Published in final edited form as:

ACS Chem Neurosci. 2016 December 21; 7(12): 1746–1752. doi:10.1021/acscchemneuro.6b00258.

Discovery of Stable Non-opioid Dynorphin A Analogues Interacting at the Bradykinin Receptors for the Treatment of Neuropathic Pain

Sara M. Hall[†], Lindsay LeBaron[†], Cyf Ramos-Colon[†], Chaoling Qu[‡], Jennifer Yanhua Xie[‡], Frank Porreca[‡], Josephine Lai[‡], Yeon Sun Lee^{*,†}, and Victor J. Hruby[†]

[†]Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States

[‡]Department of Pharmacology, University of Arizona, Tucson, Arizona 85719, United States

Abstract

Dynorphin A (Dyn A) is a unique endogenous ligand that possesses well-known neuroinhibitory effects via opioid receptors with a preference for the kappa receptor but also neuroexcitatory effects, which cause hyperalgesia. We have shown that the neuroexcitatory effects are mediated through bradykinin (BK) receptors and that intrathecal (i.th.) administration of our lead ligand **1**, [des-Arg⁷]-Dyn A-(4–11), which shows good binding affinity (IC₅₀ = 150 nM) at the BK receptors, blocks Dyn A-induced hyperalgesia in naïve animals and reverses thermal and tactile hypersensitivities in a dose-dependent manner in nerve-injured animals. However, **1** has a serious drawback as a potential drug candidate for the treatment of neuropathic pain because of its susceptibility to enzymatic degradation. In an effort to increase its stability, we modified ligand **1** using non-natural amino acids and found that analogues substituted at or near the N-terminus with a D-isomer retain binding at the receptor and provide a large increase in stability. In particular when Leu⁵ was modified, with either the D-isomer or N-methylation, there was a large increase in stability ($t_{1/2}$ = 0.7–160 h in rat plasma) observed. From these studies, we have developed a very stable Dyn A analogue **16**, [D-Leu⁵,des-Arg⁷]-Dyn A-(4–11), that binds to BK receptors (IC₅₀ = 130 nM) in the same range as ligand **1** and shows good antihyperalgesic effects in both naïve rats and L₅/L₆ spinal nerve ligation rats.

Graphical abstract

*Corresponding Author: Telephone: 1-520-626-2820. Fax: 1-520-626-2204. yeon@email.arizona.edu.

Supporting Information: The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.6b00258. Analytical data, including HRMS data, HPLC data, and aLogPs for all new compounds (PDF)

Author Contributions: The experiments were conducted by S.M.H., L.L., C.R.-C., C.Q., J.Y.X., and Y.S.L. The design of the analogues and writing of the manuscript were achieved through the contributions of S.M.H., F.P., J.L., Y.S.L., and V.J.H. All authors have given approval to the final version of the manuscript.

Notes: The authors declare no competing financial interest.

Keywords

Non-opioid dynorphin A; Bradykinin receptors; Structure; activity relationship; Peptide stability; Antihyperalgesic effect; Neuropathic pain

Peptides have recently become more prevalent as therapeutics in part because their efficacy, selectivity, and specificity are greater than those of small molecules.¹ In addition, the degradation products of peptides are less toxic and exhibit a lower level of accumulation in tissues.² One of the major obstacles in developing peptide therapeutics is their short half-lives that are caused by their susceptibility to degradation by peptidases. Peptidases from the plasma of blood, the lumen of the small intestine, and the brush border membrane of epithelial cells rapidly degrade peptides.³ Therefore, to increase their stability, various kinds of chemical modifications such as cyclization, N- or C-terminal modification, un-natural amino acid substitution, and peptide backbone modification have been successfully applied to peptides.

Neuropathic pain results from the dysfunction of the central nervous system (CNS) or the peripheral nervous system (PNS) and imposes a significant problem for the health industry.⁴ Treatment of this disease with conventional methods is difficult, partly because the mechanism is not well-known. One target for neuropathic pain treatment may be the blockade of dynorphin A (Dyn A) (H-Try-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-OH). Dyn A is a unique endogenous peptide that has neuroinhibitory effects, mediated through the opioid receptors,^{5,6} and neuroexcitatory effects, mediated through bradykinin (BK) receptors in the CNS.⁷ These neuroexcitatory effects are caused by the interaction of Dyn A or [des-Tyr¹]-Dyn A fragments with the BK receptors and cannot be blocked by opioid receptor antagonists such as naloxone, indicating non-opioid effects.⁶ Dyn A has been found to be upregulated in a neuropathic pain model and contributes to the maintenance of pain.⁷ Therefore, the development of a BK receptor antagonist to block the excitatory effects of Dyn A can result in the discovery of a novel therapeutic for chronic neuropathic pain. Because these Dyn A analogues are non-opioid ligands interacting with BK receptors, they will not develop the serious side effects caused by long-term administration of opioids but will retain potential activity for modulating Dyn A-induced hyperalgesia under pathological pain conditions.

We previously discovered a good pharmacophore, compound **1** (H-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH), for the central BK receptors, of which the structure is based on Dyn A. Intrathecal (i.th.) administration of compound **1** was able to block Dyn A-induced hyperalgesia and motor impairments in naïve animals.⁸ The compound was also found to

reverse thermal hyperalgesia and mechanical hypersensitivity in nerve-injured animals [L₅/L₆ spinal nerve ligation (SNL)] in a dose-dependent manner.⁸ While **1** is efficacious in a neuropathic pain model, this compound is susceptible to enzymatic degradation because of its composition of natural amino acids (half-life of <1 h in rat plasma). Therefore, in an effort to increase its stability, **1** was modified as follows: replacement of Ile, Leu, and Pro residues with non-natural amino acids, N-terminal acetylation, C-terminal amidation, D-amino acid substitutions, and N-methylations. From the modifications, we were able to identify a clear degradation pattern for **1**, which was different from that of Dyn A, the parent compound, and were able to develop a very stable analogue, **16**.

Here we report that analogues substituted at or near the N-terminus with a D-amino acid retain good binding affinity at the BK receptors with a large increase in stability. In particular, when Leu⁵ (position 2 of **1**) was substituted with a D-Leu residue (ligand **16**) or an NMeLeu residue (ligand **21**), this resulted in a large increase in stability (up to 160 h), which indicates that the Leu residue is involved in the degradation. More importantly, the modification with a D-Leu in **16** retained good biological activities like ligand **1** in in vitro and in vivo assays.

Results and Discussion

Peptide Synthesis

The Dyn A analogues were synthesized by standard Fmoc chemistry with 50–80% crude purity. Purification was simple and fast in part because of the short retention times (RTs) of the analogues, due to their hydrophilic nature. The peptide structures were confirmed by high-resolution mass spectrometry (HR-MS), and the final purity of all analogues was 95% (Table 1 of the Supporting Information).

Structure–Activity Relationship (SAR) at the BK Receptors

Previous studies by our group have extensively looked at the SAR and found that amphipathicity is a key component in binding at the BK receptors.^{8,9} We also found that deletion of Arg⁷ in Dyn A fragments did not affect the binding at the BK receptors.¹⁰ From these SAR studies, we discovered compound **1**, [[des-Arg⁷]-Dyn A-(4–11)], as a good pharmacophore for the BK receptors. In an effort to increase its stability, analogues substituted with non-natural amino acids were designed and synthesized. Substitution of Leu⁵ and Ile⁸ with a norleucine (Nle) residue was examined in **2** and **3**, respectively, both of which maintained the same range of good binding affinity at the receptor (IC₅₀ values of 160 nM for **2** and 190 nM for **3**) (Table 1). Like the modifications of hydrophobic amino acids, we also substituted three basic amino acid residues (Arg⁶, Lys⁹, and Lys¹¹) in **1** with three Arg residues along with double Nle substitutions, which resulted in ligand **4** maintaining good binding affinity (IC₅₀ = 86 nM). Acetylation of the N-terminus of **4** did not alter the binding affinity (IC₅₀ = 140 nM) in **5**, and the same trend was observed in other acetylated peptides (data not shown). In contrast, substitution of three basic amino acid residues with three ornithine residues resulted in ligand **6** showing decreased binding affinity (IC₅₀ = 510 nM). In our previous studies, it was shown that substitutions of hydrophobic amino acid residues and basic amino acid residues with multiple Nle residues and multiple Arg (or Lys)

residues, respectively, were well tolerated.^{8,9} The triple substitution with the ornithine residue in ligand **6** might cause less exposure of the important basic amino group due to the lack of one carbon chain and thus resulted in a decrease in the level of binding to the receptor. Pro is known to be an important amino acid residue that makes a turn structure because of its imide nature. A nuclear magnetic resonance study showed that the Pro¹⁰ residue in ligand **1** was involved in the formation of a distorted type I β -turn structure at the C-terminus.⁸ To confirm this role, un-natural amino acids were used in place of Pro¹⁰, such as the proline surrogate 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), D-Tic, and the Pro analogues octahydroindole-2-carboxylic acid (Oic) and 3-thienylalanine (Thi). The binding affinities of the resulting ligands **7–10** decreased by 3–75-fold, suggesting that the constrained Pro¹⁰ residue plays an important role in making a topographical structure for recognition at the BK receptors.

We previously observed that a complete inversion of chirality of the peptide, the so-called inversed peptide, resulted in a dramatic loss of affinity.⁹ Therefore, to investigate which amino acid residues could be replaced and tolerated, a systemic D-amino acid scan was performed (Table 1, **11–17**). In this scan, it was found that substitutions in the middle of the sequence and at the C-terminus led to a loss of affinity (**11–14**), while those substituted at the N-terminus retained high affinity at the receptor (**15–17**). This result correlates well with our previous SAR results, in that substitutions at the N-terminus were well tolerated while the C-terminus cannot be modified because of its important role in BK receptor recognition.^{8–10} Although a single D-amino acid substitution at the N-terminus was tolerated, a double substitution with the D-isomer at Phe⁴ and Arg⁶ (**18**) or Phe⁴ and Leu⁵ (**19**) completely abolished affinity at the BK receptors.

N-Methylation of the peptide backbone is known to be a good tool for increasing peptide stability, oral bioavailability, affinity, and selectivity.¹¹ For these reasons, Phe⁴ and Leu⁵ were substituted in two ligands with the N-methylated amino acid. Both of the resulting ligands (**20** and **21**) lost binding affinity (IC₅₀ values of 870 and 1500 nM, respectively).

Stability in Rat Plasma

Drugs that are bioavailable and taken orally are degraded mainly by the small intestine and/or liver.³ Most peptides are not bioavailable, and instead, the route of administration is intravenous (i.v.) or i.th. injection. Their degradation is mainly caused by proteolytic enzymes in the plasma. For these reasons, our ligands were tested in rat plasma. As suspected, lead ligand **1** was found to be very unstable in the rat plasma, being completely degraded within 4 h of incubation, and had a calculated half-life ($t_{1/2}$) of 0.7 h (Figure 1A and Table 2). To study the structure–stability relationship and thus to identify potent Dyn A analogues with enhanced stability, peptides with diverse modifications were selected for the stability test in rat plasma. Considering the high affinity (IC₅₀ = 86 nM) and the possible increase in stability from the Nle substitution, ligand **8** was selected first for the test. This ligand led to a small increase in stability with a $t_{1/2}$ of 1.2 h, which is to be expected considering the structural similarities between Nle and Leu/Ile. A further increase in stability, albeit small, was seen with N-terminal acetylation of **8**, in ligand **9** with a $t_{1/2}$ of 3.4 h. Next, the effects on stability of D-amino acid substitutions were examined. As shown in

Figure 1B, all of the D-amino acid substitutions near the N-terminus (ligands **15–17**) resulted in a large increase in stability ($t_{1/2}$ values of 0.7 h vs 31, 160, and 19 h, respectively). In particular, the substitution with D-Leu⁵ enhanced the stability dramatically from a $t_{1/2}$ of 0.7 to a $t_{1/2}$ of 160 h with 99% intact after 24 h. It has been suggested by Varshasky that the N-terminal amino acid residue determines the stability of a peptide (the N-end rule).¹² The same low stability ($t_{1/2}$ = 0.7 h) of ligand **11** (D-Lys⁹) and **1** is a good example of the N-end rule (Figure 1C). On the basis of the N-end rule, the N-terminal modification by an unnatural amino acid residue is considered to be an efficient way to optimize peptide stability by reducing susceptibility to enzymatic degradation.

Interestingly, the D-amino acid scan at all positions of ligand **1** (ligands **11–17**) resulted in a very distinct SAR. The D-amino acid substitution near the region of the C-terminus was not tolerated and decreased the binding affinity at BK receptors (**11–14**). To gain a key insight into the degradation of the Dyn A fragments, ligand **14** with a low binding affinity was tested and found to be more stable ($t_{1/2}$ = 3.3 h) but not as stable as other ligands (**15–17**). All of the D-amino acid substitutions in ligands **11–17** showed the interesting pattern of stability shown in Table 2: the stability increase depends on the location of a D-amino acid. Now it is clear that the C-terminal region, which is very important for BK receptor recognition, does not play an important role in rat plasma stability but the N-terminus does. Further support was obtained from **20** and **21**, which contain NMePhe⁴ and NMeLeu⁵, respectively (Figure 1D). These ligands do not retain high affinity at the BK receptors but were found to be stable like ligands **16** and **17** that contained D-amino acid residues. Interestingly, when Leu⁵ was methylated, compound **21** was extremely stable and was 100% intact after a 24 h incubation. The stability data of ligands **16** and **21** indicate that position 5 is the main site for degradation of Dyn A analogues in rat plasma. Taken together, these results indicate that it is possible to develop a highly potent Dyn A ligand with enhanced stability simply by modifying the N-terminal region.

In the 24 h rat plasma study, **16** and **21** were both very stable without any trace of degradation. Because there was little degradation over 24 h, the calculated half-life may not be meaningful ($t_{1/2}$ for **21** of >4000 h). To obtain a more accurate half-life, a 5 day study was conducted with **16** and **21**, in which time points were taken every 24 h. In this study, **16** was found to have a $t_{1/2}$ of 160 h, and at the last time point, 120 h, 66% of the peptide remained. On the other hand, **21**, which had the same high stability as **16** in the 24 h study, showed stability ($t_{1/2}$ = 65 h, and only 31% of the peptide remained at 120 h) lower than that of **16**. With the more precisely calculated half-lives, these compounds are still the most stable of the series. Both have modifications at Leu⁵, which points to the importance of this amino acid for stability.

Stability in Human Plasma

The most stable ligands in the rat plasma assay (**16** and **21**) were further tested in human plasma as well as **1** as a control (Figure 2 and Table 3). It was found that **1** was more stable in human plasma (2.4 h) than in rat plasma (0.7 h). After a 24 h incubation in human and rat plasma, compounds **16** and **21** were both more than 95% intact. Compound **16** had similar half-lives in both plasmas, while compound **21** was more stable in human plasma. Although

there are changes of exact half-lives in rat and human plasma, the trend is the same in both plasmas. Ligand **1** is quickly degraded, and modifications at position 5 with a D-amino acid residue or a NMeLeu residue (**16** or **21**, respectively) resulted in large increases in plasma stability.

Identification of the Degradation Site for Ligand **1**

From the rat and human plasma stability assays, it was shown that **1** is not stable and quickly degrades within 4 h of incubation. On the basis of the structure–stability relationship results, it was suggested that the degradation of ligand **1** mainly occurs at the Leu⁵ site because ligands **16** and **21**, which are modified at this residue, showed the most enhanced stability in the rat and human plasma. To identify a site for degradation, a major peak after a 30 min incubation in rat plasma was isolated by reversed-phased high-performance liquid chromatography (RP-HPLC). Liquid chromatography and mass spectrometry (LC–MS) of the peak showed an ion at m/z 782.6 (MH⁺) to indicate the existence of a [des-Arg⁷]-Dyn A-(5–11) fragment, which confirms that **1** is mainly degraded at the Phe⁴–Leu⁵ bond near the N-terminal region.

Comparison of the Stability of Ligand **1** in Rat Plasma with or without Protease Inhibitors

The LC–MS data confirmed that the degradation of compound **1** occurs at the Phe⁴–Leu⁵ bond. To investigate which proteases are responsible for the cleavage, various protease inhibitors were added with **1** to rat plasma. Because ligand **1** was degraded at the N-terminal peptide bond, an aminopeptidase may be responsible for the degradation. Bestatin is a common aminopeptidase inhibitor of leucine aminopeptidase, aminopeptidase B, and aminopeptidase N.^{14,15} Chymotrypsin is a serine protease that cleaves after aromatic amino acids such as Tyr, Phe, and Trp.¹⁶ Chymostatin is a known inhibitor of chymotrypsin, as well as cathepsin B, and cathepsin D, a cysteine protease that cleaves after a Phe residue.¹⁷ Phenyl-methanesulfonyl fluoride (PMSF) is another serine protease inhibitor that also targets chymotrypsin but will also inhibit papain and thrombin.¹⁸ Captopril is an angiotensin-converting enzyme (ACE) inhibitor, and BK, the endogenous ligand for the BK receptors, is known to be degraded by ACE.¹⁹ Even though the structures of BK and Dyn A are very different, captopril was used to make sure ACE did not degrade it as well. Ethylenediaminetetraacetic acid (EDTA) is a metal chelator, and many metalloproteases that could cleave the Xaa–Leu bond, such as neprilysin, enkephalinase, and neutral endopeptidase, use metals in their active site.³ All inhibitors were added to the test solution at an effective concentration, but there was no stability increase observed in any case (Table 4). To determine if multiple inhibitors would better prevent the degradation, all five inhibitors were added to the test sample but were not successful in increasing the half-life. This result is similar to that seen for Dyn A, for which inhibition with protease inhibitors did not improve stability.²⁰

Antihyperalgesic Effects of Ligand **16**

Ligand **1** was demonstrated to have potent antihyperalgesic effects in SNL rats and also to modulate Dyn A-induced neuroexcitatory effects in naïve rats.⁸ To determine whether ligand **16**, which showed good binding affinity with increased metabolic stability, possesses the

same antihyperalgesic effects as **1**, von Frey filament tests and Infra-Red tests were performed in naïve rats (Figure 3A) and SNL rats (Figure 3B). i.th. injection (3 nmol) of Dyn A-(2–13) into naïve rats reduced both paw withdrawal threshold and latency in the tests, which indicated its thermal and tactile hypersensitivities. The Dyn A-(2–13)-induced hypersensitivities were blocked by the co-administration of ligand **16** (3 nmol/5 μ L) in both tests. The paw withdrawal threshold and latency were maximized 40 min after injection, and their recovery at 40 min in the tests clearly confirms the antihyperalgesic effects of ligand **16**. The pattern of antihyperalgesic effects was similar to that of ligand **1** from our previous work.⁸ Ligand **16** also modulated nerve injury-related tactile hypersensitivity in SNL rats. From the in vivo tests, it was demonstrated that ligand **16** possesses potent antihyperalgesic effects like ligand **1**.

Conclusion

We had previously discovered a non-opioid Dyn A ligand **1** for the BK receptors that modulates Dyn A-induced neuro-excitatory effects in naïve animals and hypersensitivities in nerve-injured animals. This ligand was shown to be highly susceptible to enzymatic degradation, and therefore, to optimize stability, various modifications were introduced in this study. We found that modifications at the C-terminus were not well tolerated and actually did not improve the stability, whereas modifications toward the N-terminus with a D-amino acid residue led to a large increase in stability with little effect on affinity. In particular, the stability data clearly indicated the effectiveness of those modifications for Leu⁵. LC–MS data for the isolated peak confirmed that **1** is quickly degraded by the main cleavage of the Phe⁴–Leu⁵ bond at the N-terminus, which cannot be prevented by the addition of inhibitors. By modifying the crucial position for enzymatic degradation, we were able to develop **16** as a lead ligand that retains the same high affinity at the BK receptors with much increased stability in plasma (229-fold) compared to that of **1**. More importantly, **16** retained the same potent antihyperalgesic effects as ligand **1** in naïve and nerve-injured animals. This study reveals a promising treatment for neuropathic pain in developing highly potent Dyn A ligands with enhanced stability through a simple modification.

Methods

Synthesis of Dyn A Analogues

The Dyn A analogues were synthesized by standard solid-phase peptide synthesis (SPPS) using Fmoc chemistry on amino acid-preloaded Wang resin (100–200 mesh).^{21,22} The resin was allowed to swell for approximately 3 h in DCM at room temperature (rt), and Fmoc deprotection was performed using 20% piperidine in N,N-dimethylformamide (DMF). To remove side products and excess reagents, resin was washed with 3 $\times$ DMF, 3 $\times$ dichloromethane (DCM), and 3 $\times$ DMF. After deprotection, a Kaiser test was conducted using the following solutions: a 5% (w/v) ninhydrin solution in ethanol (EtOH), 80% (w/v) phenol in EtOH, and 200 nM KCN in pyridine. After a positive Kaiser test, coupling was conducted with 3 equiv of N-hydroxybenzo-triazole (HOBt), 3 equiv of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), and 6 equiv of diisopropylethylamine (DIPEA) in DMF for 50 min. HOBt was added to prevent

racemization. Deprotection and coupling steps were repeated to complete peptide chain elongation, and resin with an N-terminal free amine was cleaved with a cocktail solution of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% water for 3 h at rt. The resulting peptide was purified by RP-HPLC (Agilent 1100 instrument with a Vydac 218TP510 C-18 column) using a gradient system (from 10 to 40% acetonitrile in 15 min) to afford a purity of >95%. The peptide structure was validated by high-resolution mass spectrometry (HR-MS) and analytical HPLC for purity.

Membrane Preparations

Crude membranes were prepared by homogenization of fresh whole brains isolated from male Sprague-Dawley rats with a Polytron (Pittsburgh, PA) PT 2100 homogenizer in ice-cold buffer [50 mM Tris-HCl (pH 6.8) and 100 μ M PMSF]. After homogenization, the brains were centrifuged at 4000 rpm in a Beckman JA-18 rotor for 15 min at 4 °C, and afterward, the supernatants were collected. The procedure was repeated (homogenization and centrifugation), and finally, the supernatants were centrifuged at 17500 rpm in a Beckman JA-18 rotor for 30 min at 4 °C. The resulting membrane pellets were resuspended in the same ice-cold buffer as described above for storage at –80 °C until they were used. The Lowry method was used to determine the protein concentration.²³

Radioligand Binding Assays

The frozen membranes were thawed and resuspended in assay buffer [50 mM Tris (pH 6.8) containing 50 μ g/mL bacitracin, 10 μ M captopril, 100 μ M PMSF, and 5 mg/mL bovine serum albumin (BSA)]. To determine the binding affinity, 10 concentrations (10^{-12} to 10^{-3} M) of the test compound were each incubated with 50 μ g of membranes and 1 nM [3 H]BK. To assess nonspecific binding, 10 μ M kallidin (KD) was added to a few tubes in all assays. The assay was conducted in a shaking water bath at 25 °C for 120 min and terminated by rapid filtration through Whatman (Gaithersburg, MD) GF/B filters presoaked in 1% polyethylenimine (PEI), followed with four washes with 2 mL of cold saline. Liquid scintillation counting on a Beckman LS5000 TD instrument was used to determine radioactivity (counts per minute). The data were analyzed by nonlinear least-squares analysis using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Stability Assays

Stock solutions of compounds (50 mg/mL in water) were diluted 5-fold into rat plasma (Pel-Freez Biologicals, lot 07230) or human plasma (Lithium Heparin Innovative Research, lot 13787) and incubated at 37 °C. After 1, 2, 4, 6, 12, and 24 h, 100 μ L of the sample was added to 150 μ L of EtOH, stopping the reaction. Samples were spun down at 10000 rpm for 15 min, and 100 μ L of the supernatant was analyzed by RP-HPLC (Agilent 1100 autosampler with a YMC AA12S05-2546WT, C-18 analytical column) using a gradient system (from 10 to 90% acetonitrile in 40 min). For the stability assay, the half-life was calculated with the equation $t_{1/2} = 0.693/b$, where b is the slope found in the linear fit of the natural logarithm of the fraction remaining of the parent compound versus incubation time.¹³ The samples were tested in three independent experiments ($n = 3$).

Isolation and Validation of a Degraded Fragment from 1

1 (10 mg/mL) was added to rat plasma, and the reaction was stopped by EtOH after 30 min. After the sample was spun down, the supernatant was placed on an HPLC instrument and the fractions before the main peak corresponding to the intact peptide were collected. The collected fractions were analyzed on a LCQ classic instrument with ESI (Finnigan, Thermoelectron).

In Vivo Assays

The in vivo assays were conducted with nonfasted male Sprague-Dawley rats [250–300 g (Harlan, Indianapolis, IN)]. Prior to the assay, the rats were kept in a room at 22 ± 2 °C with a 12 h illumination. All experiments were performed using an approved protocol of the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona under policies and guidelines for laboratory animals of the International Association for the Study of Pain (IASP) and the National Institutes of Health (NIH). The rats were implanted with i.th. catheters (polyethylene 10, 7.8 cm) for drug administration, under ketamine/xylazine [80/12 mg/kg, intraperitoneal (i.p.)] anesthesia. The catheters were placed through the atlanto-occipital membrane extended to the level of the lumbar spinal cord.²⁴

To assess thermal hypersensitivity and tactile hypersensitivity, infrared (IR) and von Frey tests, respectively, were performed.^{8,25} In the tests, compounds were administered (i.th.), and behavior was assessed five times every 20 min (up to 120 min). Paw withdrawal latencies in seconds were measured after calibrating the apparatus to give a latency of approximately 20 s on the uninjured paw. To determine paw withdrawal thresholds, an “up–down” method and analysis using a Dixon nonparametric test were performed. Paw withdrawal latencies and paw withdrawal thresholds were calculated and expressed in Graph Pad Prism 7; one-way analysis of variance (ANOVA) was performed with FlashCalc (University of Arizona), and statistical significance achieved when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work has been supported by U.S. Public Health Services, the National Institutes of Health, and National Institute on Drug Abuse Grants P01DA006284 and R01DA013449.

References

1. Hummel G, Reineke U, Reimer U. Translating peptides into small molecules. *Mol BioSyst.* 2006; 2:499–508. [PubMed: 17216031]
2. Loffet A. Peptides as drugs: is there a market? *J Pept Sci.* 2002; 8:1–7. [PubMed: 11831558]
3. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. *Drug Discovery Today.* 2010; 15:40–56. [PubMed: 19879957]
4. Baron R. Mechanisms of disease: neuropathic pain—a clinical perspective. *Nat Clin Pract Neurol.* 2006; 2:95–106. [PubMed: 16932531]
5. Chavkin C, Goldstein A. Demonstration of a specific dynorphin receptor in guinea pig ileum myenteric plexus. *Nature.* 1981; 291:591–593. [PubMed: 6264311]

6. Walker JM, Moises HC, Coy DH, Baldrihi G, Akil H. Nonopiate effects of dynorphin and des-Tyr-dynorphin. *Science*. 1982; 218:1136–1138. [PubMed: 6128791]
7. Lai J, Luo MC, Chen QM, Ma SW, Gardell LR, Ossipov MH, Porreca F. Dynorphin A activates bradykinin receptors to maintain neuropathic pain. *Nat Neurosci*. 2006; 9:1534–1540. [PubMed: 17115041]
8. Lee YS, Muthu D, Hall SM, Ramos-Colon C, Rankin D, Hu J, Sandweiss AJ, De Felice M, Xie JY, Vanderah TW, Porreca F, Lai J, Hruby VJ. Discovery of amphipathic dynorphin A analogues to inhibit the neuroexcitatory effects of dynorphin A through bradykinin receptors in the spinal cord. *J Am Chem Soc*. 2014; 136:6608–6616. [PubMed: 24742335]
9. Lee YS, Hall SM, Ramos-Colon C, Remesic M, LeBaron L, Nguyen A, Rankin D, Porreca F, Lai J, Hruby VJ. Modification of amphipathic non-opioid dynorphin A analogues for rat brain bradykinin receptors. *Bioorg Med Chem Lett*. 2015; 25:30–33. [PubMed: 25434001]
10. Lee YS, Rankin D, Hall SM, Ramos-Colon C, Ortiz JJ, Kupp R, Porreca F, Lai J, Hruby VJ. Structure-activity relationships of non-opioid [des-Arg(7)]-dynorphin A analogues for bradykinin receptors. *Bioorg Med Chem Lett*. 2014; 24:4976–4979. [PubMed: 25282551]
11. Chatterjee J, Gilon C, Hoffman A, Kessler H. N-methylation of peptides: a new perspective in medicinal chemistry. *Acc Chem Res*. 2008; 41:1331–1342. [PubMed: 18636716]
12. Varshavsky A. The N-end rule: functions, mysteries, uses. *Proc Natl Acad Sci U S A*. 1996; 93:12142–9. [PubMed: 8901547]
13. Konsoula R, Jung M. In vitro plasma stability, permeability and solubility of mercaptoacetamide histone deacetylase inhibitors. *Int J Pharm*. 2008; 361:19–25. [PubMed: 18562136]
14. Suda H, Aoyagi T, Takeuchi T, Umezawa H. Inhibition of aminopeptidase B and leucine aminopeptidase by bestatin and its stereoisomer. *Arch Biochem Biophys*. 1976; 177:196–200. [PubMed: 999285]
15. Umezawa H, Aoyagi T, Suda H, Hamada M, Takeuchi T. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J Antibiot*. 1976; 29:97–99. [PubMed: 931798]
16. Kaufman S, Schwert GW, Neurath H. The specific peptidase and esterase activities of chymotrypsin. *Arch Biochem*. 1948; 17:203–5. [PubMed: 18910698]
17. Umezawa H. Structures and activities of protease inhibitors of microbial origin. *Methods Enzymol*. 1976; 45:678–695. [PubMed: 1012021]
18. Gold AM, Fahrney DE. The mechanism of reactivation of phenylmethanesulfonyl alpha-chymotrypsin. *Biochem Biophys Res Commun*. 1963; 10:55–59. [PubMed: 13948636]
19. Regoli D, Barabe J. Pharmacology of bradykinin and related kinins. *Pharmacol Rev*. 1980; 32:1–46. [PubMed: 7015371]
20. Leslie FM, Goldstein A. Degradation of Dynorphin-(1-13) by Membrane-Bound Rat-Brain Enzymes. *Neuropeptides*. 1982; 2:185–196.
21. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Pept Protein Res*. 1990; 35:161–214. [PubMed: 2191922]
22. Carpino LA, Han GY. The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J Org Chem*. 1972; 37:3404–3409.
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193:265–275. [PubMed: 14907713]
24. Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*. 1992; 50:355–363. [PubMed: 1333581]
25. Largent-Milnes TM, Yamamoto T, Nair P, Moulton JW, Hruby VJ, Lai J, Porreca F, Vanderah TW. Spinal or systemic TY005, a peptidic opioid agonist/neurokinin 1 antagonist, attenuates pain with reduced tolerance. *Br J Pharmacol*. 2010; 161:986–1001. [PubMed: 20977451]

Abbreviations

ACE	angiotensin-converting enzyme
ANOVA	one-way analysis of variance

BK	bradykinin
BSA	bovine serum albumin
CNS	central nervous system
DALKD	[des-Arg ¹⁰ ,Leu ⁹]-kallidin
DCM	dichloromethane
DMF	<i>N,N</i> -dimethylformamide
DIPEA	diisopropylethylamine
Dyn A	dynorphin A
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
EtOH	ethanol
Fmoc	9-fluorenylme-thyloxycarbonyl
HOBt	<i>N</i> -hydroxybenzotriazole
HBTU	2-(1 <i>H</i> -benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HR-MS	high-resolution mass spectrometry
i.p	intraperitoneal
i.th	intrathecal
i.v	intravenous
KD	kallidin
LC-MS	liquid chromatography and mass spectrometry
Oic	octahydroindole-2-carboxylic acid
PEI	polyethylenimine
PMSF	phenylmethanesulfonyl fluoride
PNS	peripheral nervous system
rt	room temperature
RT	retention time
SNL	L ₅ /L ₆ spinal nerve ligation
SPPS	solid-phase peptide synthesis
TFA	trifluoroacetic acid

Thi	3-thienylalanine
TIS	triisopropylsilane
Tic	2,3,4-tetrahydroisoquinoline-3-carboxylic acid

Figure 1.
In vitro stability of Dyn A analogues in rat plasma. The samples were tested in three independent experiments ($n = 3$).

Figure 2.

In vitro stability of selected Dyn A analogues in human and rat plasma. The samples were tested in three independent experiments ($n = 3$).

Figure 3.

Antihyperalgesic effects of ligand **16** in (A) naïve rats and (B) SNL rats. Statistical significance was determined by a 95% confidence interval. * $p < 0.05$ compared with Dyn A-(2-13). # $p < 0.05$ compared with vehicle. $n = 6$. Vehicle: DMSO/Tween 80/saline (1/1/8).

Table 1
Binding Affinities of [des-Arg⁷]-Dyn A-(4–11) Analogues at the BK Receptors in Rat Brain

no.	structure	BK receptors, ^a [³ H]BK	
		log[IC ₅₀] ^b	IC ₅₀ (nM)
1	H-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH	−6.83 ± 0.09	150 ^c
2	H-Phe-Nle-Arg-Ile-Arg-Pro-Lys-OH	−6.79 ± 0.10	160
3	H-Phe-Leu-Arg-Nle-Arg-Pro-Lys-OH	−7.05 ± 0.09	90
4 ^d	H-Phe-Nle-Arg-Nle-Arg-Pro-Arg-OH	−7.06 ± 0.10	86 ^e
5 ^d	Ac-Phe-Nle-Arg-Nle-Arg-Pro-Arg-OH	−7.17 ± 0.10	140 ^e
6	H-Phe-Leu-Orn-Ile-Orn-Pro-Orn-OH	−6.29 ± 0.12	510
7	H-Phe-Leu-Arg-Ile-Arg-Tic-Lys-OH	−5.15 ± 0.09	510
8	H-Phe-Leu-Arg-Ile-Arg-D-Tic-Lys-OH		>10000
9	H-Phe-Leu-Arg-Ile-Arg-Thi-Lys-OH	−5.99 ± 0.20	1000
10	H-Phe-Leu-Arg-Ile-Arg-Oic-Lys-OH	−5.82 ± 0.10	1500
11	H-Phe-Leu-Arg-Ile-Arg-Pro-D-Lys-OH	−6.03 ± 0.14	830
12	H-Phe-Leu-Arg-Ile-Arg-D-Pro-Lys-OH		>10000
13	H-Phe-Leu-Arg-Ile-D-Arg-Pro-Lys-OH	−5.23 ± 0.12	5900
14	H-Phe-Leu-Arg-D-Ile-Arg-Pro-Lys-OH		>10000
15	H-Phe-Leu-D-Arg-Ile-Arg-Pro-Lys-OH	−6.51 ± 0.19	310
16	H-Phe-D-Leu-Arg-Ile-Arg-Pro-Lys-OH	−6.90 ± 0.23	130
17	H-D-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH	−6.60 ± 0.14	250
18	H-D-Phe-Leu-D-Arg-Ile-Arg-Pro-Lys-OH		>10000
19	H-D-Phe-D-Leu-Arg-Ile-Arg-Pro-Lys-OH		>10000
20	H-NMePhe-Leu-Arg-Ile-Arg-Pro-Lys-OH	−6.06 ± 0.14	870
21	H-Phe-NMeLeu-Arg-Ile-Arg-Pro-Lys-	−6.78 ± 0.15	1500

^aCompetition assays were conducted at pH 6.8 in rat brain membranes using 1 nM [³H]BK.

^bLogarithmic values were determined by the nonlinear regression analysis of data collected from at least two independent experiments in duplicate.

^cIC₅₀ = 69 nM at pH 6.8 in the competition assay using [³H]DALKD.

^dFrom ref 8.

^eCompetition assay using 1 nM [³H]DALKD.

Table 2
Structure–Stability Relationships of Dyn A Analogues in Rat Plasma^a

no.	structure	IC ₅₀ (nM)	<i>t</i> _{1/2} (h) ^b	intact (%) ^c
1	H-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH	150	0.7	0
4	H-Phe- Nle -Arg- Nle -Arg-Pro- Arg -OH	86	1.2	0
5	Ac-Phe- Nle -Arg- Nle -Arg-Pro- Arg -OH	140	3.4	0
11	H-Phe-Leu-Arg-Ile-Arg-Pro-D- Lys -OH	830	0.7	0
14	H-Phe-Leu-Arg-D- Ile -Arg-Pro-Lys-OH	>10000	3.3	0
15	H-Phe-Leu-D- Arg -Ile-Arg-Pro-Lys-OH	310	31	62 ± 25
16	H-Phe-D- Leu -Arg-Ile-Arg-Pro-Lys-OH	130	160 ^d	99 ± 9
17	H-D- Phe -Leu-Arg-Ile-Arg-Pro-Lys-OH	250	19	44 ± 12
20	H-N MePhe -Leu-Arg-Ile-Arg-Pro-Lys-OH	870	15	31 ± 11
21	H-Phe-N MeLeu -Arg-Ile-Arg-Pro-Lys-OH	1500	65 ^d	100 ± 16

^aDetails described in Figure 1 and Table 1.

^bHalf-life (*t*_{1/2}) determined as 0.693/*b*, where *b* is the slope found in the linear fit of the natural logarithm of the fraction remaining of the parent compound vs incubation time.¹³

^cAfter a 24 h incubation.

^dCalculated from a 5 day stability test.

Table 3
Structure–Stability Relationship of Dyn A Analogues in Human Plasma^a

no.	structure	$t_{1/2}$ (h)	Intact (%) ^b	$t_{1/2}$ (h) ^c
1	H-Phe-Leu-Arg-Ile-Arg-Pro-Lys-OH	2.4	0	0.7
16	H-Phe-D-Leu-Arg-Ile-Arg-Pro-Lys-OH	150	99 ± 10	160
21	H-Phe-NMeLeu-Arg-Ile-Arg-Pro-Lys-OH	190	96 ± 5	65

^aDetails described in Figure 2 and Table 2.

^bAfter a 24 h incubation.

^cIn rat plasma.

Table 4
Rat Plasma Stability of 1 with Inhibitors

inhibitor ^a	<i>t</i> _{1/2} (h) ^b
none	0.7
60 nM bestatin	0.8
100 μ M chymostatin	1.0
1 mM PMSF	0.8
10 μ M captopril	0.6
1 mM EDTA	0.8
all five inhibitors	0.9

^aUsed effective concentration of inhibitors to inhibit protease.

^bCalculated after a 4 h incubation.