A Bivalent Ligand (KDN-21) Reveals Spinal δ and κ Opioid Receptors Are Organized as Heterodimers That Give Rise to δ_1 and κ_2 Phenotypes. Selective Targeting of δ−κ Heterodimers

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Abstract: In view of recent pharmacological studies suggesting the existence of δ−κ opioid receptor heterodimers/oligomers in the spinal cord, we have synthesized and evaluated (intrathecally in mice) a series of bivalent ligands (KDN series) containing κ and δ antagonist pharmacophores. Pharmacological and binding data have provided evidence for the bridging of spinal δ−κ receptor heterodimers by KDN-21 and for their identification as δ_1 and κ_2. The selectivity profile of KDN-21 and the apparent absence of coupled δ_1−κ_2 phenotypes in the brain suggest a new approach for targeting receptors.

Opioid receptors are members of the rhodopsin subfamily in the superfamily of G-protein-coupled receptors (GPCRs). The three major types of opioid receptors (δ, μ, and κ) share 60% amino acid identity with one another and are antagonized by naloxone.[1] Classical models of opioid receptors have been based on the assumption that they are organized as monomers. However, in view of evidence implicating dimerization/oligomerization among a variety of GPCRs[2] and recent reports on the heterodimerization of opioid receptors,[3,4] we have investigated the possibility that putative δ and κ opioid receptor subtypes may be δ−κ heterodimers.[5,6] This was accomplished by the design and synthesis of a series of bivalent ligands that contain δ and κ antagonist phamacophores. Here, we report on an opioid antagonist bivalent ligand **5** (KDN-21)[7] whose intrathecal (i.t.) antinociceptive profile in mice and binding in cultured cells suggest it selectively bridges δ−κ opioid receptor heterodimers that recognize δ_1- and κ_2-selective ligands.

The potentiation of antinociception by spinally administered δ and κ opioid receptor agonists[8] and the report on the colocalization of δ and κ opioid receptors in spinal neurons,[9] taken together with evidence for the existence of δ−κ heterodimers/oligomers in cultured cells[6] and porcine ileum,[10] have prompted us to investigate the possible organization of these receptors in the spinal cord. Very recent i.t. pharmacological studies in mice have suggested that the putative δ_1 opioid receptor subtype[11,12] actually represents an allosterically coupled δ−κ receptor heterodimer.[5] The results of these studies have far-reaching implications because heterodimers may possess pharmacological selectivity and signal transduction pathways that differ from those of homodimeric receptors.[3]

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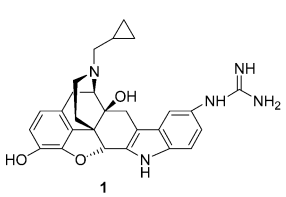
To investigate δ−κ opioid receptor heterodimers, we have explored the bivalent ligand approach for the design of receptor probes. Bivalent ligands are defined as structures that consist of two pharmacophores linked through a spacer.[13] Early studies with homobivalent ligands first suggested the existence of μ opioid receptor dimers in the guinea pig ileum preparation.[14] Structure−activity studies (SAR) revealed that peak agonist potency resided with a spacer containing 18 atoms, and this was interpreted as bridging between two neighboring opioid recognition sites. These SAR studies have served as the conceptual basis for the design of the δ−κ heterobivalent ligands disclosed in this report.

The δ−κ bivalent ligands in this report contain a κ-selective antagonist pharmacophore 5′-guanidinonaltrindole (5′-GNTI, **1**)[15] tethered through a spacer to the δ-selective antagonist pharmacophore naltrindole (NTI, **2**).[16] These pharmacophores were employed because of



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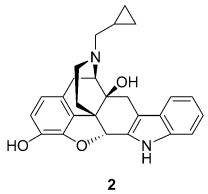

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their high affinity and selectivity. The known SAR of these ligands also permitted us to select a position for the attachment of the spacer without seriously compromising potency. Also, the chemical modification of these ligands for spacer attachment could be readily accomplished.

The constitution of the spacer was based on our desire to maintain a favorable hydrophilic−lipophilic balance coupled with flexibility to vary its length. The spacer contains glycyl units that are connected to amino groups attached to the pharmacophores and an alkyldiamine moiety that permits variation of the spacer length by one-atom increments. Monovalent ligands containing either a δ or κ antagonist pharmacophore attached to a capped spacer were synthesized as controls in an effort to factor out contributions of the spacer to activity. Earlier studies on μ−μ bivalent ligands suggested a spacer length of approximately 22 Å for maximal potency.[14] On the basis of these criteria, bivalent ligands with different spacer lengths (**3**−**7**) were synthesized along with the δ and κ monovalent ligands (**8** and **9**). The synthesis of these ligands is described in the Supporting Information.

The mouse tail-flick assay was employed to measure antinociception after i.t. administration of ligands **3**−**7**, monovalent ligands **8** and **9**, and selective standard agonists or antagonists.[17] The selective agonists employed to evaluate the antagonist selectivity of the target ligands were [D-Pen^{2,5}]enkephalin (DPDPE, δ_1),[18] [D-Ala$_2$,Glu$_4$]deltorphin (deltorphin II,[19] δ_2), U50488[20] (κ_1), and bremazocine[21] (κ_2). Only **5** (KDN-21) and its monovalent ligand **8** (KN-21) were devoid of agonist activity. The other ligands (**3**, **6**, **7**, **9**) in the series were

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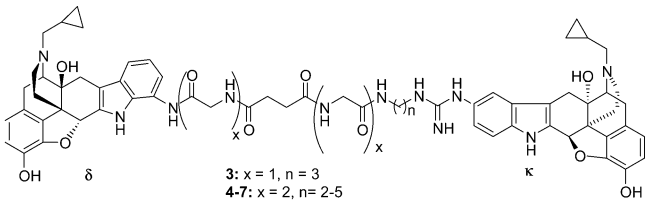
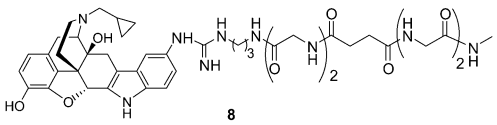
Table 1. Antagonism of Selective Opioid Agonist-Induced Antinociception upon Intrathecal Administration in Mice[a]

		agonist[b] ED_{50} ratio[c] (95% CL)[d]			
antagonist[e]	dose (pmol)	DPDPE (δ_1)	deltorphin II (δ_2)	U50488 (κ_1)	bremazocine (κ_2)
norBNI (κ)	250	12.4 (9.9−15.8)[f]	1.0 (0.6−1.8)[f]	5.3 (0.3−16.0)[f]	129.5 (75.3−237.1)
BNTX (δ_1)	25	10.6 (6.1−18.5)[f]	1.0 (0.5−2.0)[f]	0.9 (0.7−1.3)[f]	49.8 (25.5−103.3)
NTB (δ_2)	50	1.0 (0.7−1.4)[f]	9.1 (4.8−11.6)[f]	0.8 (0.3−1.5)[f]	5.8 (2.97−12.4)
KDN-15 (**3**)	1.0	2.0 (1.5−2.7)	1.9 (1.1−3.2)		2.5 (1.5−3.8)
KDN-20 (**4**)	2.5	3.4 (3.1−3.8)	1.1 (0.7−1.7)		1.7 (1.8−2.5)
KDN-21[g] (**5**)	30	8.0 (6.2−10.7)	1.2 (0.9−1.8)	1.9 (1.4−2.5)	50.4 (28.4−86.1)
KDN-22 (**6**)	2.5	6.3 (3.3−12.5)	2.2 (1.5−3.4)	0.6 (0.2−1.2)	1.7 (1.4−2.0)
KDN-23 (**7**)	2.5	2.2 (2.0−2.5)	2.1 (1.5−2.7)		4.1 (3.0−7.0)
KN-21 (**8**)	30	1.1 (0.8−1.7)	1.0 (0.4−2.3)		14.5 (7.9−30.6)
DN-21 (**9**)	25	1.1 (0.9−1.3)	2.4 (0.8−5.4)		2.4 (2.0−2.9)
8 + **9**	15	5.1 (2.8−8.7)	0.9 (0.6−1.5)	1.2(0.4−2.1)	13.6 (10.2−17.7)

[a] At least three groups of 10 male CD1 mice (Harlan Sprague Dawley) weighing between 20 and 25 g were employed in a modified tail flick assay. Antinociception was considered positive if the latency to flick its tail was more than the control latency plus 3 SD of the mean of the reaction time. [b] Peak times and control ED_{50} values (nmol/mouse) for the antinociceptive effect of the agonists (i.t.) were as follows: DPDPE, 10 min, 3.35 (3.05−3.66); deltorphin II, 10 min, 2.91 (2.18−3.93); U50488, 12 min, 20.97 (18.18−24.71); bremazocine, 20 min, 0.02 (0.01−0.03). [c] ED_{50} of agonist in the presence of antagonist divided by the control ED_{50} of agonist. [d] The parallel line assay[25] was used to calculate the ED_{50} values and the 95% confidence limits. ED_{50} ratios were considered significant when the 95% confidence intervals of the ratio were >1.0. [e] At higher doses, antinociception (ED_{50}, nmol/mouse) was observed for **3** (0.07), **6** (0.025), **7** (0.035), and **9** (0.18). KDN-20 (**4**) possessed partial agonist activity (30% antinociception at 1 nmol), whereas **5** (KDN-21) and **8** exhibited only antagonist activity. The agonist activity of **3**, **6**, **7**, and **9** was primarily at μ and κ. All compounds were evaluated for antagonist activity at doses that did not produce agonism. [f] Reported ED_{50} ratios.[5] [g] KDN-21 versus DAMGO gave an ED_{50} ratio of 2.50 (1.7−3.7) in mice that were pretreated with 5 μg i.t. dynorphin A antiserum to block the effect of dynorphin release.

therefore evaluated for opioid antagonist activity at lower doses that did not produce antinoception (Table 1).





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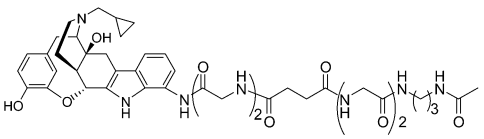

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It is noted that within the bivalent ligand series (**3**−**7**), **4**−**6** selectively antagonized DPDPE relative to deltorphin II. Since only KDN-21 (**5**) was devoid of agonist activity and was a potent antagonist of both DPDPE and bremazocine, it was considered as a candidate for selectively bridging associated δ and κ opioid receptors. Moreover, the finding that KDN-21 only weakly antagonized the κ_1 agonist U50488 or the μ agonist [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin[22] (DAMGO) suggested that heterodimerized δ and κ opioid receptors may be the origin of the phenotypic δ_1 and κ_2 activity in the cord.

As indicated by the ability of the κ antagonist norbinaltorphimine[23] (norBNI) to antagonize the δ_1 agonist DPDPE, the κ and δ opioid receptors in this putative $\delta-\kappa$ heterodimer are apparently allosterically coupled. Given that norBNI does not antagonize deltorphin II, this δ_2 agonist apparently does not interact with an allosterically coupled $\delta-\kappa$ heterodimer.[5] The potentiation of the antagonism of DPDPE by a combination of κ and δ monovalent ligands **8** and **9** is consistent with positive cooperativity between these receptors in the heterodimer (Table 1).

The evidence for spinal $\kappa-\delta$ opioid receptors prompted our evaluation of intraventricularly administered (icv) KDN-21 in order to determine whether such heterodimers are present in the brain. The icv pharmacological evaluation of KDN-21 revealed it to have agonist activity (ED_{50} = 31 pmol) and antagonist activity at lower doses.[24] In the evaluation of its icv antagonist selectivity with a dose (5 pmol) of KDN-21 that did not produce agonism, it was revealed that the selectivity profile differed from that obtained i.t. In this regard, KDN-21 antagonized only DPDPE (ED_{50} ratio of 5.52) and had no effect on deltorphin II, U,50488, or bremazocine antinociception.[24] These results suggest that κ and δ opioid receptors in the brain are not organized as $\delta-\kappa$ heterodimers. Furthermore, the data suggest that DPDPE interacts with δ receptors in the brain that differ from those in the cord.

In an effort to evaluate bridging, binding studies on the bivalent and monovalent ligands were carried out on human embryonic kidney (HEK) cells that contained singly expressed or coexpressed δ and κ opioid receptors. This included studies using a mixture of HEK cells with singly expressed δ or κ opioid receptors to distinguish between bivalent and univalent binding of the bivalent ligands. The binding data (Table 2) revealed that bivalent ligands KDN-20 (**4**) and KDN-21 (**5**) with 20- and 21-atom spacers had ∼120- and ∼200-fold higher affinity for the coexpressed $\delta-\kappa$ cell line when compared to the mixed cell lines. The bivalent ligands with shorter [KDN-15 (**3**)] or longer [KDN-22 (**6**), KDN-23 (**7**)] spacers possessed lower affinity (**3**) or similar affinity for coexpressed $\delta-\kappa$ receptors relative to a mixture of HEK cells containing singly expressed δ and κ receptors (**6**, **7**). These data strongly suggest that KDN-20 and KDN-21 bridge $\delta-\kappa$ opioid receptor heterodimers in the coexpressed cell line and that there is an optimal spacer

Table 2. Binding of Bivalent and Monovalent Ligands to δ and κ Opioid Receptors Expressed in HEK Cells

		K_i (nM)[a]	
compd	compd name	coexpressed $\delta-\kappa$[b]	mixed $\delta + \kappa$[c]
3	KDN-15	315 ± 15	6.3 ± 2.9
4	KDN-20	0.5 ± 0.1	58.8 ± 13.7
5	KDN-21	0.3 ± 0.0	63 ± 6.3
6	KDN-22	1.4 ± 0.3	1.1 ± 0.2
7	KDN-23	4.8 ± 3.2	3.3 ± 2.4
8	KN-21	>1000	54.8 ± 15.3
9	DN-21	>1000	199 ± 90

[a] Binding was conducted on intact HEK cells using [^{3}H]diprenorphine as radioligand. [b] Coexpressed δ and κ opioid receptors in HEK cells. [c] Mixed HEK cells containing singly expressed δ or κ receptors.

distance for bridging. The very low binding affinity of monovalent ligands, KN-21 (**8**) and DN-21 (**9**), for the coexpressed cell lines is consistent with this conclusion. These results support the i.t. studies that suggested that KDN-21 bridges $\delta-\kappa$ opioid receptor heterodimers. We were unable to adequately compare the antagonist selectivity of i.t.-administered KDN-20 with that of KDN-21 because the partial agonist activity associated with the former precluded testing at equal doses. Consequently, the correlation between the i.t. antagonist selectivity and binding for KDN-20 is not as robust as for KDN-21.

In conclusion, KDN-21 (**5**) is a member of a series of bivalent ligands that contains δ and κ opioid antagonist pharmacophores attached to variable-length spacers. In contrast to other members of the series, i.t.-administered KDN-21 exhibited selective δ_1 and κ_2 opioid antagonist activity and was devoid of antinociceptive activity. Considering the previously reported[5] allosteric property of spinal $\delta-\kappa$ heterodimeric opioid receptors that possess the δ_1 phenotype, we conclude that δ and κ opioid receptor recognition sites in the heterodimer are bound in the antagonist state by a single KDN-21 molecule. The substantially greater affinity of KDN-21 for coexpressed δ and κ receptors in cultured cells, when compared to its monovalent analogues, supports the bridging of one KDN-21 molecule to δ and κ recognition sites in a heterodimer. The bridging of a $\delta-\kappa$ heterodimeric opioid receptor by KDN-21, which contains a 21-atom spacer, suggests that bivalent ligands can serve as molecular rulers to obtain insight on the distance between recognition sites for the identification of possible monomer interfaces involved in GPCR dimerization. Moreover, the present study exemplifies the power of the bivalent ligand approach for the development of pharmacological tools to investigate the association of different GPCRs in an oligomeric array. Significantly, the different i.t. and icv antagonist selectivity profiles for KDN-21 suggest that δ and κ receptors in the cord are organized differently from those in the brain. This suggests the possibility of developing ligands that are selective for heterodimeric G-protein-coupled receptors as an approach for targeting different tissues (e.g., spinal cord vs brain). Finally, the finding that the δ_1 and κ_2 phenotypes reflect coupled δ and κ opioid receptors has broad implications in regard to screening paradigms of GPCRs because the pharmacological properties and signal transduction pathways may be altered by such association. In such cases, screening of coexpressed receptors may give results that are more consistent with in vivo data when compared to singly expressed receptors.

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Supporting Information Available: Experimental details including synthetic methods and physical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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