acetyl group was substituted with a heteroatom-containing group were evaluated for their opioid receptor affinity, selectivity, and efficacy. Methoxycarbonyl derivatives exhibited the highest κ opioid receptor affinity among the analogs. Additional CH₃OCO[NMePhe¹]arodyn analogs where position 3 was substituted with other aromatic or non-aromatic residues were also evaluated for κ receptor affinity, selectivity, and efficacy. [CH₃OCO-NMePhe¹]arodyn has similar κ opioid receptor affinity as [NMePhe¹]arodyn, retains high κ opioid receptor selectivity, and is a potent κ opioid receptor antagonist.

Keywords

N-methylamino acid; dynorphin analogs; arodyn; κ opioid receptor

Introduction

Clinically used drugs that interact with opioid receptors are primarily mu (μ) opioid receptor agonists (e.g. morphine) that are used as analgesics, but these agents are associated with serious side effects (respiratory depression, addiction liability and constipation).¹ Therefore, there is considerable interest in developing agents for other opioid receptors, namely kappa (κ) and delta (δ) opioid receptors, as analgesics and for the treatment of other disorders. Peripherally selective κ opioid agonists have shown promise because of the analgesic activity of these compounds in visceral pain models, especially in conditions involving inflammation,² without centrally mediated side effects (e.g. dysphoria^{3,4}).

Kappa receptor antagonists were initially used only as pharmacological tools to study the pharmacology of κ opioid receptors, but recent reports sparked interest in their potential clinical applications. Kappa receptor antagonists have demonstrated antidepressant⁵ and anti-anxiety^{6,7} activity. They also have potential use in the treatment of cocaine^{8,9} and opioid dependence.^{10,11} Several selective nonpeptide κ opioid receptor antagonists have been discovered during the past two decades. The bivalent ligand nor-binaltorphimine (nor-BNI)¹² is a potent and selective κ opioid receptor antagonist that has been used extensively in pharmacological studies.¹³ Extensive structure-activity relationship (SAR) studies of the δ opioid receptor antagonist naltrindole resulted in the identification of 5'-guanidinylnaltrindole (5'-GNTI) as a κ opioid receptor selective antagonist.¹⁴ JD₁Tic is a phenylpiperidine derivative that is also a potent antagonist for κ opioid receptors and shows very high κ opioid receptor affinity and selectivity.¹⁵ However, all three antagonists exhibit extremely long activity (ranging from several days to several weeks after a single dose),^{13,16-19} thus limiting their use as pharmacological tools and potentially as therapeutic agents.

The endogenous ligands for κ opioid receptors are peptides. We are exploring the structure-activity relationships (SAR) of the endogenous peptide Dyn A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), with an emphasis on analogs that are selective κ opioid receptor antagonists. It has been proposed that peptide and nonpeptide ligands bind to different domains of the κ opioid receptor,²⁰⁻²² and therefore selective peptide antagonists are complementary to nonpeptide antagonists. Furthermore, peptide derivatives are expected to have shorter durations of action due to metabolism by proteases, and therefore peptide antagonists could overcome the problems associated with long-acting nonpeptide antagonists.

Several derivatives of Dyn A with antagonist activity have been reported.¹ Early analogs generally exhibited weak antagonist activity and low κ opioid receptor selectivity, but recently more selective and potent analogs have been reported. [Pro³]Dyn A-(1-11)NH₂ has high affinity and selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/2110/3260) for κ opioid receptors, but this ligand exhibits only weak antagonist potency in functional assays.²³ Its Arg⁸ analog shows higher κ affinity without compromising the high κ selectivity.²⁴ Dynant¹ ([^{2S}]-Mdp¹]Dyn A-(1-11)NH₂, Mdp = 2-methyl-3-(2',6'-dimethyl-4-hydroxyphenyl)propionic acid), with modification of the first amino acid Tyr, was reported to be a potent κ receptor antagonist (K_e = 0.63 nM in the guinea pig ileum against Dyn A-(1-13)NH₂) with high affinity (K_i (κ) = 0.82 nM) and selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/259/198) for κ opioid receptors.²⁵

Our laboratory has identified several κ opioid receptor antagonists by modifying the N-terminal "message"²⁶ sequence of Dyn A. These include three peptides lacking a basic N-terminal amine.²⁷⁻²⁹ One of these peptides is aro¹dyn (**1**), which exhibits high κ opioid receptor affinity (K_i (κ) = 10 nM) and selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/174/583) and is a κ receptor antagonist.²⁸ SAR studies of aro¹dyn resulted in [NMePhe¹]aro¹dyn (**2**) which exhibits higher κ opioid receptor affinity (K_i (κ) = 4.6 nM) and selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/1100/>2170) than aro¹dyn.³⁰ This is one of the most selective peptide antagonists for κ opioid receptors.

[NMePhe¹]aro¹dyn and its analogs that are acetylated at the N-terminus, however, are all prone to loss of Ac-NMePhe under acidic cleavage conditions (see the accompanying paper). The yields of these peptides could be increased by modifying the cleavage cocktail and cleavage conditions, but even under optimized cleavage conditions, substantial degradation of these peptides still occurred. We showed that this degradation reaction could be suppressed by substituting the acetyl group with a heteroatom-containing group (e.g. methoxycarbonyl, phenyloxyacetyl, glycyl, and acetyl glycyl; see the accompanying article).

Considering the remarkable pharmacological activity of [NMePhe¹]arodyn, the pharmacological profiles of these stable analogs were examined. The SAR of arodyn indicated that Phe³ contributes to κ opioid receptor affinity, selectivity, and efficacy.³⁰ Since the substitution of Phe¹ with NMePhe can significantly increase κ opioid receptor affinity and selectivity, additional stable [NMePhe¹]arodyn analogs in which position 3 was substituted with other aromatic or non-aromatic residues were also evaluated for κ opioid receptor affinity, selectivity, and opioid activity. Here we report the pharmacological results for these stable [NMePhe¹]arodyn analogs.

Results and discussion

Analog design

We designed a number of analogs for evaluation of their pharmacological activity. First, the effects of substituting different heteroatom-containing groups at the N-terminus of the parent peptides [NMePhe¹]arodyn (**2**) and [NMePhe¹,Trp³]arodyn (**3**) were evaluated. [Trp³]arodyn (**4**), the Phe¹ analog of [NMePhe¹,Trp³]arodyn (**3**), was also prepared to evaluate the effects of N-methylation on the κ opioid receptor affinity and selectivity of [Trp³]arodyn. Thus, we synthesized [CH₃OCO-NMePhe¹,X³]arodyn (**5** and **9**), [PhOCH₂CO-NMePhe¹,X³]arodyn (**6** and **10**), [Gly-NMePhe¹,X³]arodyn (**7** and **11**), and [Ac-Gly-NMePhe¹,X³]arodyn (**8** and **12**) (where X = Phe or Trp, Table 1). Compounds **3**, **4**, and **9-12** were originally synthesized for the study of the side reaction (see the accompanying article). The methoxycarbonyl group was also incorporated in analogs where the third position was substituted with other aromatic residues (Tyr and 2-naphthylalanine (Nal(2'))) in compounds **13** and **14**, respectively) or a non-aromatic residue (cyclohexylalanine (Cha) in compound **15**). (The structures of Phe, NMePhe, Tyr, Trp, Nal(2') and Cha are shown in Figure 1.) Their corresponding unstable acetyl analogs (compounds **16-18**, Table 1) were synthesized for comparison of their κ opioid receptor affinities, selectivities, and efficacy.

Chemistry

All peptides were synthesized on the Fmoc-PAL-PEG-PS (PAL-PEG-PS = Peptide Amide Linker-poly(ethylene glycol)-polystyrene) resin by solid-phase peptide synthesis (SPPS) using Fmoc-protected amino acids according to standard procedures.²⁸⁻³⁰ After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, different acyl groups were coupled to the N-terminal free amine. After synthesizing the protected peptides on the resin, these peptides were cleaved with Reagent B (88% trifluoroacetic acid (TFA), 5% phenol, 5% water, and 2% triisopropylsilane (TIPS))³¹ or pure TFA (for compounds **2**, **3**, and **16-18**; see the accompanying article) and purified by reversed-phase high-performance liquid chromatography (HPLC). The purity of the final purified peptides was >98% as verified by two different HPLC solvent systems (MeCN/water and MeOH/water containing 0.1% TFA). The molecular weights of these peptides were verified by electrospray ionization-mass spectrometry (ESI-MS). The analytical data for the peptides is presented in Table 1.

Pharmacology

The affinities of these peptides for rat κ and μ opioid receptors expressed on Chinese hamster ovary (CHO) cells were determined by equilibrium competitive inhibition of the radioligands [³H]diprenorphine and [³H]DAMGO ([D-Ala²,MePhe⁴,glyol]-enkephalin), respectively³² (Tables 2 and 3). Since all of the arodyn analogs reported to date show very low affinity for δ opioid receptors ($K_i > 5 \mu\text{M}$), only κ and μ opioid receptor affinities were evaluated.

[NMePhe¹]arodyn (**2**) was the most potent and selective arodyn analog in the initial SAR study.³⁰ Substitution of the acetyl group in this compound with a methoxycarbonyl group resulted in compound **5** that has unchanged affinity for κ opioid receptors (Table 2). The μ opioid receptor affinity of this peptide increased by about 3-fold, however, and therefore the selectivity for κ over μ opioid receptors decreased by 3-fold. Compared with **2**, κ opioid receptor affinity decreased somewhat when the N-terminal acetyl group of **2** was substituted with other acyl groups (compounds **6-8**). Substitution of the N-terminal acetyl group in **2** with the bulky phenoxyacetyl group (compound **6**) was well tolerated by κ opioid receptors while increasing μ opioid receptor affinity by 3.5-fold; therefore, the selectivity of **6** for κ opioid receptors is 6-fold less than **2**. The largest decrease in κ opioid receptor affinity (6.3-fold) occurred when glycine was substituted for the acetyl group (compound **7**), likely as a result of the introduction of the positively charged amine group at the N-terminus. This effect has also been observed for the des-acetyl derivative of arodyn, in which the κ opioid receptor affinity decreased 4-fold.³⁰ The addition of this protonated amine also increases the affinity for μ opioid receptors slightly so that this compound shows only 106-fold selectivity for κ over μ opioid receptors. Acetylation of the N-terminal free amine of **7** to give **8** increased the affinity for κ opioid receptors 3.5-fold and decreased μ opioid receptor affinity (> 3-fold) compared to **7**, so that the resulting compound exhibits very high κ opioid receptor selectivity (K_i ratio (μ/κ) > 1240) comparable to **2**. This is one of the most selective peptide ligands for κ opioid receptors.

[NMePhe¹,Trp³]arodyn (**3**) shows similar affinity and selectivity for κ opioid receptors as arodyn (**1**) (Table 2). However, this analog exhibits about 3-fold lower affinity than [NMePhe¹]arodyn (**2**), suggesting that the larger aromatic group in position 3 in this peptide decreases κ opioid receptor affinity. Compared with **3**, its Phe¹ analog **4** shows similar affinity (Table 2) for κ opioid receptors; however, this compound exhibits about 3-fold higher affinity for μ opioid receptors, resulting in 3-fold lower selectivity for κ opioid receptors than compound **3**. These results once again indicate that the N-methyl group on Phe¹ increases κ opioid receptor selectivity, possibly due to the effect of methylation on orientation of the acetyl group. When the N-terminal acetyl group in **3** is replaced by a methoxycarbonyl group (compound **9**), the affinity at κ opioid receptors increases slightly, but the affinity at μ opioid receptors increases 2-fold, resulting in somewhat lower κ opioid receptor selectivity compared to **3**. Substitution of the N-terminal acetyl group with the larger phenoxyacetyl group (compound **10**) did not affect κ opioid receptor affinity, but resulted in a 2-fold increase in affinity for μ opioid receptors; therefore, the selectivity of this peptide for κ opioid receptors is also lower than **3**. The largest increase in μ opioid receptor affinity occurred when glycine was substituted for the acetyl group (compound **11**) as a result of the introduction of the positively charged N-terminal amine group, resulting in a compound with limited selectivity (33-fold) for κ opioid receptors. Acetylation of the free amine in this peptide to give compound **12** decreased μ opioid receptor affinity so that **12** exhibits 5-fold higher κ opioid receptor selectivity than **11**.

Thus the effects of the substitution in the two series of compounds (Phe³ and Trp³) are somewhat different. For example, compound **7** shows 6.3-fold lower affinity for κ opioid receptors than the methoxycarbonyl analog **5**, while compound **11** has similar κ opioid receptor affinity as its corresponding methoxycarbonyl derivative **9**. This substitution also has different effects on μ opioid receptor affinity. Compound **7** shows about 2-fold lower affinity for μ opioid receptors than its methoxycarbonyl analog **5**, while compound **11** shows 2-fold higher affinity for μ opioid receptors than the corresponding methoxycarbonyl derivative **9**. These results suggest that the residue in position 3 affects the orientation of the N-terminal substituent and/or N-methyl group in the binding pocket, which in turn affects the pharmacological signature.

Based on these results, the acetyl group in the parent compounds can best be substituted with a methoxycarbonyl group to maintain affinity and selectivity for κ opioid receptors. The methoxycarbonyl group has similar size and other physicochemical properties as the acetyl group, and therefore the biological activity is preserved.

These arodyn analogs were evaluated for their efficacy at 10 μ M to inhibit adenylyl cyclase (AC) using cloned rat κ opioid receptors stably expressed on CHO cells.³³ The analogs exhibited negligible or low efficacy in this assay ($\sim$ 74% of the control ($\sim$ 26% inhibition) compared to Dyn A-(1-13)NH₂, Table 2), similar to arodyn.

Stable N-terminal methyloxycarbonyl analogs with various amino acids in position 3 were then prepared for comparison to the corresponding unstable acetylated NMePhe¹ peptides (Table 3). The methoxycarbonyl analogs [CH₃OCO-NMePhe¹,Tyr³]arodyn (**13**) and [CH₃OCO-NMePhe¹,Cha³]arodyn (**15**) retain similar high affinity for κ opioid receptors as [CH₃OCO-NMePhe¹]arodyn (**5**), indicating that replacing Phe in position 3 with the phenolic amino acid Tyr or the saturated residue Cha (Figure 1) is tolerated by κ opioid receptors. [CH₃OCO-NMePhe¹,Nal(2')³]arodyn (**14**), with Phe replaced by the bulky aromatic amino acid Nal(2') (Figure 1), however, showed a 5-fold decrease in affinity for κ opioid receptors compared to **5**. This is a larger decrease than observed for the Trp-containing analog [CH₃OCO-NMePhe¹,Trp³]arodyn (**9**). Interestingly, even though [CH₃OCO-NMePhe¹,Tyr³]arodyn (**13**) has similar affinity for κ opioid receptors as [CH₃OCO-NMePhe¹]arodyn (**5**), this is not the case for the corresponding acetylated analog [MePhe¹,Tyr³]arodyn (**16**) which has 6-fold lower affinity for κ opioid receptors than the corresponding N-terminal methoxycarbonyl analog **15** (Table 3). In contrast, [MePhe¹,Nal(2')³]arodyn (**17**) has almost the same κ opioid receptor affinity as its methoxycarbonyl analog **14**. These results once again suggest that the residue in 3 affects the orientation of the N-terminal substituent in the binding pocket, which in turn affects receptor affinity.

The κ opioid receptor selectivity of these methoxycarbonyl analogs is not as high as [CH₃OCO-MePhe¹]arodyn (**5**, Table 3). [CH₃OCO-MePhe¹,Nal(2')³]arodyn (**14**) unexpectedly shows relatively high μ opioid receptor affinity (K_i = 54 nM), and therefore this compound exhibits minimal selectivity (2-fold) for κ over μ opioid receptors. Similarly, its acetylated analog **17** also has relatively high affinity for μ opioid receptors (Table 3). Unlike the other arodyn analogs, these two peptides are the exceptions that exhibit significant affinity for μ opioid receptors. The bulky hydrophobic aromatic side chain in position 3 appears to be responsible for the increased affinity for μ opioid receptors, with the Nal(2') peptides exhibiting >20-fold higher affinity for μ opioid receptors than the corresponding Trp-containing peptides. The detailed structural requirements for the μ opioid receptor affinity are currently under investigation in our laboratory.

These arodyn analogs were also evaluated for their efficacy to inhibit AC via κ opioid receptors. The four compounds with either Tyr or Cha in position 3 (**13**, **15**, **16** and **18**) exhibited significant efficacy in this assay (<60% control, >40% inhibition of AC relative to Dyn A-(1-13)NH₂, Table 3) and are therefore partial agonists for κ opioid receptors.

Because of its high affinity and selectivity for κ opioid receptors, [CH₃OCO-NMePhe¹]arodyn (**5**) was evaluated for its ability to antagonize Dyn A (1-13)NH₂ in the AC assay. Schild analysis³⁴ of peptide **5** vs Dyn A (1-13)NH₂ (Figure 2) yielded a K_B of 8.5 nM (pA_2 = 8.07; 6.97-10.8 95% confidence interval), indicating that it is a potent κ opioid receptor antagonist.

Conclusions

[NMePhe¹]arodyn (**2**) exhibits high affinity and selectivity for κ opioid receptors, but this peptide is unstable and prone to deletion of the Ac-NMePhe moiety under acidic conditions. By replacing the acetyl group with heteroatom-containing groups, stable analogs can be obtained (see accompanying article). Pharmacological evaluation showed that a methoxycarbonyl group could be substituted for the acetyl group in [NMePhe¹]arodyn without loss of κ opioid receptor affinity. [CH₃OCO-NMePhe¹]arodyn (**5**) has similar κ opioid receptor affinity as [NMePhe¹]arodyn (**2**) and retains high κ opioid receptor selectivity. For the [NMePhe¹]arodyn analogs the N-terminal substituents C₆H₅OCH₂CO and Ac-Gly (peptides **6** and **8**, respectively) were well tolerated by κ opioid receptors, with only Gly causing a substantial (6.3-fold) decrease in κ opioid receptor affinity. [Ac-Gly-NMePhe¹]arodyn (**8**) showed extremely high selectivity for κ over μ opioid receptors comparable to that of **2** due to its very low affinity for μ opioid receptors. In the [NMePhe¹,Trp³]arodyn series of analogs, substituents other than CH₃OCO resulted in similar κ opioid receptor affinity (within 2-fold) compared to **9**. As in the Phe³ series, the selectivity for κ over μ opioid receptors in this series of compounds was generally not as high as when CH₃OCO was the N-terminal substituent, except for [AcGly-NMePhe¹,Trp³]arodyn (**8**), which retained very high κ opioid receptor selectivity comparable to [NMePhe¹]arodyn (**2**).

The N-terminal methoxycarbonyl analogs with other aromatic or non-aromatic amino acids in position 3 generally retained similar affinity for κ opioid receptors as [CH₃OCO-NMePhe¹]arodyn (**5**). In contrast, [NMePhe¹]arodyn (**2**) has higher κ opioid receptor affinity than the acetylated analogs where Phe³ was replaced with other aromatic or non-aromatic amino acids (Table 3). The selectivity of the methoxycarbonyl analogs for κ over μ opioid receptors was somewhat lower than **5**. [CH₃OCO-MePhe¹,Nal(2')³]arodyn (**14**) and its acetylated analog **17** unexpectedly showed relatively high μ opioid receptor affinity, and therefore they exhibit minimal selectivity for κ over μ opioid receptors (Table 3).

Most of the analogs described here, similar to arodyn, exhibited negligible or low efficacy in the AC assay. The exceptions were the Tyr³ and Cha³ analogs **13**, **15**, **16**, and **18**. [CH₃OCO-NMePhe¹]arodyn (**5**) antagonized Dyn A-(1-13)NH₂ at κ opioid receptors in the AC assay with high potency (K_B = 8.5 nM). Thus we have successfully identified stable [NMePhe¹]arodyn analogs that are selective for κ opioid receptors and in the case of [CH₃OCO-NMePhe¹]arodyn (**5**) acts as a potent κ opioid receptor antagonist. [CH₃OCO-NMePhe¹]arodyn and selected analogs could be very useful pharmacological tools complementary to the non-peptide κ receptor selective antagonists to study the physiological processes mediated by κ opioid receptors and also as lead peptides for further modification.

Experimental section

Materials

Fmoc-Nal(2')-OH was purchased from Peptides International (Louisville, KY). Fmoc-Cha-OH was purchased from Calbiochem-Novabiochem (San Diego, CA). The sources of other materials are the same as used in the accompanying manuscript.

Peptide synthesis and cleavage

Peptide Synthesis: All peptides were synthesized on the Fmoc-PAL-PEG-PS resin (0.16-0.24 mmol/g) by standard SPPS methods (see the accompanying article for details). For the stable analogs, the peptides were cleaved from the resin and deprotected using Reagent B (85% TFA, 5% phenol, 5% water, and 2% triisopropylsilane)³¹ at room

temperature for 2 h. For the unstable analogs (i.e. **2**, **3**, and **16-18**), the peptides were cleaved from the resin using pure TFA without any scavenger at 4 °C for 3 h (see the accompanying article).

Purification and analysis of peptides

The crude peptides were purified by preparative reversed-phase HPLC (Shimadzu HPLC system equipped with a Shimadzu SPD-10A detector) on a Vydac C18 column (10 μ m, 300 Å, 21 $\times$ 250 mm) equipped with a Vydac guard cartridge. For purification, a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min, was used. The purification was monitored by UV absorbance at 214 nm.

The purity of the final peptides was verified by analytical HPLC in two different solvent systems using a Shimadzu HPLC system equipped with a Shimadzu SPD-10A detector and a Vydac C18 column (5 μ m, 300 Å, 4.6 $\times$ 50 mm) equipped with a Vydac guard cartridge. In system 1, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeCN containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used with monitoring at 214 nm. In system 2, a linear gradient of 5-50% solvent B (solvent A aqueous 0.1% TFA, and solvent B MeOH containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used with monitoring at 230 nm. However, for the more hydrophobic peptides (i.e. **6**, **10**, **14**, **15**, **17**, and **18**), a gradient of 5-86% solvent B (methanol containing 0.1% TFA) over 45 min was used as the second system for analysis. The final purity of all peptides in both analytical systems was >98% (Table 1). Interestingly, the retention times for the methoxycarbonyl analogs were longer than for the corresponding acetyl analogs. The molecular weights of the compounds were determined by ESI-MS (Waters, Q-TOF).

Radioligand binding assays

Radioligand binding assays were performed as previously described using cloned rat κ and μ opioid receptors stably expressed on CHO cells.³² [³H]Diprenorphine and [³H]DAMGO ([D-Ala²,MePhe⁴,glyol]enkephalin) were used as radioligands in the assays for κ and μ receptors, respectively. Nonspecific binding was determined in the presence of 10 μ M unlabeled Dyn A-(1-13)NH₂ or DAMGO for κ and μ receptors, respectively. IC₅₀ values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism software (GraphPad Software Co., San Diego, CA). K_i values were calculated from the IC₅₀ values by the Cheng and Prusoff equation,³⁵ using K_D values of 0.45 and 0.49 nM for [³H]diprenorphine and [³H]DAMGO, respectively. The results presented (Tables 2 and 3) are the mean $\pm$ SEM from at least three separate assays.

Adenylyl cyclase assay

The AC assay was performed using cloned rat κ opioid receptors stably expressed on CHO cells as previously described.³³ The inhibition of cAMP production was determined for each peptide at 10 μ M compared untreated controls and the reference agonist Dyn A-(1-13)NH₂; the results presented (Tables 2 and 3) are the mean $\pm$ SEM from at least three separate assays.

The antagonist activity of **5** was determined by measuring the EC₅₀ of Dyn A-(13)NH₂ in the AC assay³³ in the absence and presence of **5** at different concentrations (10 nM-10 μ M). The pA₂ was determined by Schild analysis.³⁴

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Figure 1.
Structural comparison of Phe, NMePhe, Tyr, Trp, Nal(2') and Cha

Figure 2.

Schild analysis of peptide **5** as an antagonist of Dyn A-(1-13)NH₂ at κ opioid receptors in the adenylyl cyclase assay. DR = dose ratio (EC₅₀ of Dyn A-(1-13)NH₂ in the presence of the indicated concentration of **5** divided by the EC₅₀ of Dyn A-(1-13)NH₂ in the absence of **5**). Data shown are pooled data from three separate experiments.

Table 1

Analytical data for the peptides
X-NMePhe-Phe-R-Arg-Leu-Arg-D-Ala-Arg-Pro-Lys-NH₂

Cmpd	X	R	HPLC (<i>t_R</i> , (min)) ^a		ESI-MS (<i>m/z</i>)	
			System 1 ^b	System 2 ^c	Calculated	Observed
3	Ac	Trp	24.7	41.1	[M+3H] ³⁺ =530.0	[M+3H] ³⁺ =530.0
4	Ac ^d	Trp	22.6	40.1	[M+3H] ³⁺ =526.0	[M+3H] ³⁺ =526.0
5	CH ₃ OCO	Phe	26.3	44.2	[M+3H] ³⁺ =522.6	[M+3H] ³⁺ =522.5
6	C ₆ H ₅ OCH ₂ CO	Phe	30.1	29.4 ^e	[M+3H] ³⁺ =547.7	[M+3H] ³⁺ =547.7
7	Gly	Phe	24.9	37.3	[M+3H] ³⁺ =522.0	[M+3H] ³⁺ =521.9
8	Ac-Gly	Phe	22.7	40.8	[M+3H] ³⁺ =536.0	[M+3H] ³⁺ =536.0
9	CH ₃ OCO	Trp	26.3	43.6	[M+3H] ³⁺ =535.3	[M+3H] ³⁺ =535.3
10	C ₆ H ₅ OCH ₂ CO	Trp	29.3	28.0 ^e	[M+3H] ³⁺ =561.0	[M+3H] ³⁺ =561.1
11	Gly	Trp	18.9	34.0	[M+3H] ³⁺ =535.0	[M+3H] ³⁺ =534.9
12	Ac-Gly	Trp	22.1	38.9	[M+3H] ³⁺ =549.3	[M+3H] ³⁺ =549.4
13	CH ₃ OCO	Tyr	24.3	41.0	[M+3H] ³⁺ =527.6	[M+3H] ³⁺ =527.7
14	CH ₃ OCO	Nal(2')	29.3	29.1 ^e	[M+3H] ³⁺ =539.3	[M+3H] ³⁺ =539.4
15	CH ₃ OCO	Cha	29.0	29.4 ^e	[M+3H] ³⁺ =524.3	[M+3H] ³⁺ =524.4
16	Ac	Tyr	22.8	38.4	[M+3H] ³⁺ =523.0	[M+3H] ³⁺ =522.9
17	Ac	Nal(2')	28.1	28.4 ^e	[M+3H] ³⁺ =534.0	[M+3H] ³⁺ =533.9
18	Ac	Cha	27.3	27.8 ^e	[M+3H] ³⁺ =519.3	[M+3H] ³⁺ =519.3

^aThe purity for all of the peptides by both methods was > 98%.

^bSystem 1: Solvent A = 0.1% aqueous TFA, solvent B = 0.1% TFA in acetonitrile.

^cSystem 2: Solvent A = 0.1% aqueous TFA, solvent B = 0.1% TFA in methanol. The gradient for both systems was 5-50% solvent B over 45 min at 1 mL/min.

^dPhe instead of NMePhe in position 1.

^eThe gradient was 5-86% solvent B over 45 min at 1 mL/min.

Table 2

Opioid receptor affinities and efficacies of aroclon (1) and its N-terminal substituted analogs X-NMePhe-Phe-R-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH₂^a

Peptide	X	R	K _i ± SEM (nM)		K _i ratio (μ/κ)	AC % control @ 10 μM ^b
			κ	μ		
1 , aroclon ^c	Ac ^d	Phe	10.0 ± 3.0	1750 ± 130	175	88 ± 8
2 ^e	Ac	Phe	4.56 ± 0.45	5056 ± 790	1100	86 ± 1
5	CH ₃ OCO	Phe	4.93 ± 0.15	1750 ± 100	355	74 ± 13
6	C ₆ H ₅ OCH ₂ CO	Phe	7.34 ± 1.37	1450 ± 190	198	98 ± 21
7	Gly	Phe	28.9 ± 7.5	3070 ± 130	106	75 ± 8
8	Ac-Gly	Phe	8.04 ± 3.14	>10,000	>1240	82 ± 17
3	Ac	Trp	13.0 ± 2.6	2990 ± 700	230	105 ± 16
4	Ac ^d	Trp	16.1 ± 2.1	1180 ± 290	73	108 ± 5
9	CH ₃ OCO	Trp	9.52 ± 1.73	1280 ± 280	134	98 ± 13
10	C ₆ H ₅ OCH ₂ CO	Trp	13.3 ± 3.6	1250 ± 140	94	> 90
11	Gly	Trp	15.4 ± 4.2	502 ± 145	33	90 ± 8
12	Ac-Gly	Trp	21.9 ± 4.8	3760 ± 910	172	83 ± 7

^aThe values are the mean ± SEM from at least three independent experiments.

^bRelative to the full agonist Dyn A (1-13)NH₂ (0% control, 100% inhibition).

^cFrom reference 28.

^dPhe instead of NMePhe in position 1.

^eFrom reference 30.

Table 3
Opioid receptor affinities and efficacy of [NMePhe¹]arodyn analogs where position 3 was substituted with other aromatic and non-aromatic amino acids
X-NMePhe-Phe-R-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH₂

Peptide	X	R	K _i ± SEM (nM)		K _i ratio (μ/κ)	AC % control @ 10 μM ^a
			κ	μ		
5	CH ₃ OCO	Phe	4.93 ± 0.15	1750 ± 100	355	74 ± 13
9	CH ₃ OCO	Trp	9.52 ± 1.73	1280 ± 280	134	98 ± 13
13	CH ₃ OCO	Tyr	5.80 ± 1.27	1300 ± 340	224	58 ± 10
14	CH ₃ OCO	Nal(2')	24.4 ± 7.2	53.8 ± 13.1	2	109 ± 10
15	CH ₃ OCO	Cha	3.60 ± 0.21	818 ± 253	227	55 ± 18
2 ^b	Ac	Phe	4.56 ± 0.45	5056 ± 790	1100	86 ± 1
3	Ac	Trp	13.0 ± 2.6	2990 ± 700	230	105 ± 16
16	Ac	Tyr	37.3 ± 16.0	3370 ± 570	90	52 ± 11
17	Ac	Nal(2')	27.0 ± 7.3	121 ± 35	4.5	101 ± 11
18	Ac	Cha	7.85 ± 2.74	5930 ± 1920	755	52 ± 6

^aRelative to the full agonist Dyn A (1-13)NH₂ (0% control, 100% inhibition).

^bFrom reference 30.