Published in final edited form as:

J Med Chem. 2016 November 23; 59(22): 10291–10298. doi:10.1021/acs.jmedchem.6b01411.

Structure–Activity Relationships of [des-Arg⁷]Dynorphin A Analogues at the κ Opioid Receptor

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Abstract

Dynorphin A (Dyn A) is an endogenous ligand for the opioid receptors with preference for the κ opioid receptor (KOR), and its structure–activity relationship (SAR) has been extensively studied at the KOR to develop selective potent agonists and antagonists. Numerous SAR studies have revealed that the Arg⁷ residue is essential for KOR activity. In contrast, our systematic SAR studies on [des-Arg⁷]Dyn A analogues found that Arg⁷ is not a key residue and even deletion of the residue does not affect biological activities at the KOR. In addition, it was also found that [des-Arg⁷]Dyn A-(1–9)-NH₂ is a minimum pharmacophore and its modification at the N-terminus leads to selective KOR antagonists. A lead ligand, **14**, with high affinity and antagonist activity showed improved metabolic stability and could block antinociceptive effects of a KOR selective agonist, FE200665, in vivo, indicating high potential to treat KOR mediated disorders such as stress-induced relapse.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b01411.

Analytical data and cell viability assay data (PDF)

Molecular formula strings and some data (CSV)

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

INTRODUCTION

Dynorphin A (Dyn A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln, Table 1) is one of three endogenous opioid peptides with high affinities for the μ (MOR), δ (DOR), and κ (KOR) opioid receptors, with a modest selectivity for the KOR.^{1–5} Dyn A mediates a neuroinhibitory effect through the opioid receptors in the nervous system resulting in antinociception. Dyn A and [des-Tyr¹]Dyn A fragments are also known to have neuroexcitatory effects in the spinal cord through non-opioid receptors, including the bradykinin receptor, causing hyperalgesic effects.^{6,7}

The KOR has been studied for its clinical potential in analgesia, stress-induced relapse, pruritus, and depression.⁸ KOR antagonists are known to reduce tolerance and addiction driven by MOR agonists and are also associated with the reduction of anxiety and depressed affective states that occur during opioid withdrawal.⁹ KOR selective antagonists have mainly been non-peptide molecules with very long durations of action, even some with shorter durations of action in vivo published before, lasting up to 8 weeks after a single dose.^{10,11} These have mainly been used as pharmacological tools and have reduced therapeutic potential due to their long duration of action.¹¹ In contrast, peptides may have an alternative mechanism of action that does not activate long-term JNK stimulation and therefore may be able to avoid this problem in developing potent KOR antagonists for therapeutic purpose.

Dyn A has been the subject of many KOR structure–activity relationship (SAR) studies.^{11–13} In the search for the minimum optimal sequence, peptide chain truncation and alanine scans were the most widely performed.¹⁴ From those SAR studies it had been identified that residues Lys¹³, Lys¹¹, and Arg⁷ are key for binding and function at the KOR.^{1,12} However, in our approach, which is different from previous peptide SAR strategies, we found that deleting Arg⁷ has no detrimental effect on binding and activity at the KOR. A peptide topographical structure depends on the residues (polarity and position), and their structural characteristics translate into affinity and activity at their given receptors.¹⁵ Changing or removing residues from the center of a peptide sequence can dramatically change the topographical structure adopted by the peptide upon binding and cause a lack of affinity and activity at their receptor.^{3,15} On the basis of this idea, our SAR results are very interesting and to our knowledge represent the first case demonstrating that positional residue deletion is a viable peptide SAR strategy.

Arg⁷ has been at the center of Dyn A SAR, as it is the second residue of a basic pair, which are more susceptible to enzymatic degradation.¹ A study by Kawasaki et al. showed that the

two consecutive Arg residues are not necessary for activity at the KOR by replacing one of the two consecutive Arg residues with a non-natural amino acid, Nle. The resulting ligands had high affinity at the KOR.³ This suggested that the seventh position on the Dyn A structure may not be as important for affinity or biological activity as previously published. Secondary structural analyses have shown that Dyn A adopts an α -helical structure in the presence of micelles and hydrophobic buffers.^{16–18} Dyn A residues at positions 5–9 adopt an amphipathic α -helix, which has been proposed to interact with the membrane, permitting the hydrophilic residues to interact with the KOR. This hypothesis would explain the importance of the polar Arg residues shown by others.¹⁶

In our previous studies to develop non-opioid Dyn A ligands for the bradykinin 2 receptor, it was found that deletion of one of the consecutive Arg residues is well tolerated and conducive to B2R antagonist activity.⁶ We applied the same strategy to pursue SAR studies for the development of KOR antagonists and here report the SAR results of [des-Arg⁷]Dyn A analogues at the KOR in vitro and in vivo.

RESULTS AND DISCUSSION

Design and Synthesis

Previous SAR studies from other groups on Dyn A had identified three key residues, Arg⁷, Lys¹¹, and Lys¹³, that are important for binding, selectivity, and potency at the KOR.¹ On the basis of the SAR results, many studies have modified Dyn A structure by substitutions and truncations while keeping these key residues to retain high biological activity at the KOR. However, our design approach was different in that we deleted the Arg residue at position 7, which is located in the middle of the sequence and known as a key residue, in an effort to identify a shorter peptide ligand with improved stability. The Dyn A(5–13) fragment, known as the “address” region for KOR selectivity and activity, includes four hydrophobic residues and five basic residues resulting in an amphipathic character. In reference to this, our design focused on the deletion of one of the two consecutive Arg residues, which have high potential for enzymatic degradation, while retaining the amphipathic nature of the ligand. Therefore, a series of [des-Arg⁷]Dyn A analogues was designed and synthesized by standard solid phase peptide synthesis using Fmoc chemistry with 50–80% crude purity. Crude peptides were purified by preparative RP-HPLC to afford 97% purity and validated for their structures by high-resolution mass spectrometry (HR-MS).

Binding Affinities of [des-Arg⁷]Dyn A Analogues

[des-Arg⁷]Dyn A analogues were evaluated for their binding affinities and receptor selectivity for KOR, MOR, and DOR by competition binding assays. Results showed that removal of Arg⁷ in Dyn A fragments is well tolerated for all three subtypes of opioid receptors contrary to what others have found.¹ It has been shown previously that Dyn A analogue interactions with the MOR and DOR are not affected by modifications of the “address” region, positions 5–13, and the same trend was observed in our studies. Initially, we tested [des-Arg⁷]Dyn A(1–13), analogue **1**, and [des-Arg⁷]Dyn A(1–11), analogue **2**, for their binding affinities at KOR, MOR, and DOR (Table 2). We found that Arg⁷ removal from

the fragments (**1**, $K_i = 0.39$ nM; **2**, $K_i = 0.43$ nM) did not affect KOR interaction, with similar affinities to the parent fragments ($K_i = 0.12$ nM and $K_i = 0.09$ nM for Dyn A(1–13) and Dyn A(1–11), respectively). These results demonstrated that Arg⁷ is not necessary for binding at the KOR as previously published.¹ Typically, deletion of an amino acid residue in the middle of a sequence of a biologically active peptide causes a large change in its topographical structure that results in a loss of affinity and functional activity at its receptor.¹⁵ However, in our studies, it was demonstrated that it is possible to delete an amino acid residue (maybe a fragment) in the middle of a peptide sequence without affecting binding affinity. Following these results, further SAR studies were performed on **2**.

A great number of neuropeptides possess a C-terminal amidation, which imparts critical biological functions and improved enzymatic stability.¹⁹ Therefore, [des-Arg⁷]Dyn A analogues were first modified to an amide at the C-terminus. The modification of ligand **2** ($K_i = 0.43$ nM) increased binding affinity by 6-fold in **3** ($K_i = 0.07$ nM) at the KOR. Schiller et al. demonstrated that substitution of the N-terminal amine with (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (MDP), which results in the deletion of the N-terminal amino group, reverses KOR agonist activity to antagonist activity.²⁰ In an effort to obtain KOR antagonist activity, Tyr at the N-terminus was replaced with MDP in **2** and **3**, resulting in ligands **4** ($K_i = 63$ nM) and **6** ($K_i = 230$ nM), which decreased binding affinities dramatically (534-fold and 900-fold, respectively) at the KOR. Similar modification with an acetyl (Ac) group in **5** greatly decreased affinity (3390-fold). A dramatic loss of affinity was also observed at the MOR and DOR by these modifications due to the critical role that the N-terminal amino group has in opioid receptor recognition.

Schlechtingen et al. showed that substitution of a Gly residue at the third position with a Pro residue reversed Dyn A analogue agonist activity to a weak KOR antagonist with improved selectivity at the KOR.²¹ On the basis of this, a Pro residue was substituted at position 3 resulting in **7**, showing comparable binding affinity ($K_i = 61$ nM) at the KOR with improved selectivity ($\kappa/\delta = 64$ -fold, $\kappa/\mu > 100$ -fold). In order to enhance KOR antagonist activity, **7** was acetylated at the N-terminus and the resulting analogue **8** lost binding affinity ($K_i = 730$ nM) by 12-fold at the KOR. This analogue did not bind to the MOR (no competition) and DOR ($K_i > 10$ μ M). It has also been shown that modifications of the C-terminal “address” region, particularly positions 8 and 10, can improve KOR selectivity.¹² Therefore, to improve KOR selectivity, we replaced Ile⁸ in **8** with DAla⁸, and Ile⁸ and Pro¹⁰ in **9** with DAla⁸ and DPro¹⁰, respectively. These modifications resulted in a large loss of binding affinities in **9** ($K_i = 5200$ nM) and **10** ($K_i = 3600$ nM) at the KOR. Interestingly, analogue **11**, in which a Gly residue remains in the third position with only modifications occurring at the N-terminus and “address” region, regained binding affinity ($K_i = 98$ nM) at the KOR (Table 2). Comparison of binding affinities of **9** and **10** with **11** suggests that simultaneous modifications in both “message” and “address” regions may not be preferred for opioid receptors due to serious structural changes, while separate modifications can be optimized for receptor interaction. Analogues **7** and **11** are examples of the latter case. Lemaire et al. observed that each modification by itself improved selectivity; with a combination of modifications at the N-terminus and “address” region, **11** resulted in similar KOR selectivity for the MOR and slightly reduced for the DOR compared to that of **7**.² **12** ($K_i = 4.3$ μ M)

goes a step further in substituting Arg⁶ in combination with the modifications at positions 3 and 8, and as expected, the result was unfavorable, significantly decreasing binding affinity at all three opioid receptors. Combining modifications at both the N and C terminuses indicated that these are detrimental for the binding and selectivity of [des-Arg⁷]Dyn A analogues.

The nature of the residues at either terminus of a peptide has a great effect on the binding affinities and functional activities. Many studies have shown that potent Dyn A analogues have a basic amino acid at their C-terminus. To fulfill this requirement, analogue **13** was prepared by truncating two amino acid residues from the C-terminus of **3**. This analogue represents a smaller Dyn A fragment and thus further SAR studies were done in search of a minimum [des-Arg⁷]Dyn A pharmacophore for the KOR. Analogue **13** ($K_i = 0.22$ nM) retained the same subnanomolar affinity as **3** at the KOR, suggesting that [des-Arg⁷]Dyn A(1–9) is a minimum pharmacophore for the receptor (Table 2). After modifications of the N-terminus with MDP or acetyl group, the resulting analogues **14** ($K_i = 94$ nM) and **15** ($K_i = 51$ nM) showed reduced binding affinity compared to **13** but were still in the nanomolar range. The Pro³ substitution was also introduced, and it retained binding affinity and selectivity at the KOR (**16**, $K_i = 195$ nM, $\kappa/\mu > 100$ -fold, $\kappa/\delta = 28$ -fold). The SAR results on the [des-Arg⁷]Dyn A(1–9) fragment showed the same trend as the [des-Arg⁷]Dyn A(1–11) fragment, and therefore the former fragment is considered a minimum pharmacophore that shows great binding affinity and selectivity at the KOR against MOR and slight selectivity against DOR. It is worthwhile to note that this series of analogues does not possess any of the previously claimed “key” amino acid residues, Arg⁷, Lys¹¹, or Lys¹³.

Rat Plasma Stability Studies

Dyn A's metabolic stability is well documented, but to help elucidate the effects of modification on metabolic stability, [des-Arg⁷]Dyn A analogues were tested for their metabolic stabilities in rat plasma. Basic amino acid pairs are highly susceptible to enzymatic degradation; here we explore the deletion of the second residue of an Arg pair on Dyn A (compound **2**) (Figure 1).

Removal of Arg⁷ improved the rat plasma half-life of **2** 6-fold ($t_{1/2} = 4.5$ h) (Table 3), compared to the parent fragment ($t_{1/2} = 0.7$ h). Analogue **7** with the Pro³ substitution showed slightly improved stability ($t_{1/2} = 1.4$ h). Non-natural amino acid substitutions further improved the stability profile; for example, MDP substitution significantly increased the stability from a few hours to more than 2 days (**14**, $t_{1/2} = 50$ h). Analogue **11** with the N-terminal Ac substitution and D-amino acid substitutions in the “address” region resulted in the most stable analogue, with a half-life greater than a week ($t_{1/2} = 350$ h).

These analogues were also evaluated for their cell toxicity potential after a 24 h exposure to KOR expressing cells, and no toxicity was seen. 100% cell survival was observed at the highest concentration (1 mM) in the XTT cell viability assay (Abcam) (Figure S1 in Supporting Information).

In Vitro Functional Activity Studies. GPI/LM/MP Assay

Initial [des-Arg⁷]Dyn A analogues were tested for KOR agonist activity by measuring the potential of depleting electrically stimulated muscle contractions (Table 4). Analogues **1** (IC₅₀ = 15 nM), **2** (IC₅₀ = 21 nM), and **3** (IC₅₀ = 7.0 nM) showed moderate agonist activities, as expected. In order to corroborate that these agonist activities are associated with the targeted receptor, a well-known KOR antagonist, nor-BNI (10 nM), was employed. In these assays, a shift in the dose–response curve was seen: a 34-fold shift for **1** and a 26-fold shift for **2**, confirming KOR agonist activities (Figure 2). In contrast, **4**, **5**, and **6**, designed as KOR antagonists, did not show significant responses in the assay, indicating that N-terminal substitution successfully eliminated KOR agonist activity.

[³⁵S]GTPγS Assay

We also tested [des-Arg⁷]Dyn A analogues for their agonist activities at the KOR in the [³⁵S]GTPγS assay (Figure 3A). At a high concentration (10 μM), analogues **1**, **2**, and **3** showed significant activation compared to vehicle, similar to that of U50,488, a KOR agonist. As expected, **4**, **5**, and **6** did not show KOR activation at high concentrations and thus were tested for KOR antagonist activities by measuring their abilities to block stimulation of U50,488. Analogues **4** and **6** with MDP substitution significantly blocked U50,488 activities at the high concentration (Figure 3B). Concentration–response curves were also performed where analogues **4** and **14** were identified as full antagonists (IC₅₀ = 220 nM, *I*_{max} = 110% and IC₅₀ = 750 nM, *I*_{max} = 120%, respectively) (Figure 4, Table 5).

As shown in Table 5, **7** partially blocked agonist activity (*I*_{max} = 38%) at the highest concentration (10 μM). This result coincides with what was previously observed by Schlechtingen et al., where [Pro³]Dyn A(1–11)-NH₂ was identified as a partial antagonist/agonist at the KOR in the GTPγS assay. Similarly, **16** was also identified as a partial antagonist at the KOR (*I*_{max} = 29%). This finding indicates that modification on the third position with a Pro residue can partially block and activate the KOR in both Dyn A and [des-Arg⁷]Dyn A fragments. The shorter [des-Arg⁷]Dyn A analogue **14** possesses full antagonist activity at the KOR, as observed with **4**, with a potency of IC₅₀ = 750 nM.

In Vivo Evaluation

Schlechtingen et al. showed that Pro³ substitution results in pure antagonist activity in GPI assays using animal tissue but partial agonist/antagonist activities in the GTPγS assay.²¹ Our GTPγS assay results also showed similar partial agonist/antagonist activities of the Pro³ substituted analogue **7**. To validate in vivo functional activities, tail-flick tests were performed in mice (0.1, 1.0, and 10 μg in 5 μL i.t.; *n* = 5/treatment). At 30 min after injection, the two highest doses of **7** (1.0 and 10 μg in 5 μL, i.t.) produced significantly elevated tail withdrawal latency (TWL) compared to vehicle-treated mice, showing agonist antinociceptive activity (Figure 5A).

Analogue **7** was also evaluated for its ability to block the KOR selective peptide agonist activity of DPhe-DPhe-DNle-DArg-4-picolylamide (FE200665, **17**) as a partial agonist/antagonist.^{22,23} **7** significantly blocked the antinociceptive effect of **17** at 15 min and up to 45 min compared to control (vehicle + **17**) (Figure 5C). After 45 min, antinociceptive effect

was recovered, which was considered to be caused by a short duration of antagonist activity of **7**. This observation is well correlated with its short half-life in rat plasma ($t_{1/2} = 1.4$ h).

Analogue **14** was also tested for its antinociceptive effects in the tail flick assay in mice and did not show any significant effect at all doses, demonstrating no in vivo agonist activity (Figure 5B). Although the percent antinociception was negative compared to vehicle, the value was not statistically different from vehicle. **14** was also tested in the presence of the KOR agonist **17**, in the same manner as **7**, and was found to block the antinociceptive effects of **17** until gone at 24 h (Figure 5C). It is worthwhile to note that the blockade of antinociceptive effects is well correlated to the rat plasma half-lives of the analogues. **7** and **14** clearly showed in vivo antagonist activity. **7**, with a shorter half-life ($t_{1/2} = 1.4$ h), resulted in a short duration of action (45 min) blocking **17**, and **14**, with a longer half-life ($t_{1/2} = 50$ h), had an extended duration of action. Taken together, **7** is a partial agonist/antagonist with an ability to attenuate antinociceptive effects of the KOR, and **14** is a pure antagonist.

CONCLUSIONS

To our knowledge, we are the first to demonstrate that Dyn A does not need the second residue of the two consecutive Arg residues for biological activities at the opioid receptors, as deletion of Arg⁷ does not significantly reduce receptor affinity. A series of [des-Arg⁷]Dyn A analogues showed a similar pattern of binding affinity with the parent analogues but showed enhanced metabolic stability in rat plasma. Analogue **7** exhibited nanomolar affinity with high selectivity at the KOR with partial antagonist activity in vitro and in vivo, with antagonist activity in the presence of a selective KOR agonist for up to 45 min. **7** is an example of partial agonists that can block the target receptor in the presence of agonists. Removal of the N-terminal amine by MDP substitution (**14**) reversed agonist activity to antagonist activity in both in vitro and in vivo assays. The effect of N-terminal substitution on SAR coincides with previous studies and therefore demonstrates the value of [des-Arg⁷]Dyn A as a new scaffold to replace Dyn A. These novel [des-Arg⁷]Dyn A ligands show no cell toxicity, even at 1 mM concentrations, with improved stability compared to the parent Dyn A(1–11)-NH₂ peptide. In summary, our results indicate that shorter, high binding affinity Dyn A KOR antagonists with minimal substitutions are attainable and further modifications should be assessed in order to improve selectivity for the KOR.

EXPERIMENTAL SECTION

Synthesis

Dyn A analogues were synthesized by standard solid phase peptide synthesis using *N*^α-Fmoc chemistry (Fmoc = 9-fluorenylmethyloxycarbonyl) on Rink amide resin (100–200 mesh, Novabiochem) in high yields (overall yield >30%). Coupling was performed using 3 equiv of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU)/3 equiv of *N*-hydroxybenzotriazole (HOBt)/6 equiv of diisopropylethylamine (DIPEA) in *N,N*-dimethylformamide (DMF) for 1 h at rt, and *N*^α-Fmoc group was deprotected by 20% piperidine in DMF for 20 min at rt. Crude peptides were obtained by cleavage using a 95% trifluoroacetic acid (TFA) solution containing 2.5% triisopropylsilane (TIS) and 2.5% water for 3 h in high purity (70–90%) and could be isolated with more than

97% purity by preparative reversed phase high performance liquid chromatography (RP-HPLC) using a gradient (10–40% acetonitrile in water containing 0.1% TFA in 15 min) in a short time (<15 min) because of their hydrophilic characteristics (refer to aLogP in Supporting Information). The purified Dyn A analogues were validated by analytical RP-HPLC and high resolution mass spectrometry (HR-MS) in positive ion mode.

Rat Plasma Stability Studies

A modified rat plasma stability protocol was used from Yamamoto et al.²⁴ Stock solutions of 50 mg/mL of test compounds were diluted 5-fold into rat plasma (rat plasma nonsterile heparin, Pel-Freez Biologicals) and incubated at 37 °C. 100 µL of sample was collected after several time points (1, 2, 4, 6, 12, and 24 h), and the reaction was stopped using 150 µL of EtOH except for **14** for which the reaction was stopped using 150 µL of ACN. Samples were centrifuged at 10 000 rpm for 15 min, and 100 µL of supernatant was collected and analyzed by RP-HPLC (Agilent 1100 autosampler using YMC AA 12S05-2546WT C-18 analytical column). The half-life ($t_{1/2}$) was calculated using the slope (b) found in the linear fit of the natural logarithm of incubation time vs the fraction remaining of the parent compound,²⁵ $t_{1/2} = 0.693/b$.

Radioligand Competition Binding Assays

Crude cell membrane preparations from transfected HN9.10, NG-108, or CHO cells were diluted to 50 µg of protein per sample in assay buffer (50 mM Tris-HCl, pH 7.4, containing 50 µg/mL bacitracin, 10 µM captopril, 100 µM PMSF, and 5 mg/mL BSA). The radioligands were obtained from PerkinElmer Inc. through the Office of Radiation Control at the University of Arizona. For the assay, 10 increasing concentrations (10^{-4} – 10^{-13} M) of test compounds were incubated with 50 µg of protein and 2 nM [³H]diprenorphine, 500 pM [³H]deltorphin II, and 1.5 nM [³H]U69,593, respectively, for MOR, DOR, and KOR for 2 h at rt in a shaking water bath as previously described.²⁶ Nonspecific binding was determined as that in the presence of 10 µM of naloxone. After the 2 h incubation, reaction mixtures were rapidly filtered through Whatman GF/B filters (Gaithersburg, MD) presoaked in 1 % polyethyleneimine (PEI), followed with four washes of 2 mL of cold 0.9% saline solution. Radioactivity was determined by liquid scintillation counting in a Beckman LS5000 TD. Data were analyzed by nonlinear least-squares analysis using GraphPad Prism 7. Logarithmic values were determined from nonlinear regression analysis of data collected from at least two independent experiments.

Functional Assays. GPI/LMMP Assay

Isolated guinea pig ileum/longitudinal muscle with myenteric plexus was used to evaluate opioid agonist activity at the KOR. Male Hartley guinea pigs under CO₂ anesthesia were sacrificed by decapitation and a nonterminal portion of the ileum removed. The longitudinal muscle with myenteric plexus (LMMP) was separated from the circular muscle and cut into strips as described previously.²⁷ These tissues were tied to gold chains with suture silk and mounted between platinum wire electrodes in 20 mL organ baths at a tension of 1 g and bathed in oxygenated (95% O₂/5% CO₂) Krebs's bicarbonate buffer at 37 °C, then stimulated electrically (0.1 Hz, 0.4 ms duration) at supramaximal voltage. Following an equilibration

period, compounds were added cumulatively to the bath in volumes of 14–60 μL until maximum inhibition was reached. A PL-017 dose–response curve was constructed to determine tissue integrity before analogue testing.

[^{35}S]GTP γS Assays

Assays were performed as previously described.²⁸ Previously frozen KOR-CHO pellets were homogenized with a Teflon-on-glass Dounce in buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and centrifuged at 20 000*g* at 4 °C for 30 min. Membrane pellet was resuspended in assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl_2 , 1 mM EDTA, 40 μM GDP) with a Teflon-on-glass Dounce. 15 μg membrane protein was incubated with drug or vehicle in the presence of 0.1 nM [^{35}S]GTP γS (PerkinElmer). Plates were incubated at 30 °C for 1 h, and then reactions were terminated by rapid filtration using a 96-well plate Brandel cell harvester (Brandel, Gaithersburg, MD). Plates were dried, and 40 μL of Microscint-20 (PerkinElmer) was added. Bound [^{35}S]GTP γS was measured using a TopCount scintillation and luminescence counter. Data were presented as the mean $\pm$ SEM of the percentage of U50,488 stimulation. Concentration–response curves, fit using a nonlinear three parameter inhibition curve, and efficacy and/or potency values were calculated using GraphPad Prism 7.

In Vivo Studies. Animals

Adult male ICR mice (15–20 g; Harlan, Indianapolis, IN) were kept in a temperature-controlled environment with lights on 07:00 to 19:00 with food and water available ad libitum. All animal procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health, and with approval from the Animal Care and Use Committee of the University of Arizona for the handling and use of laboratory animals.

Behavioral Assay

A mouse model of acute pain (tail flick test) was used, and the compound was administered by lumbar puncture (l.p.) to eliminate the need for intrathecal catheters, as previously described.^{29,30} Briefly, the latency to tail withdrawal from a 52 °C water bath was measured before three doses of analogue **7** or **14** (0.1, 1.0, and 10 μg , in 5 μL volume, $n = 5/\text{treatment}$) were injected into the intrathecal space (baseline values). Tail flick latencies were reassessed up to 5 times over a 90 min period. A cutoff latency of 10.0 s was implemented to prevent tissue damage to the distal third of the tail.

For the measurements in the presence of agonist, mice were pretreated at $t = -15$ min with vehicle (MPH_2O , 5 μL), **7**, or **14** (10 μg in 5 μL volume, $n = 6/\text{treatment}$) by lumbar puncture. At $t = 0$ min, **17** (10 μg in 5 μL volume, l.p.) was given.^{22,23} Tail flick latencies were reassessed up to 9 times over 24 h. A cutoff latency of 10.0 s was implemented to prevent tissue damage to the distal third of the tail. Maximal percent efficacy was calculated and expressed as % antinociception = $100 \times (\text{test latency after drug treatment} - \text{baseline latency})/(\text{cutoff} - \text{baseline latency})$

Statistics

All data were analyzed by nonparametric two-way analysis of variance (ANOVA; post hoc, Bonferroni). Areas under the curve were compared by one-way ANOVA. Differences were considered to be significant if $p \leq 0.05$. All data were plotted in GraphPad Prism 7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Gabriella Molnar for her assistance in binding assays. This work has been supported by U.S. Public Health Services, NIH, and NIDA (Grants RO1 DA 13449 and PO1 DA 006284).

ABBREVIATIONS USED

Ac	acetyl
ACN	acetonitrile
ANOVA	one-way analysis of variance
BSA	bovine serum albumin
CHO	Chinese hamster ovary
DAMGO	[D-Ala ² ,N-MePhe ⁴ ,Gly-ol]enkephalin
DCM	dichloromethane
DMF	<i>N,N</i> -dimethylformamide
DIPEA	diisopropylethylamine
Dyn A	dynorphin A
EtOH	ethanol
Fmoc	9-fluorenylmethyloxycarbonyl
GPI-LM/MP	guinea pig ileum longitudinal muscle with myenteric plexus
HOBt	<i>N</i> -hydroxybenzotriazole
HBTU	2-(1 <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HR-MS	high resolution mass spectrometry
LC-MS	liquid chromatography–mass spectrometry
lp	lumbar puncture
MDP	(2 <i>S</i>)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid
Nle	norleucine

PEI	polyethyleneimine
PMSF	phenylmethylsulfonyl fluoride
rt	room temperature
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
TIS	triisopropylsilane

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Figure 1.
Rat plasma stability studies of Dyn A(1-11) and analogues at 37 °C.

Figure 2. Rightward shift in dose-response curve in the GPI-LM/MP assay in the presence of 10 nM KOR antagonist Nor-BNI of test compounds: (A) **1**, 34-fold shift [(■) **1**, (●) **1** + 10 nM Nor-BNI]; (B) **2**, 26-fold shift [(■) **2**, (●) **2** + 10 nM Nor-BNI].

Figure 3.

(A) [35 S]GTP γ S binding assay of a 10 μ M concentration of [des-Arg 7]Dyn A analogues in KOR-CHO cells. (B) Antagonist mode GTP γ S assay in the presence of agonist U50,488 (100 nM) with test compounds (10 μ M), measuring the analogues' ability to block receptor stimulation by agonist. Analogues with MDP substitution blocked U50,488's agonist activity. Statistical significance was determined by 95% confidence interval [(**) P 0.01, (***) P 0.001].

Figure 4. [³⁵S]GTPγS assay antagonist mode dose–response curve of [des-Arg⁷]Dyn A analogues in KOR-CHO cells versus 100 nM U50,488. Data points represent the mean ± SEM of the % of U50,488 stimulation from $n = 3$ independent experiments.

Figure 5.

Antinociceptive effects of **7** and **14** on tail flick tests 30 min after lumbar puncture into the intrathecal space. (A) TWLs at 52 °C of three doses of **7** (0.1, 1.0, and 10 µg, in 5 µL volume, $n = 5/\text{treatment}$) were measured over 90 min. **7** showed significantly elevated TWL at 30 min by $39.45 \pm 6.95\%$ of max response in the 10 µg in 5 µL dose. (B) TWLs at 52 °C water, three doses of **14** (0.1, 1.0, and 10 µg, in 5 µL volume, $n = 5/\text{treatment}$) were measured over a 24 h period. (C) Percent antinociception of selective KOR agonist **17** measured after pretreatment with **7** and **14**, analyzed by nonparametric two-way analysis of variance (ANOVA; post hoc, Bonferroni). Areas under the curve were compared by one-way ANOVA. Statistical significance was determined by 95% confidence interval [(*) $P \leq 0.05$; (**) $P \leq 0.01$ vs vehicle] on GraphPad Prism 6.

Table 1Structures of [des-Arg⁷]Dyn A Analogues

compd	structure
Dyn A	H-Tyr ¹ -Gly-Gly-Phe-Leu-Arg-Arg ⁷ -Ile-Arg ⁹ -Pro-Lys ¹¹ -Leu-Lys ¹³ -Trp-Asp-Asn-Gln ¹⁷ -OH
1	H-Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH; [des-Arg ⁷]Dyn A(1–13)
2	H-Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH; [des-Arg ⁷]Dyn A(1–11)
3	H-Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-NH ₂ ; [des-Arg ⁷]Dyn A(1–11)-NH ₂
4	Mdp-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-NH ₂ ; N ^α -MDP-[des-Arg ⁷]Dyn A(1–11)-NH ₂
5	Ac-Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-NH ₂ ; N ^α -Ac-[des-Arg ⁷]Dyn A(1–11)-NH ₂
6	Mdp-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-NH ₂ ; N ^α -MDP-[des-Arg ⁷]Dyn A(1–11)
7	H-Tyr-Gly-Pro-Phe-Leu-Arg-Ile-Arg-Pro-Lys-NH ₂ ; [Pro ³ ,des-Arg ⁷]Dyn A(1–11)-NH ₂
8	Ac-Tyr-Gly-Pro-Phe-Leu-Arg-Ile-Arg-Pro-Lys-NH ₂ ; N ^α -Ac-[Pro ³ ,des-Arg ⁷]Dyn A(1–11)-NH ₂
9	Ac-Tyr-Gly-Pro-Phe-Leu-Arg-DAla-Arg-Pro-Lys-NH ₂ ; N ^α -Ac-[Pro ³ ,DAla ⁸ ,des-Arg ⁷]Dyn A(1–11)-NH ₂
10	Ac-Tyr-Gly-Pro-Phe-Leu-Arg-DAla-Arg-DPro-Lys-NH ₂ ; N ^α -Ac-[Pro ³ ,DAla ⁸ ,DPro ¹⁰ ,des-Arg ⁷]Dyn A(1–11)-NH ₂
11	Ac-Tyr-Gly-Gly-Phe-Leu-Arg-DAla-Arg-DPro-Lys-NH ₂ ; N ^α -Ac-[DAla ⁸ ,DPro ¹⁰ ,des-Arg ⁷]Dyn A(1–11)-NH ₂
12	Ac-Tyr-Gly-Pro-Phe-Leu-Orn-DAla-Arg-Pro-Lys-NH ₂ ; N ^α -Ac-[Pro ³ ,Orn ⁶ ,DAla ⁸ ,des-Arg ⁷]Dyn A(1–11)-NH ₂
13	H-Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-NH ₂ ; [des-Arg ⁷]Dyn A(1–9)-NH ₂
14	Mdp-Gly-Gly-Phe-Leu-Arg-Ile-Arg-NH ₂ ; N ^α -MDP-[des-Arg ⁷]Dyn A(1–9)-NH ₂
15	Ac-Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-NH ₂ ; N ^α -Ac-[des-Arg ⁷]Dyn A(1–9)-NH ₂
16	H-Tyr-Gly-Pro-Phe-Leu-Arg-Ile-Arg-NH ₂ ; [Pro ³ ,des-Arg ⁷]Dyn A(1–9)-NH ₂

Table 2

Binding Affinities of [des-Arg⁷]Dyn A Analogues at KOR, MOR, and DOR^a

compd	hKOR, [³ H]U69,593 ^b		hMOR, [³ H]diprenorphine ^c		hDOR, [³ H]deltorphin II ^d	
	log IC ₅₀ ^e	K _i , nM ^f	log IC ₅₀ ^e	K _i , nM ^f	log IC ₅₀ ^e	K _i , nM ^f
Dyn A(1-13)	-9.83 ± 0.10	0.09		8.0 ^g		8.3 ^g
Dyn A(1-11)	-9.96 ± 0.33	0.12		ND		ND
Dyn A(1-11)-NH ₂	-9.81 ± 0.27	0.10		5.6 ^g		3.2 ^g
1	-9.10 ± 0.22	0.39	-8.06 ± 0.09	4.1 ^h	-7.83 ± 0.04	4.5
2	-9.05 ± 0.16	0.43	-7.88 ± 0.06	6.3 ^h	-7.69 ± 0.06	6.2
3	-9.88 ± 0.14	0.07	-8.74 ± 0.22	0.94 ^h	-8.16 ± 0.11	3.6
4	-6.93 ± 0.09	63	-5.79 ± 0.10	1600 ^h	-6.38 ± 0.09	210
5	-6.80 ± 0.07	85	-5.79 ± 0.25	820 ^h	-5.31 ± 0.14	2200
6	-6.38 ± 0.08	230	-5.45 ± 0.09	1800 ^h	-5.95 ± 0.18	580
7	-6.94 ± 0.16	61		>10000	-5.11 ± 0.31	3900
8	-5.83 ± 0.18	730		NC		>10000
9	-5.04 ± 0.18	5200		NC	-4.61 ± 0.14	9700
10	-5.16 ± 0.20	3600		NC		>10000
11	-6.73 ± 0.11	98		NC	-5.06 ± 0.19	720
12	-5.15 ± 0.12	4300		NC		>10000
13	-9.26 ± 0.13	0.30	-6.19 ± 0.01	380	-7.95 ± 0.04	6.1
14	-6.90 ± 0.20	94		>10000	-6.14 ± 0.14	360
15	-7.03 ± 0.30	51	-5.02 ± 0.12	4315	-5.19 ± 0.06	3700
16	-6.40 ± 0.30	220		NC	-5.07 ± 0.15	4600

^aRadioligand competition assays were performed using membrane preparations from transfected HN9.10, NG108, or CHO cells expressed the respective receptor types. ND = not determined. NC = no competition.

^bK_d = 1.5 nM.

^cK_d = 2.81 nM.

^d[³H]Deltorphin, K_d = 0.50 nM.

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ϵ_f Logarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments GraphPad Prism 6.

f Calculated by the Cheng-Prusoff equation.

$g_{\text{Reference 21}}$

$h^{[3\text{H}]\text{DAMGO}}$, $K_D = 0.50$ nM.

Table 3

Rat Plasma Half-Life of Dyn A(1–11) Analogues

analogue	$t_{1/2}$ (h)
Dyn A(1–11)-OH	0.7
Dyn A(1–11)-NH ₂	0.7
2	4.5
7	1.4
11	350
14	50
16	2.1

Table 4GPI/LMMP Assays of [des-Arg⁷]Dyn A Analogues

compd	GPI/LMMP	
	IC ₅₀ (nM) ^a	shift with 10 nM Nor-BNI
1	15 ± 3.8	34-fold
2	21 ± 8.7	26-fold
3	7.0 ± 0.87	ND ^c
4	0% ^b	
5	0% ^b	
6	8.7% ^b	

^aConcentration at 50% inhibition of muscle contraction at electrically stimulated isolated tissues.^bPercent of agonist activity at 1 μM test compound compared to average signal 3 min prior to drug.^cND: not determined.

Table 5Antagonist Mode [^{35}S]GTP γS Assays of [des-Arg⁷]Dyn A Analogues at KOR

compd	<u>[^{35}S]GTPγS binding^a</u>	
	IC ₅₀ (nM) ^b	I _{max} (%) ^c
naloxone	140 ± 58	100
1	NC	NC
2	NC	NC
3	NC	NC
4	220 ± 28	110 ± 7
7	>2500	38 ^d
14	750 ± 137	120 ± 4
16	>2500	29 ^d

^aExpressed in KOR-CHO cells.^bConcentration to afford 50% inhibition. Data reported as the mean ± SEM normalized to 100 nM U50,488 (100% stimulation).^cData are normalized to % of naloxone.^dPercent inhibition at 10 μM; a full curve could not be fit.