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An Immunocytochemical-Derived Correlate for Evaluating the Bridging of Heteromeric Mu-Delta Opioid Protomers by Bivalent Ligands

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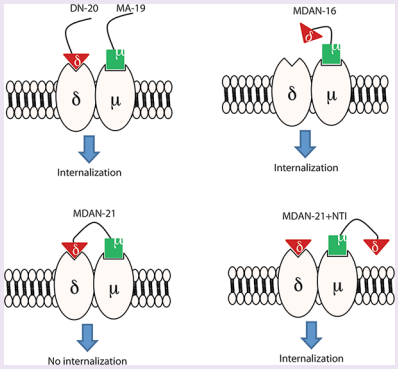
Supporting Information

ABSTRACT: Bivalent ligands that contain two pharmacophores linked by a spacer are promising tools to investigate the pharmacology of opioid receptor heteromers. Evidence for occupation of neighboring protomers by two phamacophores of a single bivalent ligand (bridging) has relied mainly on pharmacological data. In the present study, we have employed an immunocytochemical correlate to support *in vivo* biological studies that are consistent with bridging. We show that a bivalent mu agonist/delta antagonist (MDAN-21) that is devoid of tolerance due to possible bridging of mu and delta protomers prevents endocytosis of the heteromeric receptors in HEK-293 cells. Conversely, a bivalent ligand (MDAN-16) with a short spacer or monovalent mu agonist give rise to robust internalization. The data suggest that the immobilization of proximal mu and delta protomers is due to bridging by MDAN-21. The finding that MDAN-21 and its shorter spacer homologue MDAN-16 possess equivalent activity in HEK-293 cells, but produce dramatically divergent internalization of mu-delta heteromer, is relevant to the role of internalization and tolerance.



Opioid ligands, such as morphine, produce analgesia via Gi/Go G protein-coupled opioid receptors.[1,2] There are three receptor types (mu, kappa, and delta) in the opioid receptor family that are activated by such ligands.[1,2] Side effects such as tolerance and physical dependence may accompany pharmacotherapy, and several studies have suggested that both mu and delta opioid receptors are involved.[3−6] Notably, it has been shown that co-administration of the delta antagonist, naltrindole[7] (NTI), attenuates morphine-induced tolerance and dependence.[3] These seminal observations, along with the discovery that mu and delta opioid receptors oligomerize to form a heteromer,[8,9] led to the design of a series of bivalent ligands that contain mu agonist and delta antagonist pharmacophores tethered through different length spacers (MDAN series, Figure 1).[10] Significantly, members the MDAN series with spacers containing 18−21 atoms were devoid of tolerance.

The rationale behind the design of the MDAN series was based on the concept that two physically associated GPCR protomers can be bridged through binding of both pharmacophores in a single bivalent ligand.[11−14] Subsequent studies have suggested that a variety of opioid bivalent ligands having spacers ranging from 18 to 22 atoms can effectively bridge physically associated protomers.[15−17] The most convincing support for the 18−22 atom spacer requirement for bridging employed BRET technology and involved the bivalent ligand-induced association of mu and CCK_2 homomers that do not form constitutive heteromer.[18] This study revealed that bivalent ligands containing mu agonist and CCK2 antagonist pharmacophores linked through 18−22 atom spacers efficiently induced physical association of coexpressed mu and CCK2 receptors by shifting the equilibrium from homomers to a heteromer, whereas ligands with shorter spacers were not effective in this regard. That the recently reported X-ray crystal structure of the mu opioid receptor reveals that transmembrane helices 5 and 6 (TM-5,6) comprise a likely interface for dimerization, 18−22 atoms is consistent with the observed range of spacers for bridging of protomers.[19]

In the present study, we have performed immunocytochemistry and intracellular calcium release experiments in HEK-293 cells coexpressing mu and delta receptors in the presence of MDAN-21 in an effort to establish an additional correlate for bridging. Given that MDAN-21 has been reported to produce potent antinociception without tolerance, physical dependence, or place preference,[10,20] and our suggestion that this is a consequence of bridging, MDAN-21 was compared with its bivalent homologue (MDAN-16) and monovalent opioid agonist (MA-19), both of which possess the aforementioned side effects. MDAN-16 was selected because we had suggested its side effects were related to univalent interaction with opioid receptors due to its shorter spacer (16 atoms). If this is the case, MDAN-21 would be expected to affect endocytosis of mu-delta heteromer differently from MDAN-16 or MA-19.

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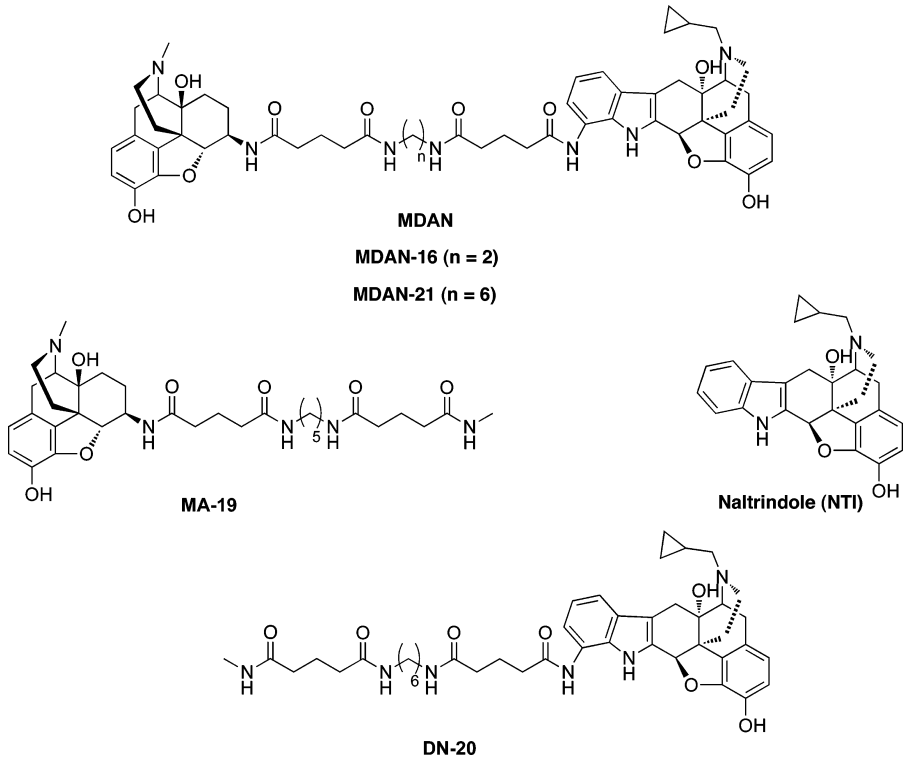

Figure 1. Structures of bivalent ligands (MDAN-16, -21), monovalent ligands (MA-19 and DN-20), and naltrindole (NTI).

HEK-293 cells containing FLAG-tagged mu (FL-mu) and hemagglutinin-tagged delta (HA-delta) were incubated with anti-FLAG and anti-HA primary antibodies (Abcam) for 2 h on ice. The cells were then treated with the respective ligands for 30 min at 37 °C, washed, and fixed with 4% paraformaldehyde for 10 min at RT. Staining was performed using the corresponding fluorophore-tagged secondary antibodies (see supporting information for full description). Primary antibodies were added to live unfixed cells so as to label receptors distributed on the plasma membrane only; fixing cells with formaldehyde kills cells makes their membranes permeable to antibodies, and as a result both plasma membrane and cytoplasmic receptors will become labeled. Antibodies were added to live cells kept on ice to prevent constitutive internalization of receptors.

Significantly, MA-19 (1 μM) produced robust co-internalization of mu and delta cell-surface opioid receptors that appear to be co-localized (Figure 2). When taken together with prior reports showing that mu and delta receptors are constitutively expressed as heteromer,[21,22] these data suggest that mu and delta receptors are physically coupled and trafficked together. Indeed, the mu agonist, DAMGO, also has been reported to co-internalize mu-delta heteromer.[22] The fact that co-administration of the monovalent delta antagonist DN-20 with mu agonist MA-19 did not block the co-internalization of mu-delta heteromers (Figure 2) suggests that univalent occupancy of the delta opioid protomer by DN-20 and the mu receptor by MA-19 does not negatively affect trafficking of the heteromer for this combination of ligands.

The finding that MDAN-21 (1 μM) did not produce significant internalization of either mu or delta receptors in the mu/delta cell line (Figure 2, Figure 3A) suggests that spacer-mediated bridging of protomers contributes to the dramatic change in trafficking. Since the bivalent ligand with a 16-atom spacer (MDAN-16) produced robust co-internalization of mu and delta receptors, this strongly suggests that MA-19 and MDAN-16 are involved in univalent interaction that leads to co-internalization of mu-delta heteromer.

Additional evidence for bridging of mu-delta protomers was obtained when cells were pretreated with the delta antagonist naltrindole (NTI) 10 min before adding MDAN-21. The fact that several punctate images of co-internalized mu and delta receptors were observed (Figure 3A, Figure 3B) suggests that the bivalent interaction was disrupted due to displacement of the delta antagonist pharmacophore of MDAN-21 by NTI. Thus, due to competition at the delta protomer by NTI, MDAN-21 functions, at least in part, univalently, which promotes endocytosis similar to that of MA-19 and MDAN-16 (Figure 4).

Given that bridging of mu-delta heteromer by MDAN-21 blocks endocytosis, we investigated whether the ability of MDAN-21 to activate the heteromer was also attenuated. In this regard, we carried out intracellular calcium release experiments in HEK-293 cells that contain stably expressed mu, delta, or mu/delta opioid receptors. These cells were transiently transfected with Δ6-$G_{\alpha qi4\text{-}myr}$,[23] a chimeric G protein that has been shown to couple opioid receptors to the calcium release mechanism.[23] We evaluated the action of bivalent ligands MDAN-16 and MDAN-21 and compared activity with that of monovalent controls, MA-19 and DN-20. In these experiments we chose to co-administer the monovalent ligands, MA-19 and DN-20, rather than pretreating with DN-20 and then adding MA-19. This was done because both pharmacophores of MDAN-21 would be able to interact with the mu and delta protomers in a concerted manner.

When tested on HEK-293 cells, MA-19 produced strong calcium release (~380 relative fluorescence units (RFU)) that was unaffected by equimolar DN-20 in either mu or mu-delta

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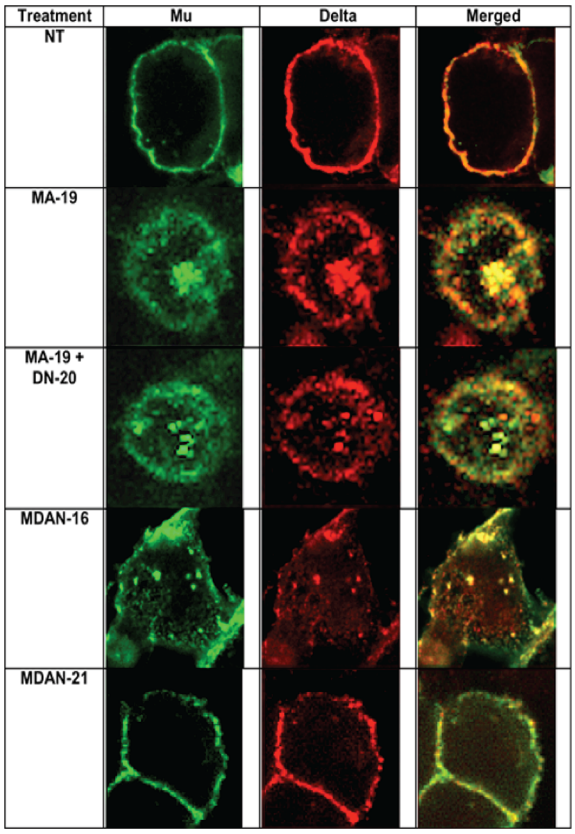

Figure 2. High magnification confocal microscopy images depicting effects of bivalent and monovalent ligands on trafficking of mu- and delta-opioid receptors coexpressed in HEK-293 cells. **NT** represents untreated cells. **MA19** produces robust internalization. **MA-19 + DN-20**: Internalization is not antagonized by co-administration of mu agonist and delta antagonist. **MDAN-16** also induces endocytosis of both mu and delta receptors. **MDAN-21** does not produce significant internalization of either mu or delta receptors.

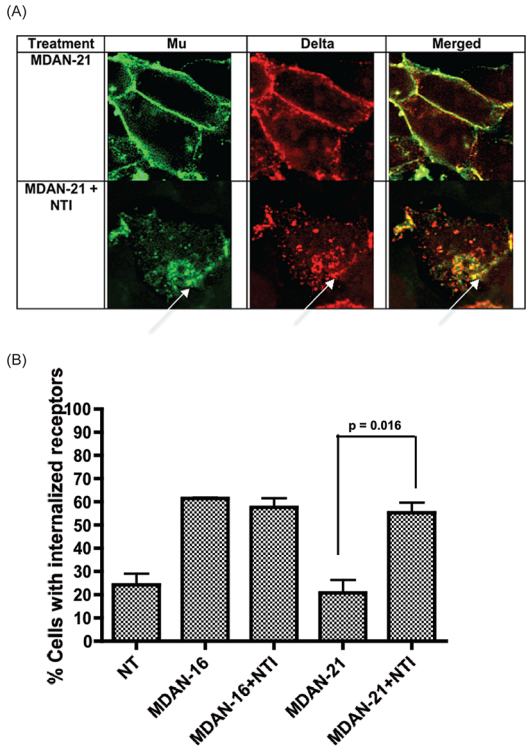

Figure 3. (A) Facilitation of internalization of mu and delta receptors upon pretreatment with delta antagonist, naltrindole (NTI), in the presence of MDAN-21. The bivalent ligand MDAN-21 alone does not produce significant internalization of either mu or delta receptor. However, upon pretreatment with NTI for 10 min MDAN-21 produced robust co-internalization of both mu and delta receptors. (B) Pretreatment with NTI (1 μM) significantly (p = 0.016, t test, two-tailed) increases the number of internalized receptors in cells treated with MDAN-21 (1 μM). On the other hand, the number of cells with internalized receptors due to MDAN-16 was unaffected with or without NTI pretreatment. A minimum of 75 cells were counted for each treatment with two independent experiments.

cells (Supplemental Figure S1, Supplemental Table 1). MDAN-16 produced slightly greater calcium release in mu/delta cells (EC_{50} = 565.7 nM, ΔRFU = 480 RFU) than in mu cells (EC_{50} = 796.6 nM, ΔRFU = 321 RFU). However, MDAN-21 was equiactive in both mu/delta (EC_{50} = 411.6 nM, ΔRFU = 468 RFU) and mu cells (EC_{50} = 850.6 nM, ΔRFU = 420 RFU). Interestingly, all three ligands had similar peak effect when added at a 10 μM concentration to cells expressing mu-delta heteromers (Supplemental Figure S1). None of the ligands tested showed any significant activity in the delta opioid cells, as all ligands tested are antagonists at delta receptors. Our data suggest that MDAN-21 does not induce endocytosis of mu-delta heteromer, despite activating the heteromer to the same extent as mu homomer.

In addition to providing a new correlate for bridging of heteromer by MDAN-21, our study is relevant to the debate concerning a possible relationship between receptor endocytosis and the development of tolerance.[24−28] In this regard, it has been proposed that the regulation of opioid receptors by endocytosis plays a significant role in the development of antinociceptive tolerance.[24−28] Thus, it has been reported that endocytosis of mu opioid receptors has an inverse relationship to tolerance,[24,25] whereas the endocytosis of delta receptors correlates with increased tolerance.[29]

In this context, our trafficking results of mu-delta heteromer are extremely relevant, as several studies have suggested that mu-delta heteromers play a critical role in tolerance development to clinically employed opioids[3−6,30] such as morphine. Since we have reported that MDAN-21 does not produce tolerance in mice,[10] and in view of the present finding that it does not promote endocytosis of mu-delta heteromer in cultured cells, this suggests that the lack of internalization of mu receptors by morphine may not be a reliable correlate of tolerance.

In conclusion, immunocytochemical trafficking and receptor activation studies in the presence of MDAN-21 in HEK-293 cells coexpressing mu and delta receptors has revealed a correlation between the absence of receptor trafficking and bridging of mu-delta heteromer. Our study shows that the lack of internalization of mu-delta heteromer in the presence of MDAN-21 is correlated with the reported absence of antinociceptive tolerance and dependence in mice. The fact that both the bivalent MDAN-16 with a shorter spacer and monovalent MA-19 produce tolerance and induce internalization suggests that the longer spacer (21 atoms) in MDAN-21 permits bridging of mu-delta heteromer. Thus, immunocytochemical trafficking data appears to be a useful approach in assessing whether bridging to a heteromer has occurred. The absence of tolerance and internalization of MDAN-21 is inconsistent with the concept that relates the lack of mu agonist-induced internalization to tolerance.

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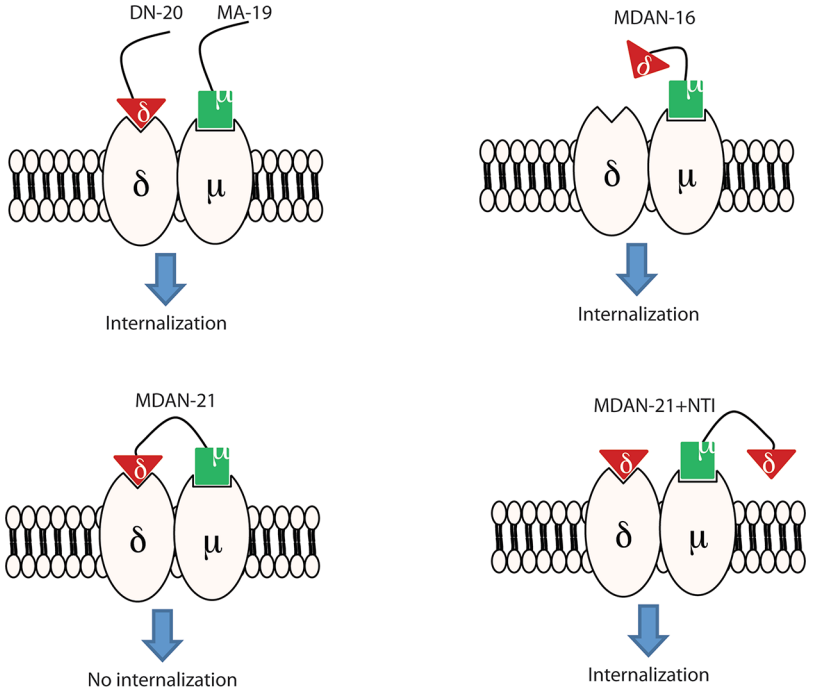

Figure 4. Effect of NTI on trafficking of mu-delta heteromers by MDAN-21. A cartoon illustrating the effect of delta antagonist on the disruption of bridging protomers in the mu-delta heteromer.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.S.Y. and P.S.P. designed the research, and A.S.Y. and A.E.K. conducted experiments. All authors analyzed the data and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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