Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series

David J. Daniels*, Natalie R. Lenard†, Chris L. Etienne*, Ping-Yee Law‡, Sandra C. Roerig†, and Philip S. Portoghese*§

*Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455; †Louisiana State University Health Sciences Center, Shreveport, LA 71103; and ‡Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455

Edited by Solomon H. Snyder, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved November 3, 2005 (received for review August 2, 2005)

Given the mounting evidence for involvement of δ opioid receptors in the tolerance and physical dependence of μ opioid receptor agonists, we have investigated the possible physical interaction between μ and δ opioid receptors by using bivalent ligands. Based on reports of suppression of antinociceptive tolerance by the δ antagonist naltrindole (NTI), bivalent ligands [μ-δ agonist-antagonist (MDAN) series] that contain different length spacers, and pharmacophores derived from NTI and the μ agonist oxymorphone, have been synthesized and evaluated by intracerebroventricular (i.c.v.) administration in the tail-flick test in mice. In acute i.c.v. studies, the bivalent ligands functioned as agonists with potencies ranging from 1.6- to 45-fold greater than morphine. In contrast, the monovalent μ agonist analogues were substantially more potent than the MDAN congeners and were essentially equipotent with one another and oxymorphone. Pretreatment with NTI decreased the ED_{50} values for MDAN-19 to a greater degree than for MDAN-16 but had no effect on MDAN-21. Chronic i.c.v. studies revealed that MDAN ligands whose spacer was 16 atoms or longer produced less dependence than either morphine or μ monovalent control MA-19. On the other hand, both physical dependence and tolerance were suppressed at MDAN spacer lengths of 19 atoms or greater. These data suggest that physical interaction between the μ and δ opioid receptors modulates μ-mediated tolerance and dependence. Because MDAN-21 was found to be 50-fold more potent than morphine by the i.v. route (i.v.), it offers a previously uncharacterized approach for the development of analgesics devoid of tolerance and dependence.

antinociception | heterodimers

Interaction between μ and δ opioid receptors was first suggested by Vaught and Takemori (1), who observed potentiation of morphine-induced antinociception by a δ agonist. Reports that followed also suggested an interaction between μ and δ opioid receptors and its possible significance in morphine tolerance and physical dependence (2–4). Studies with δ antagonists have demonstrated that the chronic effects of morphine can be blocked without significantly diminishing its antinociceptive action (4–7). Subsequent studies by using antisense oligodeoxynucleotides (8, 9) and δ opioid receptor knockout mice (10) have supported these ideas, implicating μ–δ interactions in the development of morphine tolerance and physical dependence. More recently, mixed μ agonist/δ antagonist ligands have been designed as an approach to analgesics devoid of these side effects (11–13).

It has not yet been reported whether the *in vivo* synergy between μ and δ agonists is a consequence of direct association between receptors or due to functional modulation involving neuronal circuitry. In view of evidence for μ-δ opioid receptor heterodimers in cultured cells (14–16), there is reason to believe that similar interactions may occur *in vivo*. The development of selective ligands that target associated μ-δ opioid receptors should help to further delineate the pharmacology of these receptors.

We have designed bivalent opioid ligands that contain μ agonist and δ antagonist pharamacophores to address whether physical interaction between μ and δ opioid receptors is required to attenuate μ agonist-induced tolerance and dependence in mice. Several studies have suggested that bivalent ligands may bridge associated opioid receptors. Bivalent ligands of opioid alkaloids (17–24) and peptide agonists derived from enkephalins (25–28) have been characterized as having increased opioid receptor potency and selectivity when compared to corresponding monovalent counterparts. More recently, bivalent ligands have been used as tools to further characterize δ and κ opioid receptor phenotypes. Specifically, unique bivalent probes that target δ_1-κ_2 heterodimers (29) or associated δ_2-κ_1 opioid receptors (30) have been reported.

Here, we report on the design, synthesis, and biological evaluation of the MDAN (MDAN, μ-δ agonist-antagonist) series of bivalent ligands containing a μ opioid agonist and a δ opioid antagonist pharmacophores designed to address the question as to whether μ and δ receptors mediate their actions in a physically associated state. The results of our studies suggest that opioid-induced tolerance and physical dependence are mediated through physical association of μ and δ opioid receptors as heterodimers.

Materials and Methods

Animals. Male ICR mice (Harlan Labs, Indianapolis) that weighed 25–30 g were used throughout these studies. The mice were housed in groups of five at 22–23°C in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal care facility under a 12 h-12 h light/dark cycle. Both food and water were available *ad libitum*. All procedures were approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

Acute Drug Administration. Drugs were dissolved in sterile saline (0.9% NaCl). Animals were anesthetized with isoflurane and drugs were administered i.c.v. in a volume of 4 μl into the lateral cerebral ventricle (31). For i.v. administration, animals were restrained by hand, and drugs were injected into a lateral tail vein in a volume of 100 μl.

Antinociceptive Testing. Antinociception was evaluated by the radiant heat tail-flick assay (32). Time required to flick the tail

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DN, δ antagonist; MA, μ agonist; MDAN, μ-δ agonist-antagonist; NTI, naltrindole.

§To whom correspondence should be addressed. E-mail: porto001@umn.edu.

in response to a focused beam of light was recorded. Each animal served as its own control and was used only once. Mice were tested once before injection (control time) and again once after injection (drug time) at the time of peak drug response as determined by pilot time course studies. The light intensity was adjusted so that control times were between 1.5–2.5 sec. A 10-sec cut-off drug time was set to minimize the risk of tissue damage. Percent maximum possible effect (%MPE) was calculated as: [(drug time (sec) − control time (sec))/(10 sec − control time (sec))] × 100 = %MPE.

Graded dose–response curves of at least four doses with at least eight mice per dose were generated from the %MPE data. ED_{50} values with 95% confidence intervals (C.I.) were computed with GraphPad PRISM by using nonlinear regression methods.

Chronic i.c.v. Infusion. Osmotic minipumps (model 1003D, Alzet, Durect Corp. Cupertino, CA) were filled with saline or the drug solution to be tested. The dose of each drug was 12 times its ED_{50}/h. These doses were based on methods previously developed in our laboratory for chronic i.c.v. infusion of morphine in mice (33). The minipumps were connected by a 1.6–1.8 cm length of PE-60 tubing to a 3-mm-long cannula (osmotic pump connector cannula, Plastics One, Roanoke, VA) and primed in sterile saline at 37°C overnight.

For pump implantation, mice were anesthetized with Avertin [2,2,2-tribromoethanol (370 mg/kg, i.p.)/*t*-amyl alcohol (0.16 mg/kg, i.p.)]. The scalp was shaved, an incision was made along the midline, and the skull was scraped clean of periosteum. Hemostats were used to make a pocket under the skin between the shoulder blades. A micro drill was used to drill a hole ≈1.6 mm lateral and 0.6 mm caudal to bregma. The minipump was placed in the pocket between the shoulder blades, the cannula was inserted through the drilled hole into the lateral ventricle, and the cannula pedestal was affixed to the skull with cyanoacrylate glue. The animals were allowed to recover on a heating pad and were returned to their cages in the animal facility for 3 days.

Testing for Dependence and Tolerance. On the fourth day after implantation of the minipump, mice were injected with naloxone (1 mg/kg, s.c.) and placed into Plexiglas cylinders for 10 min (34). The number of vertical jumps were counted as withdrawal signs, indicating development of physical dependence. Wet shakes were also observed in some animals but were not recorded.

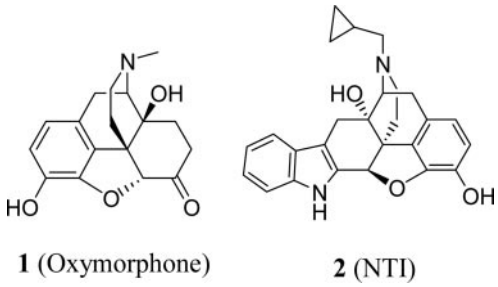
The minipumps were then removed, and mice were returned to their home cage for 4 h. To test for development of tolerance, animals were then injected i.c.v. with the test drug into the contralateral cerebroventricle and antinociception was measured with the tail-flick test at the predetermined time of peak drug activity.

Statistical Analyses. ED_{50} values were considered significantly different when the 95% C.I. intervals did not overlap.

Synthesis and Characterization. Preparation and characterization data for all of the compounds are published as supporting information, which is published on the PNAS web site.

Results

Rationale for Ligand Design and Chemistry. The pharmacophores (**1** and **2** in Fig. 1) chosen for the MDAN series incorporate the μ opioid agonist oxymorphone **1** (35) and the δ opioid antagonist naltrindole (NTI) **2** (36), linked through a variable length spacer. The NTI pharmacophore was selected because of the report that δ opioid antagonists suppress tolerance and dependence without a substantial diminution of efficacy (5). The spacer features a central diamine flanked by adjacent diglycolic acid moieties.



1 (Oxymorphone) **2** (NTI)

Fig. 1. Key pharmacophores: oxymorphone (**1**, μ agonist) and NTI (**2**, δ antagonist).

Spacer length was varied by changing the number of methylenes in the central diamine portion. Because prior studies have provided evidence for bridging of associated opioid receptors by bivalent ligands whose spacers are in the range of 22 Å, we bracketed this length with shorter and longer length spacers (22). The spacers varied from 16 atoms (MDAN-16, **3**) to 21 atoms (MDAN-21, **8**). The constitution of the spacers was motivated by our desire to maintain a favorable hydrophilic-hydrophobic balance of the bivalent ligands. Matched monovalent control compounds were synthesized for the μ monovalent series (MA-16–MA-21, **9**–**13**)[¶] (MA, μ agonist) along with a δ monovalent control (DN-20, **14**) (DN, δ antagonist) in an effort to factor out possible effects of the spacer on activity in the bivalent ligands. Final compounds are illustrated in Fig. 2.

Pharmacological Results. ***Acute i.c.v. administration.*** To determine whether spacer length affects the antinociceptive potency of our bivalent ligand series, compounds **3**–**8** (MDAN series) were acutely evaluated after i.c.v. administration to mice. These bivalent ligands were all less potent than the μ monovalent ligands **9**–**13** (MA series) (Table 1). Increasing the distance between the μ and δ pharmacophores of the MDAN ligands was associated with increased antinociceptive potency. In contrast, acute administration of the μ monovalent compounds (MA series) revealed that the spacer length did not affect potency. It should be noted that the ED_{50} values of the MA compounds were not different from that of oxymorphone. Coadministration of MA-19 and DN-21 produced an antinociceptive effect that was similar to that of MA-19 administered alone.

To determine whether pretreatment with the δ antagonist NTI could modulate the potency of the bivalent ligands, mice were pretreated with NTI (50 pmol, i.c.v.) 10 min before administration of MDAN-16, -19, or -21 (Table 2). NTI shifted the dose–response curves of MDAN-16 and -19 to the left but did not affect the response to MDAN-21. The ED_{50} for MDAN-19 was increased 8.6-fold compared to the 5.7-fold increase for MDAN-16. Interestingly, after NTI pretreatment, the ED_{50} values for MDAN-19 and -21 were not different from the ED_{50} value for the μ monovalent agonist MA-19, whereas the MDAN-16 ED_{50} value remained greater than that for MA-19. Additionally, NTI did not enhance the potency of MA-19 but actually slightly decreased its activity.

Chronic i.c.v. administration. To investigate tolerance and dependence associated with the MDAN bivalent ligands, we performed chronic i.c.v. administration studies. The compounds of interest were administered i.c.v. via a cannula by using an osmotic minipump for 3 days as described in ref. 33. After this time period, withdrawal was measured by administering naloxone (1 mg/kg, s.c.) and counting the naloxone-precipitated

[¶]MA-18 was never synthesized because the data showed that all the other μ monovalent compounds had similar potencies (Table 1).

PHARMACOLOGY

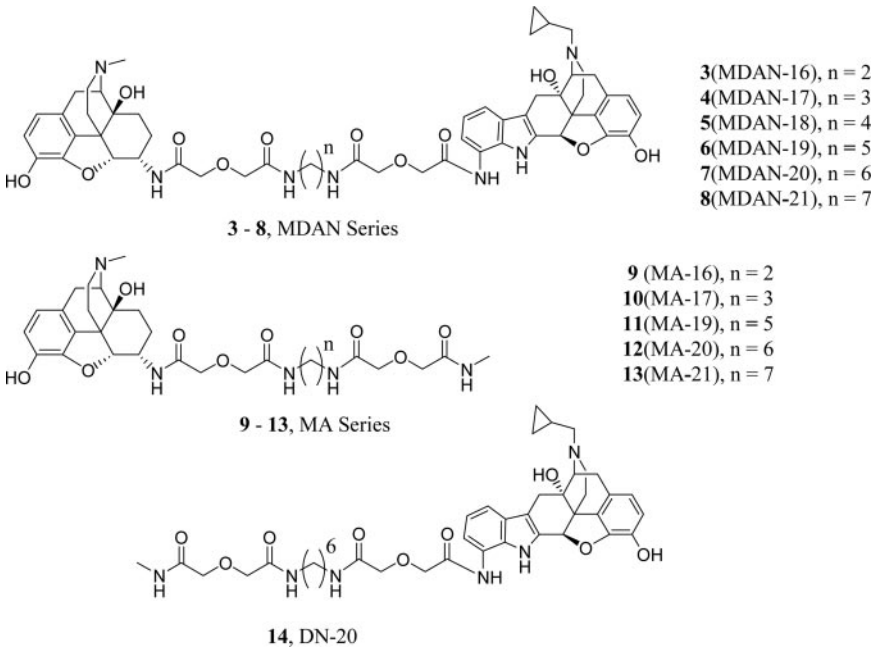


Fig. 2. Final compounds.

jumps for 10 min. Tolerance was determined after the compound was infused for 3 days and determining the chronic ED_{50} value at that time.

Results obtained after chronic i.c.v. drug administration are shown in Table 3. Morphine was tested for comparison purposes and showed a 6-fold increase in ED_{50} value (development of tolerance) and robust physical dependence (100 naloxone-induced jumps in 10 min). Chronic i.c.v. infusion with MDAN-16 produced a 2.6-fold increase in antinociceptive ED_{50} value and fewer naloxone-induced jumps. Similarly, 3 days of i.c.v. infusion with MDAN-17 or MDAN-18 resulted in 3.6- and 3.7-fold increases in ED_{50} values, respectively. However, few naloxone-induced jumps were observed in these animals. When mice were infused i.c.v. with MDAN-19, -20, or -21, the ED_{50} values were not different from those found after acute administration and naloxone administration did not induce a significant number of vertical jumps. Thus, development of tolerance and physical dependence depended on spacer length, with the shortest spacer ligands producing both the intermediate spacer ligands producing tolerance without physical dependence and the longest spacer ligands producing neither.

After chronic i.c.v. administration of the μ monovalent agonist MA-19, a 5.5-fold increase in the ED_{50} value was observed and naloxone precipitated 83 jumps (Table 3). These results are similar to those seen in mice after i.c.v. morphine treatment. Chronic coadministration of MA-19 with the δ monovalent antagonist DN-20 in a 1:1 equimolar ratio produced an 8.9-fold increase in the ED_{50} value and 29 naloxone-induced jumps. Thus, the μ agonist monovalent ligand, in the presence or absence of the δ antagonist monovalent ligand, produced both tolerance and physical dependence.

To determine whether the compounds were active after systemic administration, MDAN-21, MA-19, and morphine were administered i.v. Results in Table 4 show that MDAN-21 was >50-fold more potent and MA-19 was 100-fold more potent than morphine when administered either i.c.v. or i.v. Thus, the i.v./i.c.v. ratios for the three compounds were similar, suggesting similar access to brain opioid receptors.

Table 1. Acute antinociceptive activity of the μ-δ bivalent ligands and μ monovalent ligands in the mouse tail-flick assay after i.c.v. administration

Ligand	Spacer length,* Å	ED_{50},† nmol (95% C.I.)
3 (MDAN-16)	19.1	1.79 (1.54–2.04)
4 (MDAN-17)	20.4	1.49 (1.04–1.95)
5 (MDAN-18)	21.6	0.95 (0.68–1.23)
6 (MDAN-19)	22.9	0.43 (0.36–0.50)
7 (MDAN-20)	24.1	0.17 (0.15–0.19)
8 (MDAN-21)	25.4	0.08 (0.06–0.10)
9 (MA-16)	19.1	0.039 (0.032–0.046)
10 (MA-17)	20.4	0.040 (0.033–0.046)
11 (MA-19)	22.9	0.040 (0.023–0.050)
12 (MA-20)	24.1	0.037 (0.029–0.045)
13 (MA-21)	25.4	0.044 (0.039–0.048)
11 and **14** (MA-19 and DN-20)		0.037 (0.031–0.043)
Oxymorphone		0.043 (0.034–0.052)

*Spacer length represents the maximum linear distance between the pharmacophores.

†ED_{50} (95% C.I.) values were calculated from %MPE values with GraphPad PRISM by using nonlinear regression methods. Each ED_{50} value represents a dose–response curve with at least four doses and eight mice per dose.

Table 2. Effect of pretreatment with naltrindole on acute antinociceptive potency of selected μ-δ bivalent ligands administered i.c.v.

	ED_{50}, nmol (95% C.I.)	
Ligand	No pretreatment	NTI (50 pmol) pretreatment
3 (MDAN-16)	1.70 (1.50–2.00)	0.30 (0.19–0.43)
6 (MDAN-19)	0.43 (0.36–0.50)	0.05 (0.05–0.07)
8 (MDAN-21)	0.08 (0.06–0.10)	0.06 (0.05–0.07)
11 (MA-19)	0.04 (0.023–0.045)	0.08 (0.065–0.085)

NTI was administered i.c.v. 10 min before each compound, and tail-flick response was measured at the time of peak compound antinociceptive activity.

Table 3. Development of tolerance and physical dependence to μ-δ bivalent ligands after chronic i.c.v. administration

Ligand	ED_{50}, nmol (95% C.I.)		Ligand ED_{50}/saline ED_{50}*	No. of jumps (S.E.M.)
	i.c.v. saline	i.c.v. ligand		
Morphine	4.54 (3.51–5.56)	26.80 (20.82–32.78)	6.0	100 (15)
3 (MDAN-16)	1.62 (1.35–1.89)	4.72 (3.47–5.91)	2.8	30 (23)
4 (MDAN-17)	1.54 (0.89–2.20)	5.61 (4.39–6.83)	3.6	0.9 (0.7)
5 (MDAN-18)	1.29 (0.97–1.61)	4.75 (3.50–6.00)	3.7	8.9 (3.0)
6 (MDAN-19)	0.42 (0.37–0.47)	0.40 (0.33–0.47)	1.0	3.6 (1.7)
7 (MDAN-20)	0.17 (0.15–0.20)	0.17 (0.13–0.21)	1.0	0.4 (0.4)
8 (MDAN-21)	0.10 (0.09–0.11)	0.10 (0.09–0.11)	1.0	3.5 (1.7)
11 (MA-19)	0.04 (0.03–0.05)	0.22 (0.19–0.26)	5.5	83 (13)
11 and **15** (MA-19 and DN-20)	0.04 (0.02–0.05)	0.33 (0.28–0.37)	8.9	29 (8)

Saline or the indicated ligand were infused i.c.v. for 3 days via osmotic minipump. On day 4, naloxone (1 mg/kg, s.c.) was administered and the number of jumps was counted. The minipump was removed, and 4 h later, ligands were administered i.c.v. and antinociceptive ED_{50} values were determined.
*Ligand ED_{50}/saline ED_{50} ratio is an indicator of fold tolerance development.

Discussion

The history of the interaction between μ and δ opioid receptors spans a period of >25 years and, until recently, the nature of this interaction has remained elusive. Although converging lines of evidence from studies of coexpressed receptors (14–16) in cultured cells have provided evidence for physical interaction between μ and δ receptors arising from their association as heterodimers, there have been no conclusive reports establishing such association *in vivo*. A key obstacle to achieving this goal has been the unavailability of ligands that selectively recognize μ-δ heterodimers.

We have recently reported on ligands that selectively target δ-κ heterodimers that are localized in the spinal cord (29, 37). Here, we describe an extension of this approach for the design of bivalent ligands that target μ-δ heterodimeric opioid receptors in the brain. The principal features in this design were based on the use of μ and δ selective pharmacophores that are tethered to each other through a spacer. The pharmacophores were derived from the μ agonist, oxymorphone, and δ antagonist NTI. The rationale for using this combination of pharmacophores was based on the presumed physical interaction between μ and δ receptors that is characterized by the ability of NTI to block tolerance and physical dependence without seriously compromising antinociceptive activity (5).

A key feature of the present approach was to vary the spacer length of the bivalent ligand. We hypothesized that a transition in the behavioral pharmacology as a function of spacer length might reflect bridging of a μ-δ heterodimer by the bivalent ligand. In this connection, changes in tolerance and physical dependence as a function of spacer length could be a manifestation of bridging if the mechanisms leading to tolerance and dependence are mediated at the molecular level of the opioid receptors. For example, a bivalent ligand with a short spacer incapable of bridging μ and δ recognition sites in the same heterodimer would be expected to produce greater tolerance and/or dependence than a congener with a longer spacer capable of bridging.

Initial testing involved acute studies to define the relationship between the spacer length of bivalent ligands **3**–**8** (MDAN-16 to -21) and antinociceptive potency via i.c.v. administration (Table 1). Monovalent ligands **9**–**13** (MA-16 to -21), containing the oxymorphone pharmacophore with matched spacers, also were tested to determine whether a change in spacer length influences agonist potency. In this regard, no agonist potency change as a function of spacer length was observed, and members of the MA series were substantially more potent agonists relative to the corresponding members of the MDAN series. In view of the absence of any change in potency in the MA series, it appears that the 22-fold potency increase in ascending the MDAN series may be related to receptor-related interactions rather than to bioavailability factors. For the MDAN series, a combination of factors may contribute to the observed change in potency as a function of spacer length. These factors include suboptimal bridging of a heterodimer when the spacer is short (e.g., MDAN-16) and greater opportunity for multiple modes of bridging with longer spacers (e.g., MDAN-21).

One possible mechanism that explains why the monovalent ligands are more potent than the bivalent ligands is negative allosteric cooperativity. It has been postulated previously that associated opioid receptors may be allosterically coupled (38, 39). Through such a mechanism, NTI could negatively modulate the μ opioid receptor component of a μ-δ heterodimer, leading to lower potency of the bivalent ligand relative to the monovalent ligands. Therefore, we investigated the effect of the δ antagonist, NTI, on the acute agonist potency of MDAN-16 (**3**), MDAN-19 (**6**), and MDAN-21 (**8**). The rationale for this study was based on the idea that NTI would displace the bound MDAN δ antagonist pharmacophore when in the bridged state, thereby facilitating dissociation of the heterodimer into its monomers (or homomers). Such a change should be characterized by enhanced agonist potency of the bivalent ligand in the presence of NTI as a consequence of the dissociation of the heterodimer into monomers due to a change from a bridged to univalently bound MDAN ligand. This change would relieve the μ opioid receptor from the negative allosteric effect of the δ opioid receptor (Fig. 3).

The results of such experiments (Table 2) revealed that NTI enhanced the potency of MDAN-19, whereas no potency increase was observed with the corresponding monovalent ligand MA-19 in the presence of NTI. This difference could be explained by the aforementioned model given that the monovalent ligand does not have a δ antagonist pharmacophore to negatively modulate its activity. It can be noted that the agonist potency

Table 4. Comparison of i.v. to i.c.v. administration potencies for MDAN-21, MA-19, and morphine

Ligand	i.c.v. ED_{50}, nmol (95% C.I.)	i.v. ED_{50}, nmol (95% C.I.)	i.v./i.c.v. ratio
8 (MDAN-21)	0.08 (0.06–0.10)	3.3 (3.0–3.6)	41.3
11 (MA-19)	0.04 (0.03–0.05)	1.61 (1.29–1.92)	40.3
Morphine	4.1 (3.7–4.8)	168 (146–178)	41.0

PHARMACOLOGY

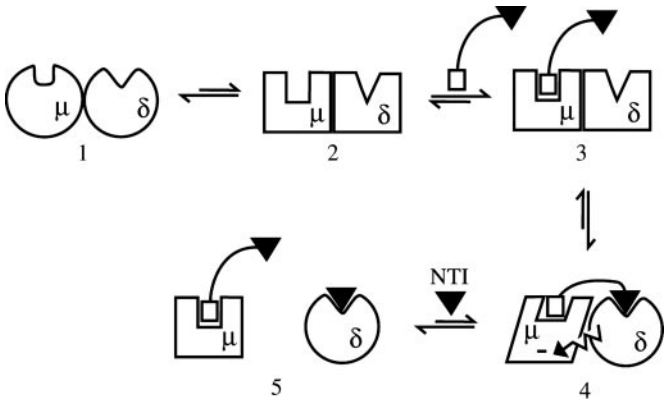


Fig. 3. Model for negative modulation of antinociception by bivalent ligands. The inactive (*1*) and active (*2*) states of the μ-δ heterodimer are in equilibrium with each other. Univalent binding of the μ agonist pharmacophore of the bivalent ligand to the μ opioid receptor recognition site leads to state *3*. Bridging with concomitant transformation of the δ receptor to the antagonist state negatively modulates the μ receptor (*4*). Displacement of the δ antagonist pharmacophore of the bridged bivalent ligand by NTI disrupts tethering of the μ and δ receptors, thereby facilitating dissociation (*5*). This model envisages the heterodimers to be either in an active or inactive state but not in a mixed state, except when the associated receptors are held together by the bridged bivalent ligand.

enhancement of MDAN-16 by NTI was less than that of MDAN-19 and that the potency of MDAN-21 under identical conditions was essentially unchanged (Table 2). One interpretation of these data is that the shorter spacer in MDAN-16 is not as efficient as MDAN-19 in bridging the heterodimer. The lack of a significant potency enhancement for bivalent ligand MDAN-21 in the presence of NTI may be due to the possibility that its longer spacer permits a greater number of bridging modes to associated μ and δ opioid receptors. It seems reasonable that many organizational arrangements exist for μ and δ opioid receptors; for example, heterodimers, homodimers, and oligomers have been suggested and/or described in refs. 14, 15, and 40. Therefore, additional binding options could include interdimer bridging between associated μ and δ opioid receptors that are not heterodimeric and, thus, not allosterically coupled. According to this model, NTI would not enhance the agonist effect of MDAN-21. Additional work is necessary to verify the validity of such a model.

Chronic studies of the MDAN series revealed that tolerance was a function of spacer length. Bivalent ligands with shorter spacers (MDAN-16, -17, and -18) exhibited tolerance that was comparable to that of the monovalent ligand MA-19 or morphine, but ligands with spacers containing 19 or more atoms (MDAN-19, -20, and -21) displayed no significant tolerance (Table 3). The absence of tolerance was most likely related to the tethering of the pharmacophores rather than to the presence of a mixture of univalently bound pharmacophores because a high degree of tolerance was produced by a mixture of monovalent ligands (coadministration of MA-19 and DN-20). The most likely explanation why the δ antagonist pharmacophore of DN-20 did not lead to a decrease in tolerance and dependence when coadminstered with MA-19 is as follows: If the receptors are organized as heterodimers, the "effective" concentration of the δ pharmacophore at the δ opioid receptor for the bivalent ligands (with spacers of optimal bridging length) should be significantly greater than when the two monovalents (MA-19 and DN-20) are coadministered. This phenomenon is due to the close proximity of the tethered pharmacophore to its associated receptor when the first pharmacophore of the bivalent ligand is in a univalently bound state. The distance requirement between the pharmacophores in the MDAN series for the blockage of tolerance strongly implicates the involvement of associated μ-δ opioid receptors in opioid-induced tolerance. This conclusion is also consistent with the report that suggests physical association between μ and δ opioid receptors may modulate μ receptor function (16).

The relationship between physical dependence and spacer length in the MDAN series, as evaluated by naloxone-induced jumping, showed a different profile from that of tolerance. In this regard, the bivalent ligand with the shortest spacer, MDAN-16, produced some dependence, whereas the remaining members of the series with longer spacers were essentially devoid of dependence. The divergent spacer length requirements for the blockage of tolerance and dependence suggest different bridging modes between μ and δ recognition sites and may reflect different underlying mechanisms for these processes. This finding is consistent with reports that suggest different mechanisms may mediate development and expression of opioid-induced tolerance and dependence (41–43), and, in one case, it has been reported that different second messenger systems may be involved in the two phenomena (42). More work is necessary to elucidate the mechanisms involved.

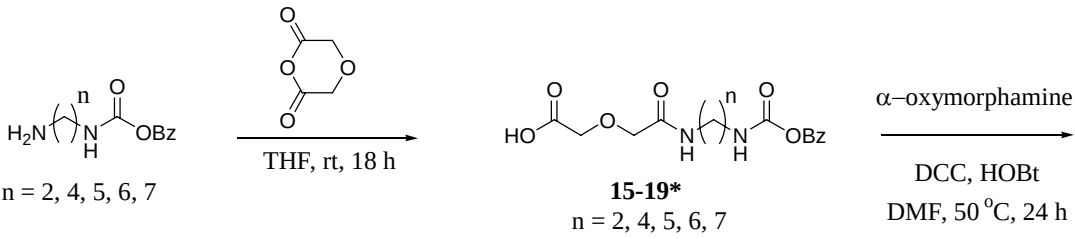
When the spacer length was longer than 22 Å (MDAN-19 to -21), neither tolerance nor dependence were observed, which is in harmony with the idea that these bivalent ligands bridge neighboring μ and δ opioid receptors effectively. But why does bridging of the μ-δ heterodimer suppress tolerance and dependence? A possible explanation is that the μ-δ heterodimer is the fundamental signaling unit that mediates tolerance and dependence through specific signal transducer(s) that recognize and couple to the heterodimer but not μ receptor monomers/homomers. The finding that δ receptor knockout mice do not become tolerant is consistent with this concept (10). Given our results, it appears reasonable that bridging μ-δ heterodimers by MDAN ligands would negatively modulate such putative transducers, thus reducing tolerance and dependence.

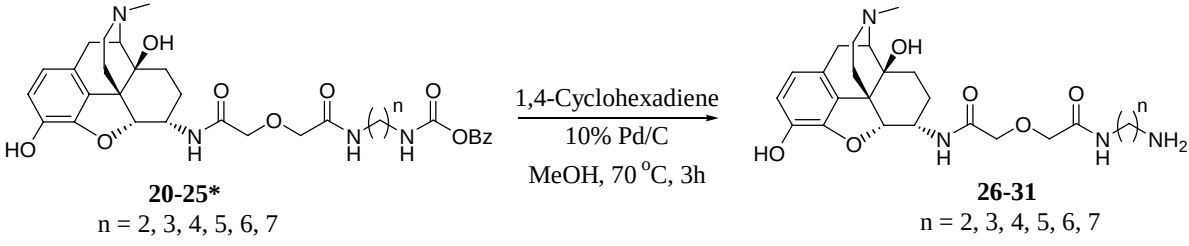
In view of the high opioid agonist potency of MDAN-21 and its absence of tolerance and physical dependence, we determined its i.v./i.c.v. potency ratio and compared it to that of morphine to estimate its relative ability to penetrate the brain. The data revealed that MDAN-21 was 50-fold more potent than morphine, whereas the i.v./i.c.v. potency ratio was identical (≈40, Table 4). These results suggest that the parenteral bioavailability of these compounds are similar. Given its much higher potency and its inability to produce tolerance and physical dependence in mice, MDAN-21 offers an approach for the development of potent, bioavailable analgesics devoid of these side effects.

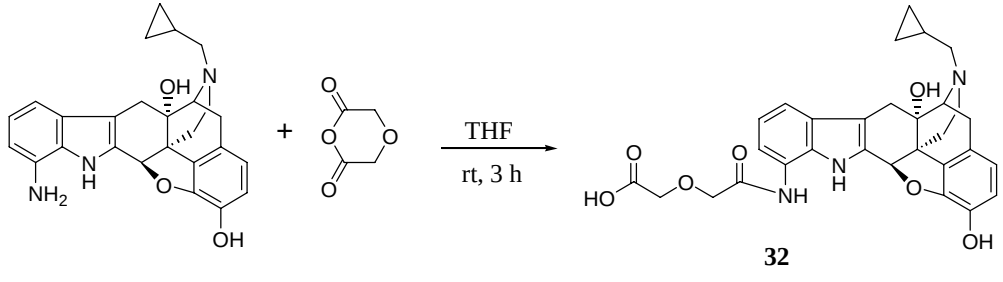
This project was supported by National Institutes of Health Research Grant DA015091 from the National Institute on Drug Abuse and Postdoctoral Fellowship DA18028 (to N.R.L.).

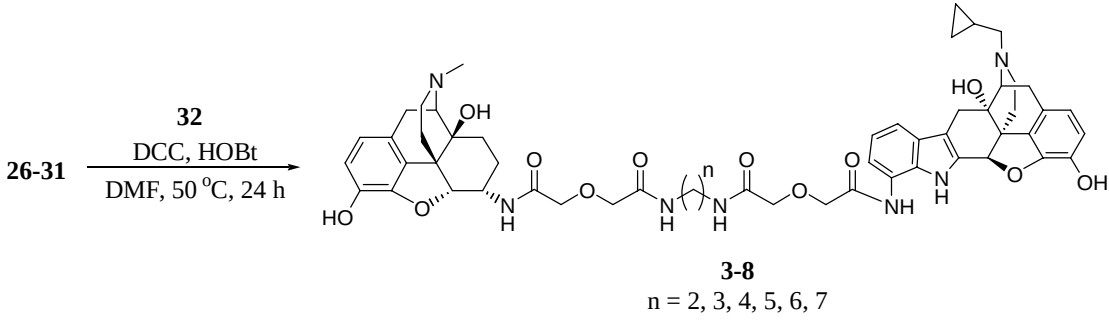
1. Vaught, J. & Takemori, A. (1979) *J. Pharmacol. Exp. Ther.* **208,** 86–94.
2. Vaught, J. L., Rothman, R. B. & Westfall, T. C. (1982) *Life Sci.* **30,** 1443–1455.
3. Heyman, J. S. & Porreca, F. (1989) *Prog. Opioid Res.* **75,** 467–470.
4. Miyamoto, Y., Portoghese, P. S. & Takemori, A. E. (1993) *J. Pharmacol. Exp. Ther.* **264,** 1141–1145.
5. Abdelhamid, E. E., Sultana, M., Portoghese, P. S. & Takemori, A. E. (1991) *J. Pharmacol. Exp. Ther.* **258,** 299–303.
6. Miyamoto, Y., Brown, W. D., Portoghese, P. S. & Takemori, A. E. (1994) *J. Pharmacol. Exp. Ther.* **270,** 37–39.
7. Fundytus, M. E., Schiller, P. W., Shapiro, M., Weltrowska, G. & Coderre, T. J. (1995) *Eur. J. Pharmacol.* **286,** 105–108.
8. Sánchez-Blázquez, P., García-España, A. & Garzón, J. (1997) *J. Pharmacol. Exp. Ther.* **280,** 1423–1431.
9. Kest, B., Lee, C. E., McLenmore, G. L. & Inturrisi, C. E. (1996) *Brain Res. Bull.* **39,** 185–189.

10. Zhu, Y., King, M. A., Schuller, A. G., Nitsche, J. F., Reidl, M., Elde, R. P., Unterwald, E., Pasternak, G. W. & Pintar, J. E. (1999) *Neuron* **24,** 243–252.
11. Schiller, P. W., Fundytus, M. E., Merovitz, L., Weltorski, G., Nguyen, T. M., Lemieux, C., Chung, N. N. & Coderre, T. (1999) *J. Med. Chem.* **42,** 3520–3526.
12. Wells, J. L., Bartlett, J. L., Ananthan, S. & Bilsky, E. J. (2001) *J. Pharmacol. Exp. Ther.* **297,** 597–605.
13. Ananthan, S., Kezar H. S., Carter, R. L., Saini, S. K., Rice, K. C., Wells, J. L., David, P., Xu, H., Dersch, C. M., Bilsky, E. J., *et al.* (1999) *J. Med. Chem.* **42,** 3527–3538.
14. George, S. R., Fan, T., Xie Z., Tse R., Tam, V., Varghese, G. & O'Dowd, B. F. (2000) *J. Biol. Chem.* **275**, 26128–26135.
15. Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, N., Nagy, V. & Devi, L. A. (2000) *J. Neurosci.* **20,** 1–5.
16. Gomes, I., Gupta, A., Filipovska, J., Szeto, H. H., Pintar, J. E. & Devi, L. A. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 5135–5139.
17. Erez, M., Takemori, A. E. & Portoghese, P. S. (1982) *J. Med. Chem.* **25,** 847–849.
18. Portoghese, P. S., Ronsisvalle, G., Larson, D. L., Yim, C. B., Sayre, L. M. & Takemori, A. E. (1982) *Life Sci.* **31,** 1283–1286.
19. Hazum, E., Chang, K.-J., Leighton, H. J., Lever, O. W., Cuatrecasas, P. (1982) *Biochem. Biophys. Res. Commun.* **104,** 347–353.
20. Portoghese, P. S., Larson, D. L., Yim, C. B., Sayre, L. M., Ronsisvalle, G., Lipkowski A. W., Takemori, A. E., Rice, K. C. & Tam, S. W. (1985) *J. Med. Chem.* **28,** 1140–1141.
21. Portoghese, P. S., Ronsisvalle, G., Larson, D. L. & Takemori, A. E. (1986) *J. Med. Chem.* **29,** 1650–1653.
22. Portoghese, P. S., Larson, D. L., Sayre, L. M., Yim, C. B., Ronsisvalle, G., Tam, S. W. & Takemori, A. E. (1986) *J. Med. Chem.* **29,** 1855–1861.
23. Portoghese, P. S., Larson, D. L., Ronsisvalle, G., Schiller, P. W., Nguyen, T. M., Lemieux, C. & Takemori, A. E. (1987) *J. Med. Chem.* **30,** 1991–1994.
24. Takemori, A. E., Yim, C. B., Larson, D. L. & Portoghese, P. S. (1990) *Eur. J. Pharmacol.* **186,** 285–288.
25. Shimohigashi, Y., Costa, T., Chen, H.-C. & Rodbard, D. (1982) *Nature* **297,** 333–335.
26. Costa, T., Shimohigashi, Y., Krumins, S. A., Munson, P. J. & Rodbard, D. (1982) *Life Sci.* **31,** 1625–1632.
27. Costa, T., Wuster, M., Herz, A., Shimohigashi, Y., Chen, H. C. & Rodbard, D. (1985) *Biochem. Pharmacol.* **34,** 25–30.
28. Sasaki-Yagi, Y., Kimura, S. & Imanishi, Y. (1991) *Int. J. Pept. Protein Res.* **38,** 378–384.
29. Bhushan, R. G., Sharma, S. K., Xie, Z., Daniels, D. J. & Portoghese, P. S. (2004) *J. Med. Chem.* **47,** 2969–2972.
30. Daniels, D. J., Kulkarni, A., Xie, Z., Bhushan, R. G. & Portoghese, P. S. (2005) *J. Med. Chem.* **48,** 1713–1716.
31. Haley, T. J. & McCormick, W. G. (1957) *Br. J. Pharmacol. Chemother.* **12,** 12–15.
32. D'Amour, F. E. & Smith, D. L. (1941) *J. Pharmacol. Exp. Ther.* **72,** 74–79.
33. Lenard, N. R. & Roerig, S. C., *Eur. J. Pharmacol.*, in press.
34. Way, E. L., Loh, H. H. & Shen, F. H. (1969) *J. Pharmacol. Exp. Ther.* **167,** 1–8.
35. Sayre, L. M. & Portoghese, P. S. (1980) *J. Med. Chem.* **45,** 3366–3368.
36. Portoghese, P. S., Sultana, M. & Takemori, A. E. (1990) *J. Med. Chem.* **33,** 1714–1720.
37. Waldhoer, M., Fonj, J., Jones, R. M., Lunzer, M. M., Sharma, S. K., Kostenis, E., Portoghese, P. S. & Whistler, J. L. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 9050–9055.
38. Portoghese, P. S. (2001) *J. Med. Chem.* **44,** 2259–2269.
39. Xie, Z., Bhushan, R. G., Daniels, D. J. & Portoghese, P. S. (2005) *Mol. Pharmacol.* **68,** 1079–1086.
40. Cvejic, S. & Devi, L. A. (1997) *J. Biol. Chem.* **272,** 26959–26964.
41. Taylor, D. A. & Fleming, W. W. (2001) *J. Pharmacol. Exp. Ther.* **297,** 11–18.
42. Aley, K. O. & Levine, J. D. (1997) *J. Neurosci.* **17,** 8018–8023.
43. Rubini, P., Schulz, R., Wuster, M. & Herz, A. (1982) *Naunyn Schmiedebergs Arch. Pharmacol.* **319,** 142–146.







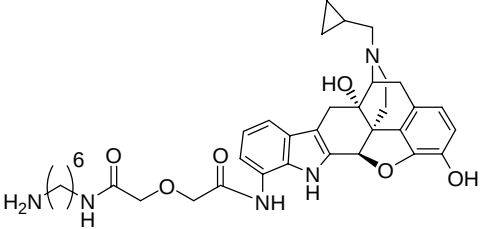


* Compounds **16** and **22** use the Boc protecting group instead of the Cbz protecting group

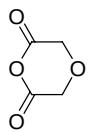
18

1. 7'-NH_2-NTI
DCC, HOBt
DMF, 50 °C, 24 h

2. 1,4-Cyclohexadiene
10% Pd/C
MeOH, 70 °C, 3h

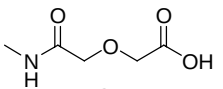


33



2M methyl amine / THF

THF, rt, 3 h

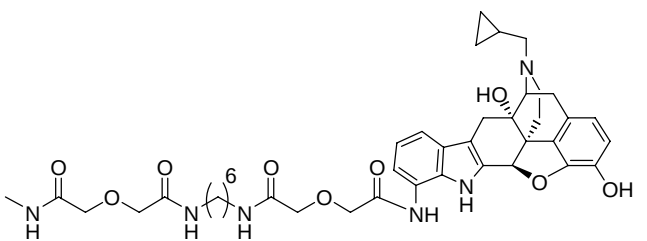


34

33

34

DCC, HOBt
DMF, 50 °C, 24 h



14