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Bivalent ligands that target mu opioid (MOP) and cannabinoid1 (CB_1) receptors are potent analgesics devoid of tolerance

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Abstract

Given that mu opioid (MOP) and canabinoid (CB_1) receptors are co-localized in various regions of the CNS and have been reported to associate as heteromer (MOP-CB_1) in cultured cells has raised the possibility of functional, endogenous MOP-CB_1 in nociception and other pharmacologic effects. As a first step in investigating this possibility, we have synthesized a series of bivalent ligands **1-5** that contain both mu agonist and CB_1 antagonist pharmacophores for use as tools to study the functional interaction between MOP and CB_1 receptors *in vivo*. Immunofluorescent studies on HEK293 cells co-expressing both receptors suggested **5** (20-atom spacer) to be the only member of the series that bridges the protomers of the heteromer. Antinociceptive testing in mice revealed **5** to be the most potent member of the series. As neither a mixture of monovalent ligands **9 + 10** nor bivalents **2-5** produced tolerance in mice, MOR-CB_1 apparently is not an important target for reducing tolerance.

Introduction

The pharmacologic interaction between opioid and cannabinoid CB_1 receptors has received considerable attention over the years. Both receptors are coupled to G_i/G_o G-protein and they are expressed in overlapping areas of the CNS.[1-5] Stimulation of either receptor with respective agonists inhibits adenylcyclase activity, blocks-voltage dependent calcium channels, activates potassium channels, and stimulates the MAP kinase cascade. Each receptor shares pharmacological properties that include analgesia, tolerance, and dependence upon chronic administration.[6-8] Due to similar distribution of both receptors in pain processing areas, their interaction can play an important role in antinociception.[9, 10, 11] For example, cross-tolerance between tetrahydrocanabinol and morphine, and the possibility that these receptors interact pharmacologically was demonstrated through antagonism of antinociception by naloxone or CB_1 antagonist.[12] Moreover, synergism between cannabinoids and opioids at sub-effective doses has been reported.[13-18] Additionally, co-administration of morphine with a CB_1 antagonist inhibited the development of both acute and chronic tolerance to morphine.[19] Other evidence for interaction was obtained from self-administration studies showing that both receptors are involved in reward processes. In this regard, both CB_1 antagonist **7** (**SR 141716**) [20] and opioid antagonist, naloxone, reduced self-administration of morphine or THC.[21, 22] Furthermore, studies on CB_1 knockout mice have shown that the dependence and reward properties were attenuated for morphine but not for

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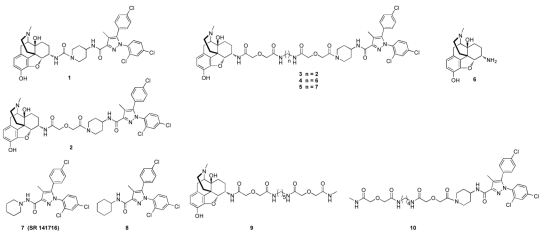
other drugs of abuse.[23] In mice lacking mu (MOP) and delta (DOP) opioid receptors, the cannabinoid withdrawal syndrome is reduced.[24]

Given that resonance energy transfer studies on co-expressing MOP and CB_1 receptors in cultured cells have suggested they are organized as heteromer,[25, 26] together with reports that these receptors are colocalized within neurons in rat nucleus accumbens[27] and dorsal horn,[28] raise the possibility that heteromeric MOP/CB_1 receptors may be involved in some of the pharmacological interactions that have been reported.

As our laboratory has developed a number of selective ligands for investigating heteromeric GPCRs[29-35], the present study was initiated to develop bivalent ligands as pharmacologic tools to study MOP-CB_1 heteromer. The bivalent ligands designed for this purpose consist of a selective mu receptor agonist connected to a CB_1 receptor-selective antagonist/inverse agonist *via* a spacer of varied length. Here we show *in vitro* evidence for bridging of protomers in a MOP-CB1 heteromer by a mu agonist/CB_1 antagonist bivalent ligands and their pharmacologic properties *in vivo*.

Design Rational and Chemistry

The design rationale for targeting neighboring protomers in a MOP-CB_1 heteromer was based on our studies with bivalent ligands that contain both agonist and antagonist pharmacophores for heteromeric opioid receptors.[34-36] The MOP agonist pharmacophore is derived from α-oxymorphamine[37] **6** which was employed previously for several bivalent series. In light of reports[38-39] that show a CB_1 antagonist is capable of eliminating morphine tolerance, dependence, and reinstatement, the CB_1 antagonist **7** was employed as a basis for the second pharmacophore in the bivalent ligand.[20]



In prior studies we have explored the relationship between spacer length and function of protomers in heteromers, with spacers ranging from 6 to 21 atoms. Given that our data is consistent with efficient bridging in the range of 18-22 atoms.[29-36, 40-43] For the present series, we have synthesized bivalent ligands that contain 2-, 6-, 15-, 19- and 20-atoms (**1**-**5**, respectively), with the expectation that **4** and/or **5** would be capable of bridging the protomers. Monovalent mu agonist **9** and CB_1 antagonist **10** served as controls in our studies. The bivalent ligands **1**-**5** consisted of a mu agonist tethered to a CB_1 antagonist pharmacophore that was modeled after **7** with a minor modification of the piperidine ring based upon reported SAR studies.[44]

The choice of **16** as a CB_1 antagonist pharmacophore and intermediate in the synthesis was based upon reported SAR studies of **7.**[44] This intermediate was prepared from **11**[45] and converted to **18** with diglycolic anhydride or activated with carbonyldiimidazole to afford **17** (Scheme 1). Intermediates **12**-**15** are described in the literature.[35] Intermediate **16** is a modified analog of **8** whose binding affinity and CB_2/CB_1 selectivity is comparable to SR141716 (**7**).

The synthesis of target compounds **1-5** and **10** is outlined in Scheme 2. Bivalent ligands **1** and **2** were prepared by reacting **6** with CB1 antagonist intermediates **17** and **18,** respectively. The intermediates (**19a**, **19b**, **19c**) for the synthesis of **3-5** were obtained by coupling **6** with the spacer components **15a**, **15b**, **15c** (Scheme 1), respectively. Condensation of **19a** with **18** afforded **3**. Intermediates **19b** and **19c** gave rise to **4** and **5**, respectively, *via* standard coupling procedure. The monovalent ligand **10** was prepared by coupling **13** (n = 4) with **18**. The monovalent ligand 9 was prepared previously.[46]

Biological studies

Immunofluorescence

Transiently co-expressing MOP and CB_1 receptors in HEK-293 cells were treated with immunofluorescent antibodies to image the receptors after treatment with monovalent (**9**, **10**) and bivalent (**2-5**) ligand. The present study was based on prior imaging studies that have revealed a lack of internalization of co-expressing MOP and DOP receptors in HEK-293 cells upon treatment with a MOP agonist/DOP antagonist bivalent ligand (MDAN-21) which is capable of bridging neighboring protomers. This was was in contrast to its homologue (MDAN-16) with a short spacer or a combination of monovalent MOP agonist/DOP antagonist ligands.[49] Thus, the internalization assay represents a valuable correlate for evaluation of agonist-antagonist bivalent ligands bridging GPCR heteromers.

Under basal conditions (no treatment) or in the presence of monovalent CB_1 antagonist **10**, MOP and CB_1 receptors were localized exclusively on the plasma membrane. (Fig. 2) However, treatment with either monovalent agonist **9**, a mixture of monovalent agonist and antagonist (**9**+**10**), or bivalent ligands (**2, 3**) with relatively short spacers, resulted in robust receptor clustering and endocytosis. A significant level of fluorescence also was observed in the cytosol when the combination of both monovalent ligands **9** and **10** were used and the internalization was similar to that observed for control ligand **9** alone. Treatment with the monovalent CB_1 antagonist alone produced no internalization. Ligand **4** did not produce as intense co-internalization as shorter bivalent ligands, especially at MOP receptor. Significantly, no co-internalization of mu and CB1 receptors was induced by the longest bivalent ligand **5** as both receptors remained located on the plasma membrane. These data are consistent with the idea that **5** with a 20 atom spacer bridges neighboring protomers. The specific number of atoms required for bridging for a particular bivalent series is dependent on both the constitution of the antagonist pharmacophore and the bound protomer.

Pharmacological evaluation

The *in vivo* profiles of bivalent ligands with 2-, 6-, 15-, 19- and 20-atom spacers (**1-5**) and monovalent ligands (**9**, **10**) were determined using the mouse tail-flick assay. The monovalent ligands were administered either singly or in combination. All compounds were evaluated for antinociception and tolerance after intracerebroventricular (i.c.v.) or intrathecal (i.t.) injection. These data are summarized in Table 1. For evaluation of tolerance, the $ED_{80–90}$ dose was measured on day one and compared to the same dose measured 24 hours later in the same mice. With the exception of the monovalent opioid agonist **9** and the bivalent ligand **1**, the bivalent compounds (**2-5**) and combination of monovalent ligands **9** and **10** were devoid of tolerance. Tolerance occurred only when the agonist **9** was applied alone[36] or when **1** was administered i.c.v.

The monovalent CB_1 antagonist **10,** like its parent compound **7** (SR141716),[44a] did not produce antinociception even at a relatively high dose (2.5 nmol/mouse). There appeared to be no significant difference in potency after acute co-administration of an equimolar dose ratio of **9** and antagonist **10** when compared to that of **9** alone. However, in contrast to **9**

alone, the co-administration with CB_1 antagonist did not produce 24 hour tolerance either by the i.c.v or i.t. routes (Table 1). This was consistent with the absence of tolerance of bivalent ligands **2-5**, presumably due to the presence of the CB_1 antagonist pharmacophore.

The i.c.v. antinociception SAR profile of bivalent ligands **1-5** was somewhat different from that obtained after i.t. administration. By the i.c.v. route, a mixture of **9**+**10** was significantly more potent than **1** or **4**, whereas this mixture was clearly less potent than **5**. When administered i.t., bivalents **2-5** were significantly more potent than **9**+**10**. Upon i.c.v. administration, **5** (20-atom spacer) exhibited a substantial 20-fold potency increase relative to its lower homologue **4** with a 19-atom spacer. By comparison, there was only a 4-fold increase by the i.t. route. Significantly, **5** not only is the most potent member of the series, but was still active (47%) 24 hours after i.c.v. admininistration. This long duration of action led us to postpone tolerance measurement to 48 hours as mentioned in Table 1.

Discussion

A key side effect of morphine and related mu agonists in the pharmacotherapy of chronic pain is the development of tolerance. Prior studies[12-18] have shown there is pharmacologic interaction between MOP and cannabinoid CB_1 receptors. In view of evidence for heteromeric MOP-CB_1 receptors in cultured cells, and the report that *i.t.* co-administration of morphine with a CB_1 antagonist prevents tolerance in rats, [19, 25, 26] we have investigated whether this effect is mediated *via* the physical association of MOP and CB_1 receptors or through other mechanisms.

Our approach to addressing this question involved the design and synthesis of bivalent ligands that contain mu opioid agonist and CB_1 antagonist pharmacophores. As we have reported that spacers in the range of 18-22 atoms can lead to bridging of protomers in a variety of heteromers containing opioid receptors in cultured cells,[32, 33, 49 , 50] such a bivalent ligand could be useful in selectively targeting a MOP-CB_1 heteromer to determine if opioid tolerance is affected.

Accordingly, we have synthesized a series of bivalent ligands containing both mu agonist and CB_1 antagonist pharmacophores that are connected by shorter spacers (**1-3**) or longer spacers (**4**, **5**). Control monovalent ligands **9** and **10** also were prepared. Bivalent ligands **1-3** would be expected to interact univalently with either MOP or CB_1 receptors, whereas **4** and **5** containing 19- and 20-atom spacers, respectively, have been reported to be in the bridging range of protomers in other heteromers. [29-36, 49, 50]

An immunocytochemical-derived correlate for establishing bridging of protomers by a bivalent ligand containing mu agonist and delta antagonist pharmacophores in MOR-DOR heteromer in HEK-293 cells has been employed.[49] In that particular study, it was determined in HEK293 cells that bivalent ligands with a 21- and 16-atom spacers (MDAN21 and MDAN16) exhibit different trafficking, in that receptors that are not bridged undergo agonist-induced endocytosis while those that are bridged remain on the cell surface.[48, 49] Thus, MDAN21 prevented internalization whereas MDAN16-treated cells exhibited endocytosis. Similar studies performed on HEK-293 cells co-expressing MOP and CB_1 receptors are consistent with the view that bridging of protomers takes place only with compound **5** (20-atom spacer), as no significant receptor internalization was observed. On the other hand, bivalent ligands with 6-, 15- and 19-atom spacers (**2**, **3**, and **4**, respectively) induced robust internalization, as did monovalent mu agonist **9**, or a mixture of **9** and monovalent CB_1 antagonist **10**. (Fig. 2)

The evaluation of antinociception and tolerance in mice using two routes of administration (i.c.v. and i.t.) revealed that bivalent ligands **2-5** and a mixture of monovalent mu agonist **9** and CB_1 antagonist **10** were devoid of tolerance. These data afforded results that are consistent with pharmacologic interaction between MOP and CB_1 receptors. It is noteworthy that bivalent ligand **1** with the shortest (2-atom) spacer possessed substantially lower potency with tolerance (Table 1) relative to other bivalent ligands **2-5** when administered i.c.v. Since **1** possesses a two-atom spacer, it is likely that the CB1 pharmacophore's antagonist activity has been compromised due to its proximity to the opioid pharmacophore. In the absence of CB1 antagonist activity, **1** would therefore produce tolerance. The reduced antinociception is likely due to steric hindrance of the opioid pharmacophore at the MOP receptor by the CB1 pharmacophore.

The finding that bivalent ligands **2-5**, whose spacers range from 6 to 20 atoms, and a mixture of monovalents (**9** + **10**) are all devoid of tolerance suggests that the ability of CB_1 antagonist to reduce or prevent opioid-induced tolerance is not necessarily mediated via a MOP-CB_1 heteromer. On the other hand, because the most potent bivalent ligand **5** is capable of bridging bridging such a heteromer in HEK293 cells, it cannot be excluded as a target by **5**.

It is perhaps significant that **5** is substantially more potent than its lower homologue **4** by a factor of 20 when given supraspinally. On the other hand, **5** is more potent than **4** only by a factor of 4 following the i.t. route. The larger potency difference i.c.v. suggests that there may be more MOP-CB_1 heteromer supraspinally. This would be consistent with our observation that the day 2 tolerance test could not be conducted because of residual antinociception (~47%). The possible supraspinal localization of MOP-CB_1 heteromer is a departure from MOP-DOP[36] and DOP-KOP[30, 51] heteromers that appear to be localized in the cord.

In conclusion, we have obtained immunofluorescence evidence in HEK293 cells that support the bridging of MOP and CB_1 protomers in a heteromer by bivalent ligand **5** that contains a mu agonist linked to a CB_1 antagonist via a 20-atom spacer. Lower homologues with 19 or fewer atoms connecting the pharmacophores do not share this property. Bivalent ligand **5** possessed the highest antinociceptive potency when administered either i.c.v. or i.t.. It was found that **5** and lower homologue bivalent ligands **2-4** that do not bridge MOP-CB_1 heteromer, as well as a mixture of monovalent ligands (mu agonist **9** and CB_1 antagonist **10**) all were free of opioid tolerance. Thus, it appears that MOP-CB_1 heteromer may not be an important exclusive target for inhibition of opioid tolerance by co-administration. These results further support the synergestic effects between the opioid and cannabinoid systems and the possible benefit of using a CB_1 antagonist component to oppose the development of tolerance induced by opioids.

Experimental section

All commercial reagents and anhydrous solvents that were purchased from vendors were used without further purification or distillation. Analytical thin-layer chromatography (TLC) was performed on EM Science silica gel 60 F254 (0.25 mm). Plates were visualized by UV light, iodine vapor, or ninhydrin solution. Flash column chromatography was performed on Fisher Scientific silica gel (230 – 400 mesh). Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. 1H NMR spectra and 13C NMR spectra were taken on a Bruker Avance 400 MHz instruments and calibrated using an internal reference. Chemical shifts are expressed in ppm and coupling constants (*J*) are in hertz (Hz). Peak multiplicities are abbreviated: broad, br; singlet, s; doublet, d; triplet, t; and multiplet, m. ESI mode mass spectra were recorded on a BrukerBioTOF II mass

spectrometer. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Analytical data confirmed the purity of the products was ≥ 95%.

General procedure for DCC/HOBt coupling

DCC (37 mg, 0.18 mmol), carboxylic acid **16** (70 mg, 0.12 mmol), and HOBt (22 mg, 0.16 mmol) were dissolved in 5 mL of anhydrous DMF. The solution was cooled to 0°C and placed under a nitrogen atmosphere. After 15 minutes at 0°C, amine (0.11 mmol) was added. The solution was sealed under a nitrogen atmosphere and was allowed to warm and to stir at room temperature overnight. The reaction mixture was filtered in order to remove dicyclohexylurea. The filtrate was poured into water (10x initial volume of DMF) and extracted with ethyl acetate. The combined organic layers were dried on magnesium sulfate, filtered and concentrated under reduced pressure. The residue was then purified by flash chromatography. The title compound was then subsequently converted into the HCl salt for biological testing.

4-(5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamido)-*N*-((4*α*S,7S,7*α*R,12*β*S)-4*α*,9-dihydroxy-3-methyl-2,3,4,4*α*,5,6,7,7*α*-octahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl)piperidine-1-carboxamide; (1)

Combining amine **6** (85 mg, 0.28 mmol), compound **17** (172 mg, 0.30 mmol) and triethylamine (48 μL, 0.34 mmol) in DCM gave the target compound which was purified by flash chromatography [MeOH/DCM/NH_4OH=3/96/1] to provide **1** as a white solid. Yield: 188 mg, 85%.

1H NMR (CD_3OD) δ 0.97 (m, 1H) ; 1.34-1.48 (m, 4H) ; 1.68-1.80 (m, 3H) ; 1.94 (m, 1H) ; 2.03 (m, 2H) ; 2.14 (s, 3H) ; 2.59 (m, 2H) ; 2.85 (s, 3H) ; 3.07-3.13 (m, 2H) ; 3.16-3.22 (m, 2H) ; 3.13 (D, 1H, *J*= 8.9 Hz) ; 3.23 (m, 1H) ; 3.83 (m, 1H) ; 4.14 (m, 1H) ; 4.35 (m, 1H) ; 4.49 (m, 1H) ; 4.64 (m, 1H) ; 6.64 (D, 1H, *J*= 8.2 Hz) ; 6.73 (D, 1H, *J*= 8.2 Hz) ; 7.21 (D, 2H, *J*= 8.5 Hz) ; 7.38 (D, 2H, J= 8.4 Hz) ; 7.45 (m, 2H) ; 7.61 (d, 1H, *J*= 2.2 Hz) ; 13C NMR δ 8.84; 22.02; 30.62; 30.75; 33.57; 42.08; 46.50; 47.19; 48.27; 55.83; 61.54; 64.27; 68.16; 71.05; 82.50; 90.38; 117.08; 119.36; 120.66; 123.34; 126.93; 128.37; 128.57; 129.30; 129.97 (2x); 130.12; 131.18; 132.29 (2x); 132.50; 134.34; 136.27; 137.35; 140.41; 143.86; 147.32; 147.78; 159.57; 165.25. Anal. Calcd. for $C_{40}H_{41}Cl_3N_6O_7$: C, 60.65 ; H, 5.22 ; N, 10.61. Found : C, 60.78 ; H, 5.23 ; N, 10.57. ESI-TOF MS calculated for $C_{40}H_{41}Cl_3N_6O_5$, *m/z* 792.212, found 793.272 $(MH)^+$

5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-*N*-(1-(2-(2-(((4*α*S,7S,7*α*R,12*β*S)-4*α*,9-dihydroxy-3-methyl-2,3,4,4*α*,5,6,7,7*α*-octahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl)amino)-2-oxoethoxy)acetyl)piperidin-4-yl)-4-methyl-1*H*-pyrazole-3-carboxamide; (2)

Compound **2** was prepared in a similar fashion as described above; combining *α*-oxymorphamine (**6**) (33 mg, 0.11 mmol), acid **18** (70 mg, 0.12 mmol) ; DCC (37 mg, 0.18 mmol), and HOBt (22 mg, 0.16 mmol) gave the target compound which was purified by flash chromatography [MeOH/DCM/NH_4OH=2/97/1] to provide **2** as a white solid. Yield: 74 mg, 78%.

1H NMR (CD_3OD) δ 1.01 (m, 1H) ; 1.25-1.36 (m, 4H) ; 1.41-1.57 (m, 4H) ; 1.80 (m, 1H) ; 2.01 (m, 2H) ; 2.17 (s, 3H) ; 2.25 (m, 2H) ; 2.35 (s, 3H) ; 2.43 (m, 1H) ; 2.64 (m, 1H) ; 2,81 (m, 1H) ; 2.97 (m, 1H) ; 3.13 (D, 1H, *J*= 8.9 Hz) ; 3.23 (m, 1H) ; 3.98-4.09 (m, 4H) ; 4.26 (m, 1H) ; 4.63 (m, 1H) ; 4.70 (m, 1H, CONH) ; 6.66 (D, 1H, *J*= 8.2 Hz) ; 6.71 (D, 1H, *J*= 8.2 Hz) ; 7.07 (D, 2H, *J*= 8.5 Hz) ; 7.11 (D, 1H, *J*= 8.4 Hz) ; 7.25 (Dd, 1H, *J*= 8.4 Hz, *J*= 2.2 Hz) ; 7.32 (D, 2H, *J*= 8.6 Hz) ; 7.45 (d, 1H, *J*= 2.2 Hz) ; 7.67 (m, 1H, CONH) ; Anal. Calcd. for $C_{43}H_{45}Cl_3N_6O_7$: C, 59.76 ; H, 5.25 ; N, 9.72. Found : C, 59.88 ; H, 5.13 ; N, 9.77. ESI-TOF MS calculated for $C_{43}H_{45}Cl_3N_6O_7$, *m/z* 864.212, Found 865.372 $(MH)^+$

5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-*N*-(1-(14-(((4*α*S,7S,7*α*R,12*β*S)-4*α*,9-dihydroxy-3-methyl-2,3,4,4*α*,5,6,7,7*α*-octahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl)amino)-5,10,14-trioxo-3,12-dioxa-6,9-diazatetradecan-1-oyl)piperidin-4-yl)-4-methyl-1*H*-pyrazole-3-carboxamide; (3)

Compound **3** was prepared in a similar fashion as described above; combining amine **19a** (51 mg, 0.11 mmol), acid **18** (70 mg, 0.12 mmol) ; DCC (37 mg, 0.18 mmol), and HOBt (22 mg, 0.16 mmol) gave the target compound which was purified by flash chromatography [MeOH/DCM/NH_4OH=2/97/1] to provide **3** as a white solid. Yield: 73 mg, 65%.

1H NMR (CD_3OD) δ 0.97 (m, 1H) ; 1.25-1.36 (m, 5H) ; 1.41-1.57 (m, 4H) ; 1.80 (m, 1H) ; 2.01-2.04 (m, 2H) ; 2.16 (s, 3H) ; 2.25-2.29 (m, 2H) ; 2.35 (s, 3H) ; 2.43 (m, 1H) ; 2.64 (m, 1H) ; 2,81 (m, 1H) ; 2.97 (m, 1H) ; 3.11 (D, 1H, *J*= 8.9 Hz) ; 3.14-3.28 (m, 4H) ; 4.00-4.08 (m, 8H) ; 4.24 (m, 1H) ; 4.59 (m, 1H) ; 6.54 (D, 1H, *J*= 8.2 Hz) ; 6.73 (D, 1H, *J*= 8.2 Hz) ; 7.04 (D, 2H, *J*= 8.5 Hz) ; 7.14 (D, 1H, *J*= 8.4 Hz) ; 7.21 (Dd, 1H, *J*= 8.4 Hz, J= 2.2 Hz) ; 7.35 (D, 2H, *J*= 8.6 Hz) ; 7.41 (d, 1H, *J*= 2.2 Hz) ; 7.86 (m, 1H, CONH) ; Anal. Calcd. for $C_{49}H_{55}Cl_3N_8O_{10}$: C, 57.56 ; H, 5.42 ; N, 10.96. Found : C, 57.72 ; H, 5.43 ; N, 10.87. ESI-TOF MS calculated for $C_{49}H_{55}Cl_3N_8O_{10}$, *m/z* 1022.368, found 1023.276 $(MH)^+$

5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-*N*-(1-(18-(((4*α*S,7S,7*α*R,12*β*S)-4*α*,9-dihydroxy-3-methyl-2,3,4,4*α*,5,6,7,7*α*-octahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl)amino)-5,14,18-trioxo-3,16-dioxa-6,13-diazaoctadecan-1-oyl)piperidin-4-yl)-4-methyl-1*H*-pyrazole-3-carboxamide; (4)

Compound **4** was prepared in a similar fashion as described above; combining amine **19b** (57 mg, 0.11 mmol), acid **18** (70 mg, 0.12 mmol) ; DCC (37 mg, 0.18 mmol), and HOBt (22 mg, 0.16 mmol) gave the target compound which was purified by flash chromatography [MeOH/DCM/NH_4OH=2/97/1] to provide **4** as a white solid. Yield: 67 mg, 57%.

1H NMR (CD_3OD) δ 0.88 (m, 1H) ; 1.24-1.34 (m, 9H) ; 1.40-1.59 (m, 8H) ; 1.79 (m, 1H) ; 1.99-2.02 (m, 2H) ; 2.16 (s, 3H) ; 2.28-2.24 (m, 2H) ; 2.36 (s, 3H) ; 2.45 (m, 1H) ; 2.61 (m , 1H) ; 2.86-2.91 (m, 2H) ; 3.09 (D, 1H, *J*= 8.8 Hz) ; 3.14-3.18 (m, 4H) ; 3.99-4.07 (m, 8H) ; 4.20 (m, 1H) ; 4.25 (m, 1H, CONH) ; 4.56 (m, 1H) ; 4.60 (m, 1H, CONH) ; 6.53 (D, 1H, *J*= 8.2 Hz) ; 6.72 (D, 1H, *J*= 8.2 Hz) ; 7.04 (D, 2H, *J*= 8.5 Hz) ; 7.15 (D, 1H, *J*= 8.4 Hz) ; 7.25 (Dd, 1H, *J*= 8.4 Hz, *J*= 2.2 Hz) ; 7.29 (D, 2H, *J*= 8.6 Hz) ; 7.44 (d, 1H, *J*= 2.2 Hz) ; 7.49 (m, 1H, CONH) ; Anal. Calcd. for $C_{43}H_{45}Cl_3N_6O_7$: C, 59.02 ; H, 5.89 ; N, 10.39. Found : C, 59.18 ; H, 5.73 ; N, 10.24. ESI-TOF MS calculated for $C_{53}H_{63}Cl_3N_8O_{10}$, *m/z* 1078.474, found 540.171 $(M/2H)^+$, 1079.366 $(MH)^+$

5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-*N*-(1-(19-(((4*α*S,7S,7*α*R,12*β*S)-4*α*,9-dihydroxy-3-methyl-2,3,4,4*α*,5,6,7,7*α*-octahydro-1*H*-4,12-methanobenzofuro[3,2-e]isoquinolin-7-yl)amino)-5,15,19-trioxo-3,17-dioxa-6,14-diazanonadecan-1-oyl)piperidin-4-yl)-4-methyl-1*H*-pyrazole-3-carboxamide; (5)

Compound **5** was prepared in a similar fashion as described above; combining amine **19c** (65 mg, 0.12 mmol), acid **18** (77 mg, 0.13 mmol) ; DCC (41 mg, 0.20 mmol), and HOBt (23 mg, 0.17 mmol) gave the target compound which was purified by flash chromatography [MeOH/DCM/NH_4OH=7/92/1] to provide **5** as a clear oil. Yield: 28 mg, 22%.

1H NMR (CD_3OD) δ 0.92 (m, 1H) ; 1.28-1.43 (m, 11H) ; 1.47-1.62 (m, 8H) ; 1.83 (m, 1H) ; 2.07-2.17 (m, 2H) ; 2.18 (s, 3H) ; 2.39-2.42 (m, 2H) ; 2.46 (s, 3H) ; 2.51 (m, 1H) ; 2.57 (m , 1H) ; 2.89-2.95 (m, 2H) ; 3.11 (D, 1H, *J*= 8.8 Hz) ; 3.15-3.21 (m, 4H) ; 4.01-4.10 (m, 8H) ; 4.23 (m, 1H) ; 4.63 (m, 1H) ; 4.71 (m, 1H, CONH) ; 6.51 (D, 1H, *J*= 8.2 Hz) ; 6.69 (D, 1H, *J*= 8.2 Hz) ; 7.04 (D, 2H, *J*= 8.5 Hz) ; 7.13 (D, 1H, *J*= 8.4 Hz) ; 7.19 (Dd, 1H, *J*= 8.4 Hz, *J*= 2.2 Hz) ; 7.23 (D, 2H, *J*= 8.6 Hz) ; 7.41 (d, 1H, *J*= 2.2 Hz) ; 7.57 (m, 1H, CONH) ; Anal.

Calcd. for $C_{54}H_{65}Cl_3N_8O_{10}$: C, 59.37 ; H, 6.00 ; N, 10.26. Found : C, 59.51 ; H, 5.97 ; N, 10.31. ESI-TOF MS calculated for $C_{54}H_{65}Cl_3N_8O_{10}$, *m/z* 1092.501, found 1093.671

5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1-(3,7,14-trioxo-5,16-dioxa-2,8,13-triazaoctadecan-18-oyl)piperidin-4-yl)-1*H*-pyrazole-3-carboxamide; (10)

Compound **10** was prepared in a similar fashion as described above; combining amine **13 (n = 4)** (24 mg, 0.11 mmol), acid **18** (70 mg, 0.12 mmol) ; DCC (37 mg, 0.18 mmol), and HOBt (22 mg, 0.16 mmol) gave the target compound which was purified by flash chromatography [MeOH/DCM= 1/99] to provide **10** as a white solid. Yield. 36 mg, 42%.

1H NMR (CD_3OD) δ 1.51-1.55 (m, 7H) ; 1.98 (m, 2H) ; 2,17 (s, 3H) ; 2.79 (s, 3H) ; 2.96 (m, 1H) ; 3.10 (m, 2H) ; 3.24-3.30 (m, 4H) ; 4.00-4.02 (m, 8H) ; 4.13 (m, 1H), 4.36 (d, 1H, *J*= 7.3 Hz) ; 4.70-4.73 (m, 2H, NH) ; 7.06 (D, 2H, *J*= 8.5 Hz) ; 7.16 (D, 1H, *J*= 8.4 Hz) ; 7.25 (Dd, 1H, *J*= 8.4 Hz, *J*= 2.2 Hz) ; 7.34 (D, 2H, *J*= 8.6 Hz) ; 7.45 (d, 1H, *J*= 2.2 Hz) ; Anal. Calcd. for $C_{35}H_{42}Cl_3N_7O_7$: C, 53.96 ; H, 5.43 ; N, 12.58. Found : C, 53.88 ; H, 5.52 ; N, 12.67. ESI-TOF MS calculated for $C_{35}H_{42}Cl_3N_7O_7$, *m/z* 779.110, Found 780.284 $(MH)^+$

5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-4-yl)-1*H*-pyrazole-3-carboxamide (16)

A mixture of the carboxylic acid **11** (1.14 g, 3 mmol) and thionyl chloride (0.8 mL, 11 mmol) in 20 mL of toluene was refluxed for 3 h and then evaporated to dryness under reduced pressure. The residue was taken up in 20 mL of toluene and the solvent was evaporated again under reduced pressure to give the crude acid chloride, which was dissolved in 15 mL of dry dichloromethane. This solution was added drop wise to a solution of *N*-Boc-4-aminopiperidine (901 mg, 4.5 mmol) and triethylamine (620 μL, 4.5 mmol) in 10 mL of dichloromethane, cooled to 0°C. After stirring at room temperature for 16 h, the reaction mixture was added to brine (30 mL) and extracted with dichloromethane. The organic layer was separated, dried (magnesium sulphate) and evaporated under reduced pressure. The residue was then taken up in DCM and TFA was added (20% volume). The mixture was stirred 16 h at room temperature. The solvent was evaporated under reduced pressure and the residue was co-evaporated with toluene (2X). The crude mixture was then purified by flash chromatography (EA) to afford compound **16** as white foam. Yield: 1.14 g, 82% (overall).

1H NMR ($CDCl_3$) δ : 1.41 (m, 2H) ; 1. 52 (m, 2H) ; 1.93 (m, 2H) ; 2.18 (s, 3H) ; 2.95 (m, 2H) ; 3.20 (m, 1H) ; 4.29 (d, 1H, NH, *J*= 7.4 Hz) ; 4.65 (d, 1H, NH, *J*= 7.2 Hz) ; 7.07 (d, 2H, *J*= 8.5 Hz) ; 7.15 (d, 1H, *J*= 8.4 Hz) ; 7.23 (dd, 1H, *J*= 8.4 Hz, *J*= 2.2 Hz) ; 7.39 (D, 2H, *J*= 8.6 Hz) ; 7.45 (d, 1H, *J*= 2.2 Hz) ; 13C NMR ($CDCl_3$) δ 163.1; 143.0; 138.5; 136.0; 135.2; 135.1; 133.1; 130.9 (x2); 130.1; 129.9; 128.7 (x2); 127.8; 12 7.2; 118.2; 56.2; 31.8 (x2); 25.8; 23.5; 8.7. mp 162-163°C (decomposition); ESI-TOF MS calculated for $C_{22}H_{21}Cl_3N_4O$, *m/z* 463.787, found 464.705 $(MH)^+$

N-(1-(1*H*-Imidazole-1-carbonyl)piperidin-4-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (17)

To a solution of piperidine **16** (200 mg, 0.43 mmol) dissolved in DCM with triethylamine (90 μL, 0.64 mmol), carbonyldiimidazole was added dropwise (83 mg, 0.52 mmol) at room temperature. The mixture was stirred overnight and was concentrated to dryness. The residue was purified by flash chromatography [MeOH/DCM=2/98] to provide **17** as a white solid. Yield: 209 mg, 87%.

1H NMR ($CDCl_3$) 1.80–1.87 (m, 2H) ; 2.25-2.29 (m, 5H with s, 3H at 2.29) ; 3.10 (t, 1H, *J*= 11.9 Hz) ; 3.41 (t, 1H, *J*= 12.2 Hz) ; 4.30 (m, 1H) ; 4.50 (d, 1H, *J*= 13.4 Hz) ; 4.95 (d, 1H,

J= 13.3 Hz) ; 7.04 (D, 2H, *J*= 8.5 Hz) ; 7.15 (D, 1H, *J*= 8.4 Hz) ; 7.18 (s, 1H) ; 7.25 (Dd, 1H, *J*= 8.4 Hz) ; 7.18 (s, 1H) ; 7.25 (Dd, 1H, *J*= 8.4 Hz, J= 2.2 Hz) ; 7.29 (D, 2H, *J*= 8.6 Hz) ;7.52 (s, 1H) ; 8.49 (s, 1H) ; 8.77 (d, 1H, *J*= 2.3 Hz) ; 13C NMR ($CDCl_3$) δ 8.89 ; 31.50. 32.76 ; 41.53 ; 46.52 ; 48.73 ; 115.97 ; 116.72 ; 121.90 (2x) ; 126.96 ; 127.90 ; 128.99 (2x) ; 129.33 ; 129.57 ; 130.60 (2x) ; 132.91 ; 135.69 ; 135.89 ; 136.32 ; 142.12 ; 146.39 ; 148.81 ; 163.78. mp 189-191°C. ESI-TOF MS calculated for $C_{26}H_{23}Cl_3N_6O_2$, *m/z* 557.859, Found 558.913 $(MH)^+$

2-(2-(4-(5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamido)piperidin-1-yl)-2-oxoethoxy)acetic acid (18)

To a solution of piperidine **16** (278 mg, 0.6 mmol) dissolved in DCM with pyridine (60 mL, 0.72 mmol), diglycolic anhydride (70 mg, 0.6 mmol) was added dropwise. The mixture was stirred at room temperature overnight. After disappearance of starting material, the solvent was concentrated to dryness; the residue was co-evaporated with toluene (3X) to afford **18** as a white solid. Yield: 347 mg, quantitative. Compound **18** was used without further purification.

1H NMR ($CDCl_3$) δ : 1.59 (m, 2H) ; 2.03 (m, 2H) ; 2.18 (s, 3H) ; 3.06 (m, 1H) ; 3.32 (m, 1H) ; 4.07-4.24 (m, 5H) ; 4.33 (m, 1H) ; 4.65 (m, 1H) ; 7.07 (D, 2H, *J*= 8.4 Hz) ; 7.16 (D, 1H, *J*= 8.3 Hz) ; 7.24 (Dd, 1H, *J*= 8.4 Hz, *J*= 2.2 Hz) ; 7.29 (D, 2H, *J*= 8.6 Hz) ; 7.43 (d, 1H, *J*= 2.2 Hz) ; ^{13}C NMR ($CDCl_3$) δ 171.9; 169.5; 163.7; 143.0; 138.8; 136.7; 136.6; 135.3; 133.7; 130.6 (x2); 130.5; 130.3; 128.9 (x2); 127.8; 127.1; 118.2; 72.3; 68.4; 57.2; 46.28 (x2); 32.9; 31.3; 8.9; mp 227-228°C; ESI-TOF MS calculated for $C_{26}H_{25}Cl_3N_4O_5$, *m/z* 579.860, found 578.673$(MH)^-$

Biology

Immunofluorescence protocol

To determine the effects of the MOP-CB_1 bivalent ligands on the trafficking of MOP and CB_1 receptors, we utilized the immunofluorescence technique[49] in HEK-293 cells transiently expressing the receptors. HEK-293 cells were transfected with cDNA for hMOPR and hCB_1 (16 μg/ 2 million cells) and seeded 24 hours later into 8-well chamber slides. After allowing for a further 24 hours for the cells to settle and equilibrate, they were incubated with the different ligands (1 μM) for 30 minutes. The cells were then washed, fixed with 4% paraformaldehyde and incubated with the mu (Neuromics Inc.) and CB_1 selective primary antibodies (R&D Systems) overnight at 4°C. The cells were then washed the next day and stained with respective fluorescent secondary antibodies (red for MOP, green for CB_1). Images were acquired using a Olympus BX10 confocal microscope and analyzed using ImageJ software (NIH).

Animals

Male ICR-CD1 mice (17 – 25g; Harlan, Madison, WI), are housed in groups of 8 in a temperature- and humidity-controlled environment with unlimited access to food and water. They are maintained on a 12 h light/dark cycle. All experiments are approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Minneapolis, MN).

Antinociceptive Testing

The tail flick assay described by D'Amour and Smith[52] and modified by Dewey *et. al*. was used to test for antinociception.[53] For the measurement of the tail-flick latency, mice are held gently in one hand with the tail positioned in the apparatus (Tail Flick Analgesia Meter, Columbus Instruments, Columbus, Ohio) for radiant heat stimulus. The tail-flick response is

elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat is set so that the mouse flicks its tail within 2 to 3 s. The test latency is measured before drug treatment (control) and again after the drug treatment (test) at the peak time of the compound, a 10 s maximum cut-off time is used to prevent damage to the tail. Antinociception is quantified according to the method of Harris and Pierson[54] as the percent maximal possible effect (%MPE) which is calculated as: %MPE = (Test - Control/10 – Control) × 100. At least three groups of eight to ten mice were used for each dose response curve, and each mouse is used only once. ED_{50} values with 95% confidence intervals (C.I.) are computed with GraphPad Prism 4 by using nonlinear regression methods.

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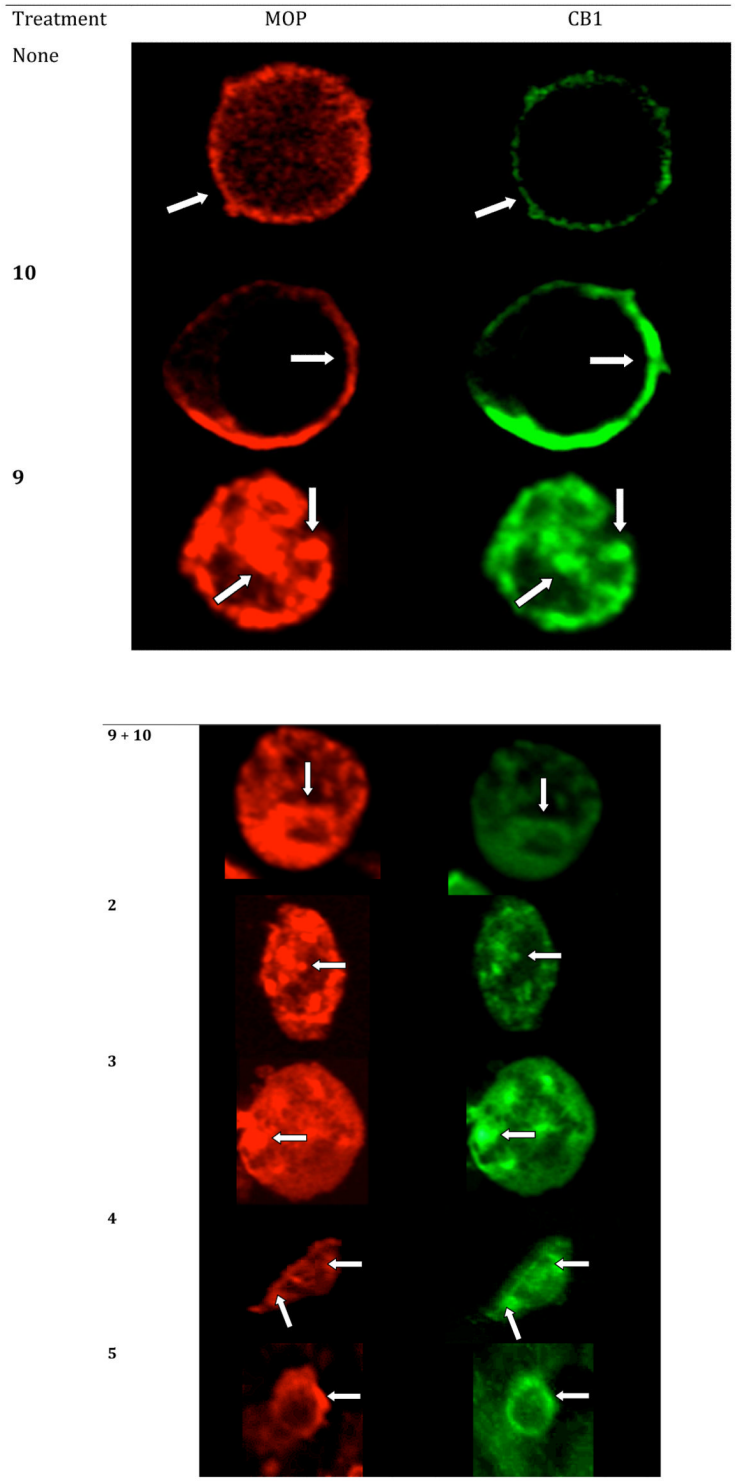
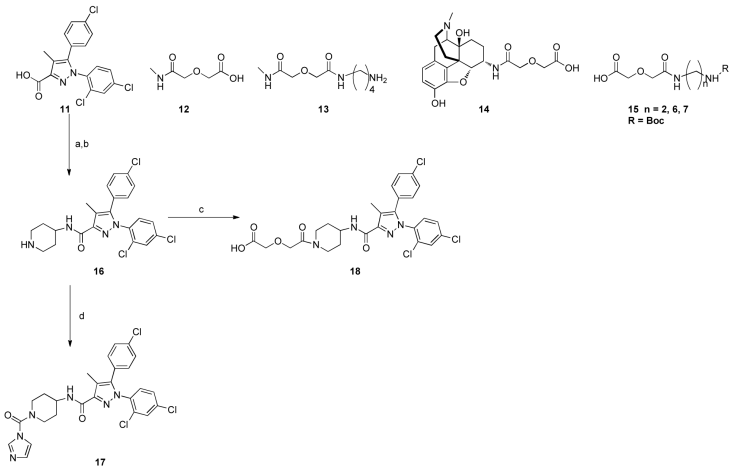


Figure 2.

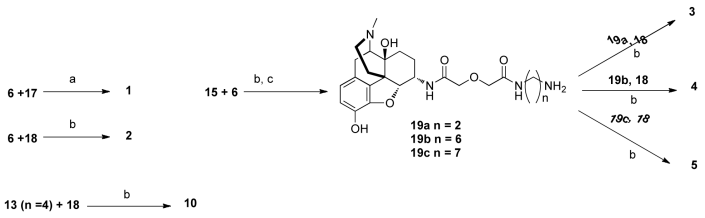
Single cell high-magnification images of double-labeling immunofluorescence for co-expressed MOP and CB_1 receptors on HEK-293 cells. The identical cells are shown labeled for MOP (red fluorescence) and CB_1 (green fluorescence) receptors. Arrows depict profiles double-labeled for MOP and CB1.



a) i. $SOCl_2$; ii. 4-Amino-1-Boc-piperidine, triethylamine; b) TFA; c) Diglycolic anhydride; d) Carbonyldiimidazole, triethylamine.

Scheme 1.

Intermediates for synthesis of bivalent ligands **1-5** and monovalent ligands **9** and **10**



a) Triethylamine; b) DCC/HOBt. c) TFA

Scheme 2.
Synthesis of target compounds (**1-5**, **10**)

Table 1

Antinociception and 24 hour tolerance[a] evaluation after i.c.v. and i.t. administration of compounds **1-5** and **9-10** in ICR-CD1 mice

Cmpd	I.C.V.		I.T.		Ratio i.c.v./i.t.
	ED_{50} (95% C.I.) pmol/mouse	**24hr tolerance**	**ED_{50} (95% C.I.) pmol/mouse**	**24hr tolerance**	
1	1219 (859- 1730)	Day 1: 64.27 ± 6.55 Day 2: 33.85 ± 11.38 Tolerance	559 (372-840)	Day 1: 73.55 ± 10.93 Day 2: 66.01 ± 6.81 No Tolerance	2.18
2	523 (223-1228)	Day 1: 77.72 ± 9.78 Day 2: 63.70 ± 16.48 No Tolerance	27 (13-54)	Day 1: 81.72 ± 9.48 Day 2: 73.41 ± 11.49 No Tolerance	19.48
3	55 (37-82)	Day 1: 83.47 ± 11.59 Day 2: 66.99 ± 9.55 No Tolerance	42 (31-58)	Day 1: 98.33 ± 1.79 Day 2: 72.5 ± 11.22 No Tolerance	1.31
4	189 (132-270)	Day 1: 95.43 ± 2.93 Day 2: 86.33 ± 7.88 No Tolerance	19 (11-34)	Day 1: 78.51 ± 10.44 Day 2: 79.58 ± 9.82 No Tolerance	9.86
5	9 (6- 12)	Day 1[b]: 89.75 ± 10.95 Day 3: 72.81 ± 12.69 No Tolerance	5 (3- 8)	Day 1: 92.20 ± 8.34 Day 2: 87.91 ± 6.76 No Tolerance	1.8
9	108 (83 - 141)	Day 1: 82.15 ± 9.67 Day 2: 24.14 ± 11.92 Tolerance	106 (41 - 273)	Day 1: 75.98 ± 12.73 Day 2: 39.54 ± 14.28 Tolerance	1.01
10	NS[c]	NA[d]	NS	NA	
9+10 [e]	78 (55-110)	Day 1: 68.84 ± 11.94 Day 2: 43.92 ± 15.20 No Tolerance	130 (97-173)	Day 1: 78.00 ± 11.62 Day 2: 74.83 ± 8.45 No Tolerance	0.60

[a] 24 hour tolerance was calculated using the highest dose of the dose-response curve on day 1 and repeated on day 2. If there was no significant difference between the two days the animals were said to be not tolerant. Compounds **9+10** (equimolar dose), **1-5** were administered at 2500, 250, 1000 and 125 pmol per mouse.

[b] As there was 47% antinociception 24 hours after the initial 15 pmol/mouse (i.c.v.) dose, a 24 hour tolerance test could not be completed. However, after 48 hours the antinociception had returned to baseline and, at this time tolerance was measured by injection of a subsequent 15 pmol/mouse.

[c] NS: not significant; compound **10** did not show any antinociceptive activity (only 20-30%) when given at 250 or 2500 pmol per mouse.

[d] Not applicable.

[e] Doses were based on a 1:1 mixture of compounds **9+10**.