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Identification of Arodyn, a Novel Acetylated Dynorphin A-(1−11) Analogue, as a κ Opioid Receptor Antagonist

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Abstract: Arodyn (*aro*matic *dyn*orphin) is a novel analogue of the opioid peptide dynorphin A with a nonbasic N-terminus that exhibits nanomolar affinity (K_i = 10 nM) and remarkable selectivity for κ opioid receptors (K_i ratio (κ/μ/δ) = 1/174/583). Arodyn completely reverses the agonism of dynorphin A (1−13)NH_2 in a concentration-dependent manner in the adenylyl cyclase assay. Thus arodyn is a novel κ opioid receptor selective antagonist that will be useful to study these receptors.

Activation of opioid receptors attenuates the transmission of pain signals to the spinal cord,[1] and opioid agonists such as morphine are important clinical agents for the treatment of severe pain. However, serious side effects, namely respiratory depression, addiction liability, and gastrointestinal effects, are associated with the use of morphine and other agents activating mu (μ) opioid receptors. Therefore there is considerable interest in identifying ligands for other types of opioid receptors, namely kappa (κ) and delta (δ) receptors, both as pharmacological tools and as potential therapeutic agents.[2]

We are interested in designing potent and selective peptide antagonists for κ opioid receptors. Starting from a novel chimeric peptide extacet (*ext*ended *acet*alin, Ac-[Arg[1],Phe[2],Met[3],Trp[4],Met[5],D-Ala[8]]dynorphin A-(1−11)NH_2),[3] we utilized a combinatorial library to discover a novel selective antagonist for these receptors. Here we describe the identification and pharmacological activity of the novel peptide antagonist arodyn (*aro*matic *dyn*orphin).

Kappa opioid receptor antagonists are useful pharmacological tools to study κ opioid receptor involvement in physiological processes, and recently the use of these antagonists has been proposed for the treatment of opioid dependence.[4] While the nonpeptide κ-selective antagonist nor-binaltorphimine (norBNI)[5] has been extensively used to study κ opioid receptors, its pharmacological properties are not optimal, and it exhibits a much longer than expected half-life in vivo.[6] Recently, C5′-guanidinylnaltrindole (GNTI, the 5′-guanidine derivative of naltrindole)[7,8] was identified as a nonpeptide antagonist for κ opioid receptors that has some advantages (i.e., increased potency) in vivo over norBNI,[9] but GNTI also has a slow onset and long half-life in vivo.[9]

It has been proposed that peptides and nonpeptides bind to different domains of the κ opioid receptor.[10−13] The second extracellular loop (EL2) of κ receptors appears to be important for the binding of dynorphin A (Dyn A), an endogenous opioid peptide agonist at these receptors;[10,11] in contrast the κ-selective nonpeptide agonists U50,488 and U69,593 appear to require most of the κ receptor for binding except the transmembrane (TM) 4-EL2-TM5 region[11] or TM5 to the C-terminus.[12] Therefore selective peptide antagonists are complimentary to nonpeptide antagonists as pharmacological tools to study κ opioid receptors, and studying these peptides may reveal distinct receptor−ligand interactions that can be utilized in future ligand design.

Reports of potent and selective κ opioid receptor peptide antagonists have been very limited, however. Our laboratory reported the peptide antagonist Ac-[Lys[2],Trp[3,4],D-Ala[8]]Dyn A-(1−11)NH_2 (JVA-901),[14] a chimeric derivative of Dyn A, which demonstrated the application of the 'message-address' concept[15,16] in designing peptide antagonists for κ opioid receptors. [Pro[3]]Dyn A-(1−11)NH_2 was reported to have high affinity and selectivity for κ opioid receptors, but this ligand exhibited only weak κ antagonist potency in functional assays.[17] Recently dynantin ([(2S)-Mdp[1]]Dyn A-(1−11)NH_2, Mdp = 2-methyl-3-(2′,6′-dimethyl-4-hydroxyphenyl)propionic acid) was reported to be a potent antagonist with high binding affinity and selectivity for κ opioid receptors.[18]

Our laboratory also used the 'message-address' concept[15,16] to design the novel Dyn A acetylated analogue extacet.[3] Dyn A shares a common N-terminal 'message' sequence with most mammalian opioid peptides and has a unique C-terminal 'address' sequence.[16] The 'message' sequence has been reported to be important for receptor recognition and activation while the 'address' sequence was postulated to be important for enhancing potency for a particular receptor.[16] To develop antagonists based on Dyn A, the N-terminal 'message' sequence was replaced by a small peptide exhibiting antagonist activity at opioid receptors. The acetalins (Ac-Arg-Phe-Met-Trp-Met-X-NH_2, where X = Arg, Lys, or Thr) were reported to be potent μ opioid receptor peptide antagonists in the guinea pig ileum assay by Houghten and co-workers.[19] These peptides lack the positively charged N-terminal amine that is important for the opioid activity of the endogenous opioid peptides. Replacing the N-terminal 'message' sequence of [D-Ala[8]]Dyn A (1−

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Table 1. Opioid Receptor Binding Affinity of Arodyn and [D-Ala^8]Dyn A-(1−11)NH_2 [a]

peptide	$K_i \pm$ SEM (nM)			K_i ratio ($\kappa/\mu/\delta$)
	κ	μ	δ	
arodyn	10.0 ± 3.0	1740 ± 130	5830 ± 1960	1/174/583
[D-Ala^8]Dyn A-(1−11)NH_2	0.19 ± 0.08	1.97 ± 0.05	12.2 ± 3.0	1/10/64

[a] The binding assays were performed using cloned opioid receptors expressed on CHO cells as previously described[22] using [^{3}H]diprenorphine for κ, [^{3}H]DAMGO ([D-Ala^2,NMePhe^4,glyol]enkephalin) for μ, and [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin) for δ receptors. K_i values are the average ± SEM of 3−6 independent experiments.

11)NH_2 [20] with [Arg^6]acetalin resulted in extacet. In radioligand binding assays, extacet displayed a 65-fold increase in affinity for κ opioid receptors (K_i = 6.6 nM) compared to [Arg^6]acetalin, while retaining high affinity for μ opioid receptors (K_i = 1.12 nM).[3]

To identify κ opioid receptor selective analogues we used a mixture-based combinatorial library to explore the structure−activity relationships (SAR) of extacet.[21] This library was small, containing only 256 peptides with four different residues incorporated in the first four positions. The mixtures were screened and deconvoluted using affinity for κ and μ opioid receptors stably expressed on Chinese hamster ovary (CHO) cells. Changing the first residue from Arg to Phe was key to identifying κ opioid receptor selective peptides from this library. The final purified peptides were evaluated for affinity in radioligand binding assays using κ, μ, and δ opioid receptors. One of these peptides, an analogue containing Met in position 5 as well as three Phe residues in the N-terminal 'message' sequence, was identified from the library which exhibited high affinity and selectivity for κ opioid receptors.[21]

Since Met is prone to oxidation, prior to further exploration of the SAR of the triple Phe analogue, the Met^5 residue was replaced with Leu resulting in arodyn (Figure 1). The synthesis of arodyn employed the Fmoc (9-fluorenylmethoxycarbonyl) synthetic strategy for solid-phase peptide synthesis.[23] The peptide was assembled on a PAL-PEG (peptide amide linker-poly(ethylene glycol)) resin (PerSeptive Biosystems Inc., Framingham, MA) with Fmoc-protected amino acids. 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetrauronium hexafluorophosphate (HBTU) and *N*-methylmorpholine (NMM) were used for loading of the first amino acid on the resin and subsequent couplings of amino acids; acetylation of the N-terminus was effected using acetic anhydride. The peptide was cleaved from the resin with Reagent B (88% TFA, 5% phenol, 5% H_2O, and 2% triisopropylsilane)[24] and purified to greater than 98% purity using reversed phase preparative HPLC and a binary solvent system consisting of aqueous 0.1% trifluoroacetic acid (TFA) and 0.1% TFA in acetonitrile. The molecular weight of the peptide was verified by electrospray ionization mass spectrometry (ESI-MS), and the purity by analytical reversed phase HPLC.[25]

In radioligand binding assays using CHO cells stably expressing κ, μ, and δ opioid receptors arodyn displayed nanomolar affinity and very high selectivity for κ opioid receptors (Table 1). The selectivity of this lead peptide for κ over either μ or δ opioid receptors was much greater than that of [D-Ala^8]Dyn A-(1−11)NH_2 (Table 1).

In adenylyl cyclase assays using CHO cells stably expressing κ opioid receptors, arodyn exhibited no concentration-dependent agonism. Therefore arodyn

[D-Ala^8]Dyn A-(1-11)NH_2:
H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Extacet:
Ac-Arg-Phe-Met-Trp-Met-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Arodyn:
Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Figure 1. Peptide sequence comparison of extacet and arodyn with the agonist [D-Ala^8]Dyn A-(1−11)NH_2. Postulated 'message' sequences of the peptides are underlined.

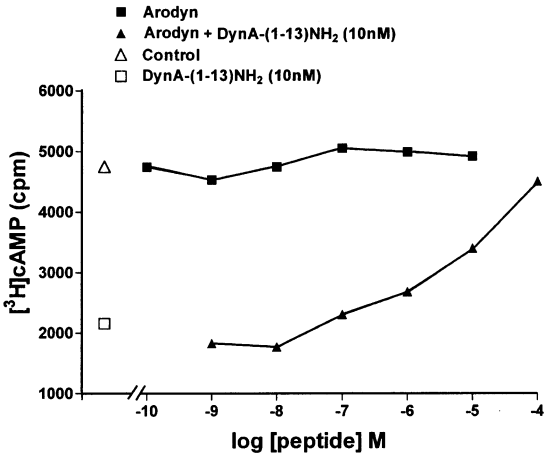

Figure 2. Inhibition of cAMP production by arodyn (■) and Dyn A (1−13)NH_2 (□) in CHO cells expressing κ opioid receptors and reversal by arodyn (▲) of the inhibition of cAMP production produced by 10 nM Dyn A (1−13)NH_2.

was examined for antagonist activity in this assay. Arodyn was able to completely reverse the agonist activity of 10 nM of Dyn A-(1−13)NH_2 in the adenylyl cyclase assay in a concentration-dependent manner (Figure 2), demonstrating that arodyn and Dyn A-(1−13)NH_2 bind in a mutually exclusive manner with arodyn acting as a κ opioid receptor antagonist.

Thus arodyn is a novel Dyn A analogue exhibiting antagonist activity and high selectivity for κ opioid receptors. The selectivity of this lead peptide is remarkable, particularly given the limited SAR that was explored in the small combinatorial library used to identify the predecessor of arodyn. Compared to the acetylated Dyn A analogue Ac-[Lys^2,Trp^{3,4},D-Ala^8]Dyn A-(1−11)NH_2 (JVA-901),[14] arodyn has 2-fold higher κ opioid receptor affinity, much greater κ opioid receptor selectivity, and lower efficacy in the adenylyl cyclase assay. Arodyn has similar selectivity for κ over μ opioid receptors and greater selectivity for κ over δ opioid receptors than the antagonist dynantin (K_i ratio ($\kappa/\mu/\delta$) = 1/259/198).[18] This is particularly noteworthy since arodyn is the initial lead structure which has not been optimized for κ receptor affinity, selectivity or antagonist activity, while dynantin was obtained by systematic

modification of a lead peptide [Hpp[1]]Dyn A-(1−11)NH_2 (Hpp = 3-(4-hydroxyphenyl)propionic acid) which exhibited only modest κ receptor affinity and selectivity (K_i = 30.7 nM, K_i ratio ($\kappa/\mu/\delta$) = 1/9/49).[18] Also arodyn does not have an aromatic residue in position 4 which is present in the antagonist analogues [Pro[3]]Dyn A-(1−11)NH_2[17] and dynantin.[18] Thus arodyn represents a novel acetylated Dyn A analogue which deserves further investigation. Exploration of the SAR of this lead peptide is currently being pursued in our laboratory.

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(25) HPLC (Vydac 218-TP 4.6 X 250 mm column) t_r = 21.8 min (5−80% aqueous MeCN with 0.1% TFA over 50 min, 1 mL/min), purity = 100%; t_r = 31.4 min (10−80% aqueous MeOH with 0.1% TFA over 46.7 min, 1 mL/min), purity = 99.4%; ESI-MS *m/z* 1535.5 $[M + H]^+$ calcd 1534.9.

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