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Design, Synthesis, and Opioid Activity of Arodyn Analogs Cyclized by Ring-Closing Metathesis involving Tyr(Allyl)

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Abstract

Kappa (κ) opioid receptor selective antagonists are useful pharmacological tools in studying κ opioid receptors and have potential to be used as therapeutic agents for the treatment of a variety of diseases including mood disorders and drug addiction. Arodyn (Ac[Phe¹⁻³,Arg⁴,D-Ala⁸]Dyn A-(1-11)NH₂) is a linear acetylated dynorphin A (Dyn A) analog that is a potent and selective κ opioid receptor antagonist (Bennett et al. *J. Med. Chem.* 2002;45:5617–5619) and prevents stress-induced reinstatement of cocaine-seeking behavior following central administration (Carey et al. *Eur J Pharmacol* 2007;569:84–89). To restrict its conformational mobility, explore possible bioactive conformations and potentially increase its metabolic stability we synthesized cyclic arodyn analogs on solid phase utilizing a novel ring-closing metathesis (RCM) reaction involving allyl-protected Tyr (Tyr(All)) residues. This approach preserves the aromatic functionality and directly constrains the side chains of one or more of the Phe residues. The novel cyclic arodyn analog **4** cyclized between Tyr(All) residues incorporated in positions 2 and 3 exhibited potent κ opioid receptor antagonism in the [³⁵S]GTP γ S assay (K_B = 3.2 nM) similar to arodyn. Analog **3** cyclized between Tyr(All) residues in positions 1 and 2 also exhibited nanomolar κ opioid receptor antagonist potency (K_B = 27.5 nM) in this assay. These are the first opioid peptides cyclized via RCM involving aromatic residues, and given their promising pharmacological activity represent novel lead peptides for further exploration.

Graphical abstract

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3 $R_1 = \text{Ac}$, $R_2 = \text{Phe-Arg-Leu-Arg-Arg-Ile-Arg-Pro-LysNH}_2$

4 $R_1 = \text{Ac-Phe}$, $R_2 = \text{Arg-Leu-Arg-Arg-Ile-Arg-Pro-LysNH}_2$

Keywords

arodyn; ring closing metathesis; peptide cyclization; kappa opioid receptor antagonists; dynorphin A analogs; tyrosine(allyl)

1 Introduction

Our laboratory is interested in the design and synthesis of antagonists for kappa (κ) opioid receptors. In addition to their use as pharmacological tools, κ opioid receptor antagonists have the potential to be used therapeutically¹ in the treatment of cocaine^{2, 3} and opioid dependence,^{4, 5} and also have antidepressant^{6, 7} and antianxiety⁷ activity. The selective κ opioid receptor antagonists nor-binaltorphimine (nor-BNI),⁸ 5'-guanidinylnaltrindole (5'-GNTI),⁹ and JD₁Tic¹⁰ have been used extensively in pharmacological studies during the past two decades. However, these prototypical antagonists all exhibit extremely long activity, i.e. ranging from several days to several weeks after a single dose,^{11–15} complicating their use. In recent years other small molecule κ opioid receptor selective antagonists have been identified that are shorter acting.^{16, 17}

We are exploring peptide antagonists for κ opioid receptors based on the structure-activity relationships (SAR) of the endogenous peptide dynorphin (Dyn) A. Peptide antagonists are complementary to small molecule antagonists and can be important pharmacological tools to study κ opioid receptors. They have been shown to have shorter durations of action than the long-acting small molecule antagonists nor-BNI and JD₁Tic,^{3, 18} and therefore can be utilized in studies where the prototypical small molecule antagonists aren't applicable.

Our laboratory has identified several κ opioid receptor antagonists^{19–21} by modifying the N-terminal "message"²² sequence (which is responsible for opioid receptor activation) of Dyn A. The novel analog arodyn (**1**, Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-LysNH₂) exhibits high κ opioid receptor affinity (K_i (κ) = 10 nM) and selectivity (K_i ratio ($\kappa/\mu/\delta$) = 1/174/583), is a κ receptor antagonist,²⁰ and prevents stress-induced cocaine-seeking administration following intracerebroventricular injection.³ As a linear peptide, arodyn is very flexible and can adopt numerous conformations, and also can be metabolized by proteases. Cyclization generally reduces conformational mobility, which is especially useful for the study of possible bioactive conformation(s) and receptor-ligand interactions^{23–25} and can increase stability to peptidases^{26–28} as well as membrane transport.

^{29, 30} For example, the cyclic Dyn A analog zyklophin ([N^α-benzyl-Tyr¹, *cyclo*(D-Asp⁵, Dap⁸)]Dyn A-(1–11)NH₂ (Dap = 2,3-diaminopropionic acid) can cross the blood-brain barrier to antagonize κ opioid receptors in brain.¹⁸ Hence cyclic peptides can represent promising lead compounds for future study and development.

Although a variety of conformationally constrained peptide agonists for κ-opioid receptors have been identified,^{25, 30–32} the search for conformationally constrained peptide antagonists for κ receptors has been quite limited. To date the only cyclic Dyn A analogs reported with antagonist activity at κ opioid receptors are the novel N-terminal cyclized derivative *cyclo*^{N-5}[Trp³, Trp⁴, Glu⁵]Dyn A-(1–11)NH₂,²¹ based on the acetylated chimeric Dyn A analog venorphin,¹⁹ and zyklophin,³³ cyclized in the “address”²² domain (the portion of the peptide targeting the peptide to κ opioid receptors), along with its analogs modified at the N-terminus.³⁴

Ring-closing metathesis (RCM) has emerged as a very useful method of making cyclic organic compounds as well as cyclic peptides.^{35–39} Compared with the traditional approach of preparing cyclic peptides by disulfide or amide bond formation between amino acid side chains, cyclization by RCM has some advantages. In contrast to side chain disulfide or amide bonds, it is possible to maintain side chain functionalities when using RCM. The majority of RCM cyclizations of peptides to date, however, have involved noncritical residues, i.e. the side chain of an aliphatic ω-alkene such as allylglycine (AllGly), and/or an N-terminal alkene. In contrast, we explored a novel approach that utilizes a derivative of an aromatic amino acid, namely the side chain of allyl-protected tyrosine (Tyr(All)),³⁵ for RCM.

Arodyn has three Phe residues in the N-terminus, making it a promising initial peptide to study the scope and limitations of RCM cyclizations involving Tyr(All). Phe¹ and Phe³ in the N-terminal “message” sequence of arodyn contribute to the interactions between arodyn and κ opioid receptors (substitution of these residues by Ala decreased κ opioid receptor affinity 3- to 5-fold), while Phe² is not important for the κ opioid receptor affinity of arodyn.⁴⁰ By using Tyr(All) for RCM we can constrain important residue(s) while maintaining the aromatic functionality of Phe¹ and Phe³, and examine the effects of such a constraint on pharmacological activity.

We investigated two cyclization approaches; the first one involved the side chain of a Tyr(All) residue in one of the first three residues and AllGly in position 5, and the second one involved the side chains of two Tyr(All) residues in two of the first three residues. This paper describes the results for these initial cyclic peptides along with linear arodyn analogs containing Tyr(All).

2. Results and discussion

2.1 Synthesis

The peptides were synthesized by solid phase synthesis using Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) amino acids. Upon completion of assembly of the peptide chains, second generation Grubbs' catalyst was used to cyclize the peptides on the solid

support (Scheme 1). A mixture of dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) (4/1, v/v) was used as the solvent for the RCM reaction.⁴¹ These reactions proved to be challenging because of a side reaction involving loss of the allyl group from one or both of the Tyr(All) residues (the details of these synthetic studies will be described elsewhere), and some peptides of interest could not be synthesized. Table 1 shows the three cyclic peptides successfully obtained. Interestingly, unlike Dyn A-(1–11)NH₂ analogs cyclized between two allylglycine residues⁴¹ where both the *cis* and *trans* isomers were obtained, only the *trans* isomers were obtained (determined by NMR) for all of successfully synthesized cyclic peptides containing Tyr(All).

The peptides were purified by preparative high-performance liquid chromatography (HPLC) system, and the purity of the final peptides (>98%) verified by analytical HPLC in two different solvent systems (Table 2). The molecular weights of the compounds were determined by electrospray ionization mass spectrometry (ESI-MS, Waters, Q-TOF).

2.2 Pharmacology

The cyclic and linear Tyr(All) arodyn analogs were evaluated for their binding affinity at κ and μ opioid receptors using Chinese hamster ovary (CHO) cells stably expressing cloned opioid receptors (Table 3) as described previously.⁴² (Since arodyn has low affinity for the δ opioid receptor ($K_i > 5 \mu\text{M}$),¹⁹ the analogs were not evaluated for affinity for this receptor.)

The κ opioid receptor affinities of the cyclic arodyn analogs varied depending on the positions involved in the cyclic constraint. Cyclization between the side chains of a Tyr(All) and an AllGly residue in positions 3 and 5, respectively, to give **2**, decreased affinity 40-fold compared to arodyn (**1**, Table 3), suggesting that the conformation(s) induced by this cyclization is not optimal for κ opioid receptor binding. In contrast, cyclizations of arodyn involving residues 1 and 2, or 2 and 3 using RCM between two Tyr(All) residues was tolerated by κ opioid receptors, with analogs **3** and **4** showing reasonable affinities for κ opioid receptors ($K_i = 71.7$ and 55.4 nM, respectively). These are the first two arodyn analog cyclized by RCM that exhibit reasonable affinity for κ opioid receptors and are promising lead peptides for further study. In the linear analogs incorporation of Tyr(All) in position 1 or 2 resulted in peptides (**5** and **6**, respectively) with similar κ opioid receptor affinities as the cyclic analogs **3** and **4**, but substitution of Tyr(All) in position 3 (peptide **7**) resulted in a much larger (13-fold) decrease in κ opioid receptor affinity.

The analogs exhibited micromolar μ opioid receptor affinities that were within 2-fold that of arodyn. Because of the reductions in κ opioid receptor affinities there were substantial reductions in the selectivity of both the cyclic and linear analogs for κ opioid receptors compared to arodyn.

These arodyn analogs were also evaluated for both agonist and antagonist activities at κ opioid receptors in the [³⁵S]GTP γ S assay using membranes from the same cells as used in the binding assay. Similar to arodyn the analogs exhibited negligible efficacy in this assay (10% inhibition compared to Dyn A-(1–13)NH₂) when screened for efficacy at 10 μM , Table 4). The cyclic analogs **3** and **4** were subsequently evaluated for antagonist activity by Schild analysis (Figure 1). Analog **4** exhibited potent κ opioid receptor antagonism against

Dyn A-(1–13)NH₂ (K_B = 3.17 nM, 95% confidence interval 0.24–11.6 nM) similar to that of arodyn (K_B = 17.0 nM, 95% confidence interval 0.07–122 nM), while analog **3** appeared to be a somewhat weaker antagonist (K_B = 27.5 nM, 95% confidence interval 8.6–83.4 nM) than **4**. **3** (A) and **4** (B) in the [³⁵S]GTPγS assay. DR = dose ratio (EC₅₀ of Dyn A-(1–13)NH₂ in the presence of the indicated concentration of the antagonist divided by the EC₅₀ of Dyn A-(1–13)NH₂ in the absence of the antagonist). Data shown are pooled data from three separate experiments.

Previous reports of peptides cyclized by RCM involving Tyr(All) have been limited to small peptides (or peptidomimetics). Tripeptides cyclized between Tyr(All) and AllGly exhibited weak to negligible antibacterial activity against *Staphylococcus aureus*,⁴³ while this approach yielded tripeptide derivatives that were submicromolar inhibitors of the cysteine protease calpain.⁴⁴ RCM was also used to cyclize Tyr(All) acylated with an O-allyl benzoic acid derivative to yield α2β2 antagonists with nanomolar potencies.⁴⁵ Studies of peptides cyclized by RCM between two Tyr(All) residues has been limited to the synthesis of a model dipeptide that was not evaluated for biological activity³⁵ and a tripeptide with modest antibacterial activity against *Staphylococcus aureus*.⁴³ All of these small peptides were synthesized and cyclized in solution. The application of RCM to opioid peptides has been limited to cyclizations utilizing AllGly residues^{41, 46, 47} and have not involved aromatic residues.

3. Conclusions

Here we report the synthesis and pharmacological activities of initial arodyn analogs cyclized utilizing a RCM reaction involving the side chain of Tyr(All). Two cyclic analogs (compounds **3** and **4**) cyclized between two Tyr(All) residues retain reasonable affinity for κ opioid receptors (K_i = 72 and 55 nM, respectively), although the lower κ opioid receptor affinities decreased their selectivity for these receptors over μ opioid receptors compared to arodyn. Similar to arodyn, all of the analogs synthesized exhibit minimal stimulation of [³⁵S]GTPγS binding. The cyclic analogs **3** and **4** antagonized Dyn A-(1–13)NH₂ at κ opioid receptors in a concentration-dependent manner, with analog **4** exhibiting potent antagonism (K_B = 3.2 nM) similar to arodyn.

This is the first report of this type of peptide RCM involving Tyr(All) residues cyclized on a solid support and the first report of an opioid peptide cyclized via RCM involving aromatic residues.⁴⁸ These analogs represent interesting lead compounds for further characterization of structure-activity relationships for arodyn at κ opioid receptors. Further studies of these and additional arodyn analogs cyclized by RCM involving Tyr(All) are ongoing in our laboratory.

4. Materials and methods

4.1 Materials

Fmoc-Tyr(All)-OH was purchased from Senn Chemicals (San Diego, CA); other reagents were obtained as previously described.⁴¹

4.2 Peptide synthesis, purification and analysis

The peptides were synthesized on an Fmoc-PAL-PEG-PS resin (PAL-PEG-PS = peptide amide linker polyethylene glycol polystyrene, 300 mg, 0.19–0.21 mmol/g) using a CS Bio automated peptide synthesizer, except for the coupling of Fmoc-AllGly-OH (2 equiv) which was performed manually. The coupling of this amino acid was generally performed for 2 h or until completion of reaction as determined by the ninhydrin test.⁴⁹ The linear precursor was first assembled on the resin by standard solid-phase peptide synthesis (SPPS)⁴¹ with the Fmoc-protected amino acids coupled to the growing peptide chain using PyBOP (benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate), HOBt (1-hydroxybenzotriazole), and *N,N*-diisopropylethylamine (4/4/10 relative to the resin substitution) in DMF (2 mL) for 2 h. The side chain of Lys was protected by Boc (*tert*-butyloxycarbonyl) and Arg by Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). For the cyclization reaction the resin was mixed with 40 mol% second-generation Grubbs' catalyst (3 mM) in DCM/DMF (4/1, v/v) under reflux conditions (60 °C) for 2 d. The resin was then washed with DCM (10 × 5 mL) to remove the catalyst. Finally, the resin was shrunk by washing with methanol and dried under vacuum.

The peptides were cleaved from the resin by treating with 5 mL Reagent B (88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) for 2 h⁵⁰ and isolated as previously described.⁴¹ Both mono- and bis- desallyl linear peptides were formed, decreasing the yields of the desired cyclic peptides (which were 50%, 38% and 64% for peptides **2**, **3** and **4**, respectively, as determined by analytical reversed phase HPLC). The crude peptides were purified by preparative reversed-phase HPLC using a linear gradient of 15–50% MeCN containing 0.1% TFA over 35 min at a flow rate of 20 mL/min; the purification was monitored by UV absorbance at 214 nm.

The purity of the final peptides was verified by analytical HPLC using two solvent systems, generally with a linear gradient of 5–50% or 25–70% solvent B (solvent A was aqueous 0.1% TFA and solvent B was MeCN or MeOH containing 0.1% TFA) over 45 minutes, at a flow rate of 1 mL/min with monitoring at 214 and 230 nm, respectively. The final purity of all of the peptides in both solvent systems was greater than 98%. Molecular weights of the peptides were determined by ESI-MS (Waters Q-TOF).

The configuration of the double bond in the cyclic RCM peptides was determined by NMR analysis. ¹H NMR spectra of the cyclic peptides (2–5 mg) dissolved in 0.7 mL DMSO-*d*₆ were obtained at 25 °C on a Bruker AVANCE DRX-500 spectrometer (500.13 MHz proton frequency) equipped with a 5 mm z-gradient Cryoprobe. ¹H chemical shifts, referenced to the residual DMSO signal at 2.49 ppm, and coupling constants were extracted from 1D spectra. Large coupling constants were observed for the vinyl protons which are consistent with the *trans* configuration: **2**: 5.51 (*J* = 15.3 Hz) and 5.58 (*J* = 16.3 Hz); **3**: 5.81 (*J* = 16.0 Hz) and 5.86 (*J* = 16.6 Hz); and **4**: 5.81 (*J* = 15.3 Hz) and 5.89 (*J* = 16.1 Hz).

4.3 Pharmacological evaluation

4.3.1. Opioid receptor binding assays—Radioligand binding assays were performed as previously described using cloned rat κ and μ opioid receptors stably expressed on CHO

cells.⁴² [³H]Diprenorphine and [³H]DAMGO ([D-Ala²,MePhe⁴,glyol]enkephalin) were used as radioligands in the assays for κ and μ receptors, respectively. Nonspecific binding was determined in the presence of 10 μ M unlabeled Dyn A-(1–13)NH₂ and DAMGO for κ and μ receptors, respectively. Binding assays were carried out under standard conditions in the presence of peptidase inhibitors (10 μ M bestatin, 30 μ M captopril, and 50 μ M L-leucyl-L-leucine) and 3 mM Mg²⁺. IC₅₀ values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism software (GraphPad Software Co., San Diego, CA). K_i values were calculated from the IC₅₀ values by the Cheng and Prusoff equation,⁵¹ using K_D values of 0.45 and 0.49 nM for [³H]diprenorphine and [³H]DAMGO, respectively. The results presented are the mean $\pm$ SEM from at least three separate assays.

4.3.2 Functional assay—The binding of [³⁵S]GTP γ S to membranes from the CHO cells expressing κ opioid receptors was assayed as previously described.^{52, 53} Efficacy was determined relative to the full agonist Dyn A-(1–13)NH₂. The antagonist potencies of **3** and **4** were determined by measuring the EC₅₀ values of Dyn A-(1–13)NH₂ in the absence and presence of four different concentrations (1–1000 nM) of the peptide, performed in triplicate. The pA₂ values were determined by Schild analysis⁵⁴ using the pooled results from three experiments (two experiments for aroclor).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schild analysis of the antagonism of Dyn A-(1–13)NH₂ at κ opioid receptors by analogs

Structures of arodyn analogs cyclized by RCM involving the side chain of Tyr(All) and linear Tyr(All) analogs^a

Peptide	Structure
1, arodyn	Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-LysNH ₂
Cyclic arodyn analogs	
2
3
4
Linear arodyn analogs	
5	Ac-Tyr(All)-Phe-Phe-Arg-Leu-Arg-Arg-Ile-Arg-Pro-LysNH ₂
6	AcPhe-Tyr(All)-Phe-Arg-Leu-Arg-Arg-Ile-Arg-Pro-LysNH ₂
7	Ac-Phe-Phe-Tyr(All)- Arg-Leu-Arg-Arg-Ile-Arg-Pro-LysNH ₂

^aThese analogs contained Ile in position 8, as is found in Dyn A.

Table 2HPLC and MS data of purified peptides **2–7**

Peptide	HPLC t_R (min) ^a		ESI-MS (m/z)	
	System 1 ^b	System 2 ^c	Calculated	Observed
2	20.94	38.68	[M+4H] ⁴⁺ =398.0 [M+3H] ³⁺ =530.3	[M+4H] ⁴⁺ =398.0 [M+3H] ³⁺ =530.3
3	27.11	23.13 ^d	[M+4H] ⁴⁺ =416.2 [M+3H] ³⁺ =554.7	[M+4H] ⁴⁺ =416.2 [M+3H] ³⁺ =554.7
4	24.87	44.44	[M+3H] ³⁺ =554.7 [M+2H] ²⁺ =831.0	[M+3H] ³⁺ =554.7 [M+2H] ²⁺ =831.0
5	26.74	31.05 ^d	[M+4H] ⁴⁺ =409.0 [M+3H] ³⁺ =545.0	[M+4H] ⁴⁺ =409.0 [M+3H] ³⁺ =545.0
6	27.57	31.53 ^d	[M+4H] ⁴⁺ =409.0 [M+3H] ³⁺ =545.0	[M+4H] ⁴⁺ =409.0 [M+3H] ³⁺ =545.0
7	27.57	30.29 ^d	[M+4H] ⁴⁺ =409.0 [M+3H] ³⁺ =545.0	[M+4H] ⁴⁺ =409.0 [M+3H] ³⁺ =545.0

^aThe purity for all of the peptides by both methods was >98%.^bSystem 1: Solvent A = 0.1% aqueous TFA, solvent B = 0.1% TFA in acetonitrile.^cSystem 2: Solvent A = 0.1% aqueous TFA, solvent B = 0.1% TFA in methanol. The gradient for both systems was 5–50% solvent B over 45 min at 1 mL/min.^dSolvent A= 0.1% aqueous TFA, solvent B= 0.1% TFA in methanol, gradient 25–70% solvent B over 45 min.

Table 3Opioid receptor affinities of the arodyn analogs cyclized by RCM and linear Tyr(All) analogs^a

Peptide	K _i ± SEM (nM)		K _i ratio (μ/κ)
	κ	μ	
1 , arodyn ^b	10.0 ± 3.0	1750 ± 130	175
2	398 ± 40	3460 ± 300	8.7
3	71.7 ± 5.2	1920 ± 150	27
4	55.4 ± 4.1	903 ± 34	16
5	83.3 ± 9.0	1170 ± 300	14
6	66.3 ± 2.8	1460 ± 130	22
7	134 ± 9	1430 ± 91	11

^aThe values are the mean ± SEM from at least three independent experiments.^bFrom ref.²⁰.

Table 4Inhibition of [³⁵S]GTPγS binding by the arodyn analogs cyclized by RCM and the linear Tyr(All) analogs^a

Compound	% inhibition at 10 μM
2	3.3 ± 2.5%
3	6.2 ± 1.3%
4	-2.1 ± 3.0%
5	-2.5 ± 2.0%
6	6.1 ± 1.5%
7	3.2 ± 6.4%

^aRelative to 100 nM Dyn A-(1-13)NH₂. The values are the mean ± SEM from three independent experiments.