Narcotic Antagonistic Potency of Bivalent Ligands Which Contain β-Naltrexamine. Evidence for Bridging between Proximal Recognition Sites

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Two bivalent ligands (P-X-P) containing the β-naltrexamine pharmacophore (P) and a connecting oligoethylene glycol spanner (X) were synthesized and evaluated for narcotic antagonistic activity in the guinea pig ileum (GPI) and mouse vas deferens (MVD). The bivalent ligand **2** whose spanner contains three ethylene units possessed 10-fold greater antagonistic potency than its monovalent analogue (**4**) in antagonizing the effects of ethylketazocine (EK) on the GPI, while no differential antagonism of morphine was observed among the compounds. In the MVD, **2** was not substantially more potent than **4** as an antagonist against [D-Ala^2,D-Leu^5]enkephalin (DADLE). The bivalent ligand **3**, whose spanner contains six ethylene units, exhibited 15 times greater potency in antagonizing the agonist effects of DADLE on the MVD than its monovalent ligand **4**. No marked increase in the ability of **3** to antagonize the effects of morphine or EK on the GPI was observed. The data indicate that μ, κ, and δ opioid receptors exhibit different selectivity toward bivalent ligands whose spanner lengths differ. The enhanced potency associated with different receptor interactions is consistent with simultaneous occupation of proximal recognition sites. Whether such proximal recognition sites are identical or different remains to be clarified. The distance between proximal sites appears to depend on the opioid receptor subtype involved.

Although extensive structure-activity data are available for many classes of ligands whose actions are mediated through opioid receptors, there is little information concerning the proximity of such receptors in their occupied state. Some data consistent with allosteric coupling between receptors have been reported,[1] but no estimates of interreceptor distance have emanated from such studies.

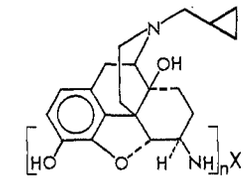
We have addressed the question of interreceptor distance by employing bivalent ligands as opioid receptor probes. Bivalent ligands are molecules which contain two pharmacophores (P) linked by a spanner (X). The general structure for bivalent ligands is P-X-P. For example, bis-onium cholinergic blocking agents[2] may be thought of as bivalent ligands.

A bivalent ligand would be expected to exhibit greatly enhanced potency relative to its monovalent analogue P-X when the spanner X is of sufficient length to permit simultaneous interaction of both pharmacophores with proximal recognition sites.

In this paper we report on studies to evaluate bivalent ligands as probes to investigate the distance between opioid receptors. Since it is possible that opioid receptor subtypes[3] (e.g., μ, κ, and δ receptors) possess different interreceptor distances, it is conceivable that high selectivity for a single subtype can be achieved by the incorporation of a specific-length spanner into the bivalent ligand. Thus, an additional objective of this study was concerned with the feasibility of developing bivalent ligands that are highly selective for an opioid receptor subtype. The results of this initial study indicate that bivalent ligands which contain the β-naltrexamine[4] (**1**) pharmacophore and different-length oxyethylene spanners possess greatly enhanced ability to selectively block opioid receptors. Moreover, the particular subtype of receptor that is blocked is dependent on spanner length.

Design Considerations and Chemistry. The pharmacophore which was used in the elaboration of the bivalent ligands is β-naltrexamine[4] (**1**), a narcotic antagonist. A consideration in employing **1** was based on the presence of the 6-amino group as a point of attachment for the spanner chain.

The spanners employed in the present study are composed of repeating oxyethylene units in order to minimize changes in the partition coefficient upon varying the spanner length. For example, lengthening an aliphatic chain would give rise to incremental increases in lipophilicity with each addition of CH_2. In this initial study to test the feasibility of the bivalent ligand approach, spanners containing three and six ethylene units were employed. For comparison purposes, this included the preparation of the monovalent ligand in order to evaluate the contribution of the spanner.



	n	X
1	1	H
2	2	$CH_2CH_2OCH_2CH_2OCH_2CH_2$
3	2	$(CH_2CH_2OCH_2CH_2OCH_2CH_2)_2O$
4	1	$CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$
5	1	$CH_2CH_2OCH_2CH_2OCH_2CH_2OTs$

The bivalent ligands **2** and **3** were synthesized by slow addition of a diglyme–toluene solution of 1 equiv of tri- or hexaethylene glycol ditosylate[5,6] to 2 equiv of β-naltrexamine (**1**). An inverse order of addition using 25× excess of triethylene glycol ditosylate afforded a preponderance of the monotosylate intermediate **5**. The monovalent ligand **4** was prepared by treatment of **5** with NaOMe in MeOH.

Pharmacology. Compounds **2–5** were evaluated on the electrically stimulated longitudinal muscle of the guinea

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Table I. Narcotic Antagonistic Activity of Bivalent and Monovalent Ligands

	GPI IC_{50} ratio[a]		MVD IC_{50} ratio[a]
no.	morphine	EK	DADLE
2[b]	3.24 ± 0.57	16.91 ± 3.07	7.17 ± 2.69
3[c]	5.75 ± 1.21	2.61 ± 0.52	30.67 ± 2.98
4[b]	3.45 ± 1.10	1.63 ± 0.50	2.09 ± 0.47
5[c]	3.78 ± 0.20	3.96 ± 1.23	4.29 ± 0.22

[a] The ratio ± SE represents the IC_{50} of the agonist in the presence of antagonist divided by the IC_{50} in the absence of antagonist in the same preparation. [b] Tested on the GPI at 2×10^{-8} M and on the MVD at 1×10^{-7} M. [c] [c] Tested on the GPI at 1×10^{-8} M and on the MVD at 1×10^{-7} M.

Table II. Partial Agonist Activity of Monovalent and Bivalent Ligands

	% max agonist response (concn, M)	
no.	GPI	MVD
2	10 (1×10^{-8})	32 (1×10^{-7})
3	60 (5×10^{-7})	26 (1×10^{-7})
4	67 (1×10^{-6})	49 (1×10^{-6})
5	57 (3×10^{-8})	27 (1×10^{-7})

pig ileum (GPI)[7] and on the mouse vas deferens[8] (MVD) preparations (Table I). The antagonistic potency is expressed as the ratio of the IC_{50} of the agonists, morphine, ethylketazocine (EK), or [D-Ala^2,D-Leu^5]enkephalin (DADLE), in the presence and in the absence of the test compound on the same preparation. These agonists were employed because of their relative selectivity for μ, κ, and δ opioid receptor subtypes, respectively.[3]

When tested on the GPI, the bivalent ligand **2** exhibited enhanced antagonism (~10×) to EK relative to the monovalent ligand **4** (Table I). The bivalent ligand **3** possessing the longer spanner showed no significant increase. All of the ligands (**2**–**5**) exhibited similar ability to antagonize morphine. Since the partial agonistic activity of **3** and **5** at 2×10^{-8} M interfered with the evaluation of their antagonism of morphine and EK, a concentration of 1×10^{-8} M was employed.

In the MVD preparation, only bivalent ligand **3** appeared to have greatly enhanced (~15×) antagonism to the agonist effect of DADLE. A small potency increase (~3×) occurred with **2**.

In addition to their antagonistic activity, both the monovalent and bivalent ligands behaved as partial agonists in the GPI and MVD, with maximal responses ranging from 10 to 60% (Table II).

Discussion

The two pharmacophores of a bivalent ligand, if separated by a polymeric spanner, should behave independently. From a statistical standpoint, the $\Delta G°$ for such binding would be expected to be about twice that of the monovalent analogue if identical sites are occupied. On the other hand, when the spanner is sufficiently short to preclude simultaneous interaction of both pharmacophores with proximal recognition sites, it is expected that the potency would be close to that of the monovalent ligand provided that one of the pharmacophores does not interfere in the overall binding process.

At some spanner length between these two extreme cases, one would expect the bivalent ligand to possess an affinity considerably greater than double that of the monovalent ligand. Thus, if the binding of a bivalent ligand to proximal recognition sites occurs in a stepwise fashion (Figure 1), then proceeding from binding state B to the bridged state C should involve a more positive entropy change, ΔS, than in the univalent binding depicted in state D. In terms of the Gibbs free energy relationship, $\Delta G° = \Delta H° - T\Delta S°$, this should be reflected in the more facile association of the second pharmacophore of the bivalent ligand relative to the first. Accordingly, at a minimum spanner length which permits facile bridging between proximal recognition sites, the spanner can exert a directive influence in guiding the unbound pharmacophore (state B) to the unoccupied proximal site leading to state C. The resulting affinity increase can be viewed as arising from the higher local "concentration" of unbound pharmacophore when the bivalent ligand is in binding state B (Figure 1). This is a consequence of the confinement of the unbound pharmacophore to the environs of the unoccupied proximal site by virtue of the spanner attachment to the bound pharmacophore. The ratio of the bridged state C to the univalently bound state D therefore is a function of spanner length.

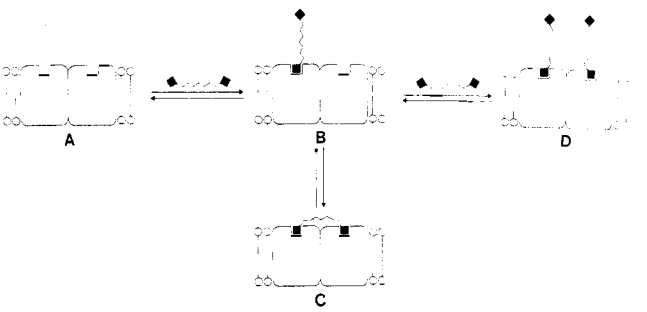

Figure 1. A schematic illustration of the steps involved in the binding of a bivalent ligand to proximal recognition sites on associated receptor subunits in a membrane bilayer. A represents two unoccupied proximal sites; B represents the univalent binding of a bivalent ligand; C depicts bridging of proximal sites; D illustrates the occupation of proximal recognition sites by individual bivalent ligands. The proximal sites may or may not be identical.

The data in Table I is in harmony with the idea that the bridging of a bivalent ligand between proximal recognition sites (Figure 1, C) leads to enhanced affinity. The considerably greater narcotic antagonistic activity of the bivalent ligands (**2** and **3**) over their monovalent analogues (**4** and **5**) appears to be dependent on spanner length, on the test system, and on the agonist employed (Table I). The fact that the potency of the bivalent ligand **2** in blocking the agonistic effect of EK, a κ-selective opioid receptor agonist, is tenfold greater than that of the monovalent ligand **4** suggests that bridging has occurred with κ-receptor involvement. While the greater affinity of **2** is a likely manifestation of such bridging, the present study does not allow us to distinguish whether these proximal recognition sites are identical or different.

Consistent with the idea of receptor bridging is the observation that the bivalent ligand **3**, having the longer spanner, shows no increase in antagonistic potency at κ receptors. This is most likely a consequence of increasing the spanner in **3** beyond the minimum critical length required for bridging, thereby leading to less favorable entropy changes for binding. That the potency increase for **2** is of a specific nature is suggested by the inability of the second aromatic ring in the monovalent ligand **5** to substantially increase activity. Thus, merely placing an aromatic moiety at the end of the spanner does not substantially facilitate the recognition process.

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Bivalent ligands **2** and **3** do not possess enhanced antagonistic activity against morphine, a μ-selective agonist on the GPI. The similar magnitude of the antagonistic effect among all four ligands (**2**–**5**) suggests the absence of significant binding interaction with a site proximal to the μ opioid receptors.

When tested on the MVD preparation, bivalent ligand **3** exhibited a 15-fold increase over **4** in reversing the effect of DADLE, an agonist which is selective for δ opioid receptors. Only a small (3×) potency increase was observed with the bivalent ligand **2** containing the shorter spanner. Since a similar increase also was observed with monovalent ligand **5**, it is possible that this is not the consequence of a specific interaction. Thus, it appears that δ opioid receptors are blocked to a greater degree by the bivalent ligand **3**, which contains the longer spanner. This is in contrast to the κ receptor interaction in the GPI, where the bivalent ligand **2** with the shorter spanner is a more effective blocker. These data therefore suggest that the intersite bridging distance is greater for δ than for κ receptors.

In summary, this study demonstrates that bivalent ligands (**2** and **3**) which contain narcotic antagonist pharmacophores can possess much greater narcotic antagonistic potency than their monovalent congeners (**4** and **5**). Moreover, the data reveal that μ, κ, and δ opioid receptors exhibit different selectivity toward the bivalent ligands, suggesting that different bridging distances are associated with different receptors. In this regard, the extended spanner length in **2** is ~9 Å, while that in **3** is ~20 Å. This is consistent with a shorter bridging distance for sites proximal to κ receptors as compared to δ receptors. The extended spanner length is undoubtedly greater than the linear intersite distance because sufficient translational mobility of the unbound pharmacophore is expected to be required for recognition by the proximal site. In other words, there should be enough slack in the spanner to permit binding of the second pharmacophore. Presently, it is not clear if such proximal recognition sites are identical or different. Finally, the bivalent ligand approach offers a new avenue for the design of agents which are highly selective for a specific subpopulation of opioid receptors.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. All analytical results were within ±0.4% of the theoretical values. IR spectra were recorded on a Perkin-Elmer 281 spectrophotometer. NMR spectra were recorded on either a Varian A-60D, Varian FT-80, or Brucker WM-250 spectrophotometer, with tetramethylsilane as internal standard. Mass spectra were obtained on an AEI MS-30 (EI, 20 eV) or Finnigan 4000 (CI, NH_3, positive or negative). HPLC was carried out on a Beckman 110A instrument with a 254-nm UV detector (column ODS, 5-μm particles, 125-cm long, 4.6-mm internal diameter, flow rate 0.8 mL/min). The eluent was a mixture of buffer (KH_2PO_4, 0.03 M, pH 4), 65%, and methanol, 35%.

1,8-Bis(β-naltrexamino)-3,6-dioxaoctane (2). Triethylene glycol ditosylate[5] (0.92 g, 2 mmol) was dissolved in hot toluene (13 mL) and dropped during a 2-h period with stirring into 12 mL of a refluxing toluene–diglyme (1.4:1) solution of β-naltrexamine[4] (1; 1.32 g, 3.9 mmol) containing Na_2CO_3 (2.6 g). The reaction, which was conducted under N_2, was refluxed for an additional 3 h with monitoring by TLC. After filtration and removal of solvents in vacuo, the product was chromatographed on silica gel by gradient elution using EtOAc–MeOH–NH_4OH (95:5:0.5 to 80:20:18). The product (1.0 g, 60%) was crystallized from EtOH–Et_2O–petroleum ether (30–60 °C) to afford **2**: mp 132–136 °C; R_f (silica gel; EtOAc–MeOH–NH_4OH, 70:30:4) 0.45. The purity of **2** was verified by HPLC (0.03 M KH_2PO_4/MeOH, 65:35). CIMS, m/e 798 (M^+·); NMR ($CDCl_3$) δ 6.62 and 6.56 (4 H, d, $J_{1,2}$ = 8 Hz, Ar), 4.84 (2 H, d, $J_{5,6}$ = 7.5 Hz, C-5 H), 3.66 (8 H, s and t, CH_2O). Positive $FeCl_3$ test. Anal. ($C_{46}H_{62}N_4O_8$) C, H, N.

1,17-Bis(β-naltrexamino)-3,6,9,12,15-pentoxaheptadecane (3). Hexaethylene glycol ditosylate[6] (1.18 g, 2.0 mmol) in toluene (10 mL) was dropped during a 1-h period into 17 mL of a toluene–diglyme (2:1) solution of **1** (1.32 g, 3.9 mmol) at 100 °C. After refluxing for 7 h, during which time the reaction was monitored by TLC, the mixture was filtered and the solvent was removed by evaporation in vacuo. The crude product (2.1 g) was chromatographed on silica gel with EtOAc–MeOH–NH_4OH (90:10:0.6 to 80:20:20) to afford 0.32 g of **3** contaminated with **1**. Further chromatographic purification, which was conducted on 0.14 g of the above mixture, afforded 0.075 g of **3** as a colorless oil. The hydrochloride salt **3**·4HCl was extremely hygroscopic and liquified soon after formation. EIMS did not give the m/e 931 peak, but a fragment (m/e 588), Nal – $NH(CH_2CH_2O)_5CH_2CH_2$, was obtained. NMR (D-exchanged salt in CD_3OD) δ 6.20 (4 H, Ar), 4.62 (2 H, d, C-5 H), 3.66 (20 H, CH_2). Anal. ($C_{52}H_{74}O_{11}$·4HCl·$4H_2O$) C, H.

1-Methoxy-8-naltrexamino-3,6-dioxaoctane (4). To a refluxing toluene (12 mL) solution of triethylene glycol ditosylate (1.15 g, 2.5 mmol) containing $NaHCO_3$ (1.04 g) was added over a 2-h period 0.70 g (2 mmol) of **1** in 13 mL of toluene–diglyme (2:1). The mixture then was refluxed for an additional 3 h. The reaction was conducted under N_2. The filtered mixture, which consisted of an oil (1.72 g) containing **1**, **2**, and **5** in a ratio of 0.3:1:1, was purified by gradient elution chromatography using silica gel and EtOAc–MeOH–NH_4OH (99:1:05 to 80:20:1). The monotosylate intermediate **5** (0.24 g, 11 mmol), R_f 0.67 (silica gel; EtOAc–MeOH–NH_4OH, 70:30:4) 0.67, was obtained in a yield of 0.24 g (11 mmol): CIMS, m/e 626 (M – 2); NMR (D-exchanged in $CDCl_3$) δ 6.69 and 6.64 (2 H, q, Ar), 7.80 and 7.38 (4 H, q, Ts Ar). The intermediate **5** (108 mg, 0.2 mmol) in MeOH (6 mL) was added to 3 mL of methanolic NaOMe (240 mg, 4 mmol) over a period of 10 min, followed by 3 h of reflux. The reaction mixture was cooled, saturated aqueous $NaHCO_3$ (25 mL) was added, and the product was extracted with EtOAc (4 × 50 mL). The EtOAc phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to yield 0.083 g (100% based on **5**) of **4**. Gradient elution chromatography on silica gel using EtOAc–MeOH–NH_4OH (96:4:0.5 to 90:10:0.5) afforded 0.075 g of pure **4** as an oil: EIMS, m/e 488 (M^+). Anal. ($C_{27}H_{40}N_2O_6$) C, H, N.

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