Published in final edited form as:

Bioorg Med Chem Lett. 2015 January 1; 25(1): 30–33. doi:10.1016/j.bmcl.2014.11.026.

Modification of Amphipathic Non-opioid Dynorphin A Analogues for Rat Brain Bradykinin Receptors

Yeon Sun Lee^{a,*}, Sara M. Hall^a, Cyf Ramos-Colon^a, Michael Remesic^a, Lindsay LeBaron^a, Ann Nguyen^a, David Rankin^b, Frank Porreca^b, Josephine Lai^b, and Victor J. Hruby^{a,*}

^aDepartment of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721, USA

^bDepartment of Pharmacology, University of Arizona, Tucson, AZ 85721, USA

Abstract

It has been shown that under chronic pain or nerve injury conditions, up-regulated dynorphin A (Dyn A) interacts with bradykinin receptors (BRs) to cause hyperalgesia in the spinal cord. Thus BRs antagonist can modulate hyperalgesia by blocking Dyn A's interaction with the BRs in the central nervous system. In our earlier structure-activity relationship (SAR) study, [des-Arg⁷]-Dyn A-(4-11) **13** was discovered as a minimum pharmacophore for rat brain BRs with its antagonist activity (anti-hyperalgesic effect) in in vivo tests using naïve or injured animals. We have pursued further modification on the [des-Arg⁷]-Dyn A analogues and identified a key insight into the pharmacophore of the rat brain BRs: amphipathicity.

Keywords

Dynorphin A; Non-opioid; Amphipathicity; Bradykinin receptors; Anti-hyperalgesic effect

Dynorphin A (Dyn A, H-Tyr¹-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln¹⁷-OH) interacts with three subtype (μ , δ , and κ) opioid receptors to inhibit nociception in the nervous system. On the other hand, Dyn A and [des-Tyr¹]-Dyn A bind to other non-opioid receptors such as bradykinin receptors (BRs) to stimulate nociception in the central nervous system (CNS), which results in motor impairment, paralysis, and hyperalgesia.^{1–6} These excitatory effects are non-opioid effects, because naloxone, an opioid antagonist, cannot block these effects.

On the basis of this fact, we hypothesized that BRs antagonists can block the Dyn A's interaction with the receptors in the CNS to modulate the excitatory effects. To develop potent BRs antagonists we have been utilizing the Dyn A structure. In earlier studies, we had identified a good pharmacophore of the Dyn A for the BRs antagonism in rat brain: [des-

© 2014 Elsevier Ltd. All rights reserved.

*Corresponding authors. Tel.: +1 520 621 6332; fax: +1 520 621 8407. yeon@email.arizona.edu (Y.S. Lee), hruby@email.arizona.edu (V.J. Hruby).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Arg⁷]-Dyn A-(4-11) (**13**).⁷ Ligand **13** inhibited the hyperalgesic and motor effects that are caused by Dyn A-(2-13) in the CNS.^{7,8} We also demonstrated that the deletion of Arg⁷ residue in Dyn A analogues does not affect binding affinities at all while maintaining an amphipathic property.⁹ Therefore, we pursued further modifications of amphipathic Dyn A analogs and gained a key insight into the pharmacophore for the receptors.

The modifications of the Dyn A analogues were performed based on the scaffold in Table 1, in which AA¹ and AA² are a hydrophobic amino acid (or peptide fragment) and a basic amino acid (or peptide fragment), respectively, and m is the number for the amphipathic moiety that is composed of a hydrophobic amino acid (or peptide fragment) and a basic amino acid (or peptide fragment). For the clarity, all of the Dyn A analogues were categorized by number “m” into five groups (m = 0, 1, 2, 3, and 4). Syntheses of the Dyn A analogues were performed by standard solid phase peptide synthesis using *N*^α-Fmoc-chemistry on amino acid pre-loaded Wang resin (100–200 mesh) in high yields (overall yields > 40%).⁷ Coupling reactions were performed using HBTU/HOBt/DIPEA (3 equiv/3 equiv/6 equiv) in DMF for 1 h at rt and *N*^α-Fmoc-group was deprotected by 20% piperidine in DMF for 20 min at rt. After completing chain-elongation, resin was dried under vacuum for 3 h and cleaved by TFA cocktail (TFA/TIS/water = 95/2.5/2.5) for 3 h at rt. In most cases, crude peptides were obtained in high purity (70 – 90%) and could be isolated with more than 97% purity by preparative RP-HPLC using gradient (10 – 40% acetonitrile in water containing 0.1% TFA in 15 min) in a short time (< 15 min) owing to their hydrophilic characters (Table 2). The purified Dyn A analogues were validated by analytical RP-HPLC and HR MS, and tested for their binding affinities by radioligand competition analysis using [³H]DALKD in rat brain membranes where non-specific binding is defined by 10 μM kallidin.⁷

In our earlier studies, it was shown that the BRs recognition of the Dyn A analogues is predominantly dependent upon the basicity of the C-terminal amino acid residue, and that modification of the C-terminal carboxylate group to an amide reduced the binding affinity dramatically.⁷ In this work, we confirmed that ligands **2**, **3**, and **23 – 25**, which do not include a C-terminal basic amino acid, bound to the BRs with very low affinity (ex. IC₅₀ = 6300 nM for **23**) regardless of the length of the peptides. The vital role of a basic amino acid residue at the C-terminus for the binding again suggests that the BRs recognition is mainly through the electrostatic interactions between the receptors and ligands.

Ligand **1**, which is without an amphipathic moiety (m = 0) in the scaffold, showed relatively low binding affinity (IC₅₀ = 950 nM) even with a basic amino acid residue at the C-terminus. This result indicated that the amphipathic moiety also plays an important role in receptor recognition along with a C-terminal basic amino acid, and thus, at least one amphipathic moiety (m = 1) is necessary for receptor recognition. It is worthwhile to note that still ligand **1** retained relatively higher binding affinity comparing to ligands **2** (IC₅₀ = 2300 nM) and **3** (IC₅₀ = 4800 nM). This comparison suggested that the most important pharmacophoric feature for the receptor recognition might be the basicity at the C-terminus.

As shown in Table 1, binding affinities of ligands (**4 – 22**) were slightly enhanced by the increase of amphipathic moiety at 1 ≤ m ≤ 3. The average binding affinities of ligands with

$m = 1, 2$, and 3 were 240 nM, 130 nM, and 80 nM, respectively. In fact, at $m = 1$, all ligands except **4** were tested for their binding to the BRs at higher pH (7.4) than the pH (6.8) for the other ligands ($m = 2 - 4$). Therefore, their binding affinities at pH 6.8 could be better than these at pH 7.4 because binding affinities of Dyn A analogues were shown to be pH sensitive and to be increased at the lower pH.^{7, 9} The pH condition might cause relatively higher average binding affinities compared to the others. All ligands with $m = 2$, except **14** ($IC_{50} = 460$ nM) showed the same range of binding affinities (average affinity: 78 nM) as ligands **19** – **22** with $m = 3$. Comparison of the binding affinity of ligand **10** ($m = 2$, $IC_{50} = 58$ nM) with ligands **20** ($m = 3$, $IC_{50} = 83$ nM) and **21** ($m = 3$, $IC_{50} = 65$ nM) indicated that there was no enhancement of binding affinity by the addition of an amphipathic moiety. From this result, it is clear that ligands can retain high binding affinity at the BRs when including a basic amino acid at the C-terminus and an amphipathic moiety ($m > 1$).

Among ligands **4** – **8**, ligand **7**, which possesses a Pro residue at the N-terminus and a Nle residue at position 3 exhibited similar good binding affinity ($IC_{50} = 71$ nM at pH 7.4) to the other potent ligands. Comparisons of ligands **4** – **6** with **7** and **8**, especially **4** with **7**, indicated that the replacement of a Leu residue at position 3 with a Nle residue increased the binding affinities more than 3 fold. However, in ligands **11** and **12** ($m = 2$) there was no Nle-replacement effect on the binding affinity observed. Likewise, the replacement of the N-terminal hydrophobic moiety did not change binding affinities in those ligands with $m = 2$. Even the truncation of N-terminal Ile in ligand **10** could retain the same high binding affinity ($IC_{50} = 58$ nM) as that of ligand **9** ($IC_{50} = 49$ nM). Ligands **15** – **17**, which contain two amino acid residues ($l = 2$) at the N-terminal hydrophobic site, showed the same range of binding affinities (**15**: $IC_{50} = 110$ nM, **16**: $IC_{50} = 100$ nM, **17**: $IC_{50} = 87$ nM) regardless of the substituted residue (Phe-Ile, Phe-Leu, or Phe-Nle). Ligands **19** – **21** ($m = 3$), which have different lengths of hydrophobic moieties ($l = 4, 1$, and 0) compared to each other, exhibited the same high binding affinities (**19**: $IC_{50} = 41$ nM at pH 7.4 , **20**: $IC_{50} = 83$ nM, **21**: $IC_{50} = 65$ nM) at BRs. Therefore it is clear that the hydrophobic moiety at the N-terminus does not play an important role in the BRs recognition, even though there were slight changes of binding affinities observed by the modifications, particularly, in ligands of short length ($m = 1$).

Ligand **9** [Dyn A-(8-13)] bound to the BRs with the same high affinity ($IC_{50} = 49$ nM) as **13** ([des-Arg⁷]-Dyn A-(4-11), $IC_{50} = 69$ nM), which was identified as a lead ligand in our previous work.⁷ Simple modification of ligand **9** with two Lys residues and two Nle residues afforded the simple amphipathic ligand **11** (AA² = only Lys), which retained high binding affinity ($IC_{50} = 78$ nM). Further replacement of two Lys residues with two Arg residues in ligand **12** again retained the high binding affinity ($IC_{50} = 83$ nM). There was no substantial alteration in the binding of ligands **9** – **10**, which is consistent with the amphipathic pharmacophore suggested in our study. The replacement of C-terminal Lys residue with an Arg residue in ligands **15** – **17** exhibited the same range of binding affinities as **13**, supporting our suggestion of the amphipathic pharmacophore for BRs as another good fit. However, ligand **14**, in which two Arg residues were replaced by two Lys residues, reduced the binding affinity ($IC_{50} = 460$ nM) up to 9 fold compared to the other ligands in the third category ($m = 2$), whereas the replacements were tolerated well in ligands **11** ($IC_{50} = 78$

nM) and **22** ($IC_{50} = 85$ nM). It is not clear why the replacement resulted in the loss of affinity in ligand **14**, but not in ligands **11** and **22**. This may be due to the altered position of a turn structure by the Pro residue, which can result in differentiation of two basic residues, Lys and Arg, from a topographical perspective.

Retro-inverso modification, which is a reversal of the direction of the primary peptide sequence with inversion of the chirality of each amino acid residue, has been shown to retain strong topographical correlation to the parent peptide with an increase of stability.^{11, 12} This is attributed to similar side chain dispositions and reduced enzymatic degradation. Thus, this modification has been frequently used for the development of potent peptide ligands possessing good bioavailability. For the same purpose, retro-inverso modification was applied to ligand **17**, resulting in ligand **27**, and loss of binding affinity (no competition: $IC_{50} > 10,000$ nM). The loss of the binding affinity was considered to be caused by the lack of a basic amino acid residue at the C-terminus which is a key structural feature. To fulfil this important structural requirement, simple inverso modification was performed for a ligand which possesses a Lys at the C-terminus, but the resulting ligand **18** also lost binding affinity at BRs. From these results, it is clear that *D*-amino acid residues cannot be tolerated in the amphipathic pharmacophoric structure for BRs recognition.

Contrary to the retro-inverso and inverso modifications, retro modification of lead ligand **13** maintained the same good binding affinity ($IC_{50} = 78$ nM) in ligand **26**. This is remarkable because ligand **26** has a hydrophobic Phe residue at the C-terminus instead of a basic amino acid residue, which was considered as a key structural feature for BRs recognition. This extraordinary result suggested that BRs recognition may need only one terminal basic amino acid residue (*C*-terminal or *N*-terminal), and thus ligand **26** can satisfy this requirement. To explore this possibility, the C-terminal Phe residue in ligand **26** was truncated to afford ligand **25**, which possesses one basic amino acid at the *N*-terminus. Contrary to our suggestion, ligand **25** had 19 fold reduced binding affinity ($IC_{50} = 930$ nM). The comparison of ligand **26** with ligands **23** and **25** clearly revealed that our suggestion was incorrect. The other suggestion is that due to the electron rich aromatic moiety, the C-terminal Phe residue could be involved in electrostatic interaction with BRs like Lys or Arg residue.

The retro modification was also applied to longer Dyn A fragments, [des-Arg⁷]-Dyn A-(2-13) and Dyn A-(2-13), and shown to have the same range of binding affinities in ligands **29** ($IC_{50} = 150$ nM) and **30** ($IC_{50} = 78$ nM) as their parent ligands ($IC_{50} = 41$ nM for [des-Arg⁷]-Dyn A-(2-13); $IC_{50} = 21$ nM for Dyn A-(2-13)). Even though there were slight decreases in the binding affinities of retro modified ligands, it is evident that the retro modification of the longer Dyn A analogues can be tolerated for the receptor recognition. Interestingly, ligands **28** – **30** do not include a basic amino acid residue or a Phe residue at the C-terminus, but showed good binding affinities at the BRs. This SAR may be applied only to the longer chain of peptide ligands which have more tendencies to adopt an ordered structure. In the circumstance, a key pharmacophoric structure, the amphipathic moiety in this case, may be assisted to have proper disposition for receptor recognition. In contrast, the structural flexibility of shorter chain of peptides may be a reason for their serious C-terminal basic amino acid dependence for the BRs recognition. Comparison of ligand **30** with **28** and

29 confirmed that there is no marked effect by the increase of an amphipathic moiety and by the truncation of an Arg residue.

In summary, we have synthesized a series of amphipathic Dyn A analogues and tested their binding affinities at rat brain BRs. Present SAR studies confirmed that non-opioid Dyn A analogues recognize the BRs mainly through electrostatic interactions, and the C-terminal basic amino acid and the amphipathic moiety of more than 1 are the key structural feature for eliciting good binding affinity at the BRs. On the basis of the SAR results, the amphipathic pharmacophore of Dyn A, as shown in Figure 1, could be identified as a scaffold for the BRs.

Acknowledgments

This work has been supported by U.S. Public Health Services, NIH, and NIDA P01DA006284 and R01DA013449.

Abbreviations

BK	bradykinin
BR	bradykinin receptor
CNS	central nervous system
DALKD	[des-Arg ¹⁰ , Leu ¹⁰]-kallidin
DIPEA	diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
Dyn A	dydnorphin A
HR MS	high resolution mass spectroscopy
Fmoc	9-fluorenylmethoxycarbonyl
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HOBt	N- hydroxybenzotriazole
RP-HPLC	reverse phase high performance liquid chromatography
rt	room temperature
SAR	structure-activity relationship
TFA	trifluoroacetic acid
TIS	triisopropylsilane

References and notes

1. Faden AI, Jacobs TP. *Br J Pharmac.* 1984; 81:271–276.
2. Friederich MW, Friederich DP, Walker JM. *Peptides.* 1987; 8:837–840. [PubMed: 2893358]
3. Long JB, Martinez-Arizala A, Echevarria EE, Tidwell RE, Holaday JW. *Eup J Pharm.* 1988; 153:45–54.
4. Walker JM, Moises HC, Coy DH, Baldrighi B, Akil H. *Science.* 1982; 218:1136–1138. [PubMed: 6128791]

5. Tang Q, Lynch RM, Porreca F, Lai J. *J Neurophysiology*. 2000; 83:2610–2615.
6. Lai J, Luo MC, Chen Q, Ma S, Gardell LR, Ossipov M, Porreca F. *Nat Neurosci*. 2006; 9:1534. [PubMed: 17115041]
7. Lee YS, Muthu D, Hall SM, Ramos-Colon C, Rankin D, Hu J, Sandweiss A, Felice MD, Xie JY, Vanderah TW, Porreca F, Lai J, Hruby VJ. *J Am Chem Soc*. 2014; 136:6608. [PubMed: 24742335]
8. Bannister K, Lee YS, Goncalves L, Porreca F, Lai J, Dickenson AH. *Neuropharmacology*. 2014; 85:375. [PubMed: 24937046]
9. Lee, YS.; Rankin, D.; Hall, SM.; Ramos-Colon, C.; Ortiz, JJ.; Kupp, R.; Porreca, F.; Lai, J.; Hruby, VJ. *Bioorg Med Chem Letts*. 2014. <http://dx.doi.org/10.1016/j.bmcl.2014.09.033>
10. Guptasarma P. *FEBS*. 1992; 310:205.
11. Chorev M, Goodman M. *Acc Chem Res*. 1993; 26:266.
12. Guichard G, Benkirane N, Zeder-Lutz G, VanRegenmortel MH, Briand JP, Muller S. *Proc Natl Acad Sci*. 1994; 91:9765.

AA^1 = a hydrophobic amino acid (or peptide fragment);

AA^2 = a basic amino acid (or peptide fragment);

$1 \leq 0; m \leq 1$)

Figure 1.

Amphipathic Pharmacophore of Dyn A for BRs

AA¹ = a hydrophobic amino acid (or peptide fragment);

AA² = a basic amino acid (or peptide fragment);

l = 0, 1; m = 0 - 4; n = 0, 1

ligand	AA ¹	m	(AA ² -AA ¹) _m	AA ²	BR ^a [³ H]DALKD	
					Log[IC ₅₀] ^b	IC ₅₀ (nM)
19	Gly-Gly-Phe-Leu		Arg-Ile-Arg-Pro-Lys-Leu	Lys	-7.39±0.34	41 ^d
20	Phe		Arg-Ile-Arg-Pro-Lys-Leu	Lys	-7.08±0.15	83
21			Arg-Ile-Arg-Pro-Lys-Leu	Lys	-7.19±0.04	65
22			Lys-Nle-Lys-Pro-Lys-Leu	Lys	-7.07±0.16	85 ^d
23			Lys-Leu-Lys-Pro-Arg-Ile		-5.20±0.10	6300
24	Phe-Leu	3	Arg-Arg-Ile-Arg-Pro-Lys-Leu		-5.28±0.10	5200
25			Lys-Pro-Arg-Ile-Arg-Leu		-6.03±0.21	930
26			Lys-Pro-Arg-Ile-Arg-Leu	Phe ^e	-7.30±0.09	50
27			DArg-DPro-DArg-DNle-DArg-DNle	DPh ^e	n.c.	n.c.
28	Leu-Leu		Lys-Pro-Arg-Ile-Arg-Arg-Leu	Phe-Gly-Gly ^e	-7.03±0.10	93
29			Lys-Leu-Lys-Pro-Arg-Ile-Arg-Leu	Phe-Gly-Gly ^e	-6.82±0.15	150
30		4	Lys-Leu-Lys-Pro-Arg-Ile-Arg-Arg-Leu	Phe-Gly-Gly ^e	-7.11±0.06	78

^a Competition assays were carried out at pH 6.8 using rat brain membranes.

^b Logarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments in duplicate.

^c pH 7.4.

^d For the comparison, IC₅₀ Values of respective Dyn A fragments in reference 7 or 9 are shown in this column.

^e The definition of AA² does not applied for this case.

n.c. no competition.

Table 2

Analytical Data of Dyn A analogs

no	molecular formula	LowMS ^a		HRMS ^b		HPLC ^c		aLogP ^{s,d}
		observed	observed	calcd.	error (ppm)	(t _R , min)		
1	C ₂₃ H ₃₇ N ₇ O ₅	492.3	492.2924	492.2929	1.0	8.7	8.7	-2.32
2	C ₃₃ H ₆₀ N ₁₂ O ₇	761.4	761.4775	761.4781	0.7	10.1	10.1	-2.19
3	C ₃₃ H ₅₇ N ₁₁ O ₆	704.4	352.7321 ^e	352.7319 ^e	-0.4	9.2	9.2	-2.03
5	C ₂₇ H ₄₆ N ₆ O ₅	535.4	535.3600	535.3602	0.2	10.8	10.8	-1.20
6	C ₂₇ H ₄₇ N ₇ O ₅	550.3	550.3711	550.3711	0.0	7.2	7.2	-1.72
7	C ₂₃ H ₄₄ N ₆ O ₅	485.3	485.3440	485.3446	0.6	7.5	7.5	-1.85
8	C ₂₄ H ₄₈ N ₆ O ₅	501.3	501.3755	501.3759	0.7	9.9	9.9	-1.41
9	C ₃₅ H ₆₇ N ₁₁ O ₇	754.5	377.7691 ^e	377.7692 ^e	0.2	6.8	6.8	-1.82
10	C ₂₉ H ₅₆ N ₁₀ O ₆	641.5	641.4455	641.4457	0.3	6.0	6.0	-2.16
12	C ₃₅ H ₆₇ N ₁₃ O ₇	782.5	782.5352	782.5359	0.8	8.2	8.2	-2.09
14	C ₃₅ H ₆₇ N ₉ O ₇	726.4	363.7654 ^e	363.7655 ^e	-0.1	7.9	7.9	-1.42
15	C ₄₄ H ₇₆ N ₁₆ O ₈	957.4	957.6101	957.6104	0.4	9.9	9.9	-1.85
16	C ₄₄ H ₇₆ N ₁₆ O ₈	957.5	957.6102	957.6104	0.3	10.8	10.8	-2.10
18	C ₄₄ H ₇₆ N ₁₄ O ₈	929.6	465.3058 ^e	465.3058 ^e	-0.1	11.1	11.1	-1.62
20	C ₅₀ H ₈₈ N ₁₆ O ₉	1057.7	1057.6986	1057.6993	0.6	10.2	10.2	-1.61
21	C ₄₁ H ₇₉ N ₁₅ O ₈	910.7	910.6317	910.6308	-1.0	8.3	8.3	-2.07
23	C ₃₅ H ₆₇ N ₁₁ O ₇	754.5	754.5314	754.5298	-2.2	9.9	9.9	-2.04
24	C ₅₆ H ₉₉ N ₁₉ O ₁₀	1198.7	599.8981 ^e	599.8984 ^e	0.5	11.4	11.4	-1.53
25	C ₃₅ H ₆₇ N ₁₃ O ₇	782.5	782.5372	782.5359	-1.6	9.9	9.9	-2.03
26	C ₄₄ H ₇₆ N ₁₄ O ₈	929.7	929.6040	929.6043	0.3	14.5	14.5	-1.63
28	C ₆₆ H ₁₁₆ N ₂₂ O ₁₃	1425.5	n.d.	1425.9171	n.d	12.6	12.6	-1.23
29	C ₆₀ H ₁₀₅ N ₁₉ O ₁₂	1284.7	642.9168 ^e	642.9161 ^e	1.4	12.1	12.1	-1.28
30	C ₆₆ H ₁₁₇ N ₂₃ O ₁₃	1440.8	720.9705 ^e	720.9673 ^e	-4.4	11.6	11.6	-1.49

^a (M + H)⁺, ESI method (Finnigan, Thermoelectron, LCQ classic).

^b (M + H)⁺, FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF (Bruker Ultraflex III).

^c Performed on a Hewlett Packard 1100 (C-18, Microsorb-MV™, 4.6 mm × 250 mm, 5 μm) using gradient system (10 – 90% of acetonitrile containing 0.1% TFA within 40 min and up to 100% within additional 5 min, 1 mL/min).

^d <http://www.vcclab.org/lab/alogps>.

^e (M+2H)²⁺.