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Molecular characterization of eluxadoline as a potential ligand targeting mu-delta opioid receptor heteromers

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ABSTRACT

Eluxadoline, an orally active mixed μ opioid receptor (μOR) agonist δ opioid receptor (δOR) antagonist developed for the treatment of diarrhea-predominant irritable bowel syndrome, normalizes gastrointestinal (GI) transit and defecation under conditions of novel environment stress or post-inflammatory altered GI function. Furthermore, compared to loperamide, which is used to treat non-specific diarrhea, the effects of eluxadoline on GI transit occur over a wider dosage range. However, the mechanisms of action of eluxadoline are unclear. In this study, we compared the ability of eluxadoline and loperamide to activate G-protein- and β-arrestin-mediated signaling at μOR homomers or μOR-δOR heteromers in heterologous cells. We also examined the ability of both compounds to reduce castor oil induced diarrhea in wild type (WT) and mice lacking δOR. We find that eluxadoline is more potent than loperamide in eliciting G-protein activity and β-arrestin recruitment in μOR expressing cells. However, in cells expressing μOR-δOR heteromers, the potency of eluxadoline is higher, but its maximal effect is lower than that of loperamide. Moreover, in these cells the signaling mediated by eluxadoline but not loperamide is reduced by μOR-δOR heteromer-selective antibodies. We find that in castor oil-induced diarrhea eluxadoline is more efficacious compared to loperamide in WT mice, and δOR appears to play a role in this process. Taken together these results indicate that eluxadoline behaves as a potent μOR agonist in the absence of δOR, while in the presence of δOR eluxadoline's effects are mediated through the μOR-δOR heteromer.

1. Introduction

Opioid receptors are therapeutic targets for the treatment of pain. Morphine, the prototypic opioid, targets the mu opioid receptor (μOR) and is clinically preferred for the treatment of chronic pain [1]. However, chronic morphine administration leads to a number of side-effects including development of analgesic tolerance and constipation. Studies seeking to decrease the side-effects associated with chronic morphine use found that delta opioid receptor (δOR) antagonists could enhance morphine-induced analgesia while preventing the development of tolerance to this drug [2–6] which suggested interactions between μOR and δOR. These interactions were examined using cells heterologously expressing either μOR or δOR or a combination of both receptors and showed that δOR selective antagonists, irrespective of their nature (peptidic or non-peptidic), could enhance μOR selective ligand binding and signaling only in cells co-expressing both receptors [7,8]. Moreover, these *in vitro* studies showed that the δOR antagonist decreased the dissociation rate of radioligand bound to μOR [9]. These data supported the idea that the δOR antagonist allosterically enhances μOR ligand binding leading to potentiation of μOR-mediated signaling and antinociception. One

Abbreviations: GI, gastrointestinal; IBS-d, irritable bowel syndrome with diarrhea; μOR, mu opioid receptor; δOR, delta opioid receptor; βgal, beta-galactosidase; GTPγS, guanosine 5′-O-(3-thiotriphosphate); DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; CB1R, cannabinoid receptor type1; AT1R, angiotensin II receptor type 1; ELISA, enzyme-linked immunosorbent assay; EC_{50}, 50% effective concentration; E_{max}, maximum effective concentration; WT, wild-type; −/−, knockout; eGFP, enhanced green fluorescent protein.

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way in which allosteric modulation of μOR properties by δOR could occur is via the formation of μOR-δOR heteromers; μOR-δOR heteromerization is supported by studies using antibodies that selectively target the heteromer [10] or TAT peptides that can disrupt the formation of μOR-δOR heteromers [11]. Ligands targeting μOR-δOR heteromers either by having μOR agonist/δOR antagonist activity such as bivalent ligands or ligands possessing mixed μOR agonist and δOR antagonist activity have been generated [12–17]. Studies using a bivalent ligand comprising of a μOR agonistic pharmacophore separated by a 21-atom spacer arm from a δOR antagonistic pharmacophore (MDAN21) [15,17] showed that it exhibited 100-times higher antinociceptive potency compared to morphine without significant development of tolerance or dependence [15]. Similarly, studies using ligands possessing mixed μOR agonist/δOR antagonist activity show that their chronic administration leads to lesser side-effects compared to morphine [13]. Taken together these results suggest that targeting the μOR-δOR heteromer could lead to the development of drugs that are likely to have lower side effects than drugs targeting μOR alone.

As mentioned above, one of the severe side-effects associated with chronic morphine use is constipation; this suggests that opioid receptors in the gastrointestinal (GI) tract could be targeted for the treatment of GI tract disorders [18] such as diarrhea. This led to the development of loperamide, a peripherally active μOR agonist, as a therapeutic agent for the treatment of diarrhea [19,20]. However, one of the side-effects associated with the use of loperamide is the development of constipation [21,22]. The possibility that drugs having μOR agonist/δOR antagonist activity could have lesser side effects led to the synthesis of eluxadoline [14,16]. Recent studies show that eluxodaline is a locally acting μOR agonist/δOR antagonist that can normalize GI transit in stressed animals over a wide dose range [16]. Eluxadoline has limited systemic bioavailability which could potentially reduce its effects on the central nervous system and consequently prevent the development of side-effects associated with therapies currently used to treat irritable bowel syndrome with diarrhea (IBS-d). Currently, eluxadoline has completed Phase II [23] and is undergoing Phase III clinical trials for treatment of IBS-d. While *in vivo* preclinical studies indicate that eluxadoline modulates GI motility and decreases intestinal pain or visceral hyperalgesia without the constipation associated with drugs that activate μOR [16], its mechanism of action is not clear. Since eluxadoline is a mixed μOR agonist/δOR antagonist [14,16,23], it is possible that it may mediate its effects by targeting μOR-δOR heteromers. Therefore, in this study we examined the mechanism of the *in vitro* effects of eluxadoline by comparing its activity in cell lines (using an assay that specifically examines heteromer signaling) and in tissues from wild-type (WT) and knockout mice ($\delta OR^{-/-}$ or $\mu OR^{-/-}$). Furthermore, we evaluated the extent to which eluxadoline affects GI transit in WT and $\delta OR^{-/-}$ mice in a castor oil induced model of diarrhea. We find that eluxadoline-mediated signaling can be significantly, albeit partially, blocked by an μOR-δOR heteromer selective antibody in cells co-expressing both receptors. We also find that eluxadoline is more effective in blocking castor oil-induced diarrhea in WT mice as compared to $\delta OR^{-/-}$ mice. These results suggest that eluxadoline, at least in part, mediates its effects by targeting μOR-δOR heteromers.

2. Methods

2.1. Cell culture

$\mu^{\beta gal}$OR and $\mu^{\beta gal}$OR-δOR expressing U2OS cells were a kind gift from DiscoveRx (Fremont, CA, USA). $\mu^{\beta gal}$OR cells expressing μOR tagged with a ProLink/β-galactosidase (βgal) donor (PK) fragment at the C-terminal region and β-arrestin tagged with a complementary βgal activator (EA) fragment were grown in MEM alpha (Life Technologies, Grand Island, NY, USA) containing 10% FBS (Biowest SAS, Nuaille, France), streptomycin-penicillin (Life Technologies), 500 μg/ml geneticin (Life Technologies) and 250 μg/ml hygromycin (Life Technologies). $\mu^{\beta gal}$OR-δOR cells expressing wild-type δOR, μOR tagged with the PK fragment at the C-terminal region and β-arrestin tagged with the EA fragment were grown in MEM alpha containing 10% FBS, streptomycin-penicillin, 500 μg/ml geneticin, 250 μg/ml hygromycin and 0.25 μg/ml puromycin (Life Technologies).

2.2. [^{35}S]GTPγS binding

Membranes were prepared from the spinal cord of either WT (Jackson Laboratories, Sacramento, CA, USA), $\delta OR^{-/-}$ (Charles River Laboratories, Kingston, NY, USA), $\mu OR^{-/-}$ (a gift from Dr. Charles Mobbs, Ichan School of Medicine at Mount Sinai, NY, USA) or from the ileal longitudinal muscle (containing myenteric plexus) of WT mice as described previously [24,25]. Membranes (10 or 20 μg) were subjected to a [^{35}S]GTPγS binding assay using DAMGO (R&D Systems, Minneapolis, USA), loperamide (Toronto Research Chemicals Inc., Ontario, Canada), eluxadoline (Furiex, Morrisville, NC, USA) (0–10 μM final concentration) in the presence or absence of TIPPψ (10 nM final concentration) (a gift from Dr. Peter Schiller, Institut de Reserches Cliniques de Montreal, Montreal, ON, Canada) as described previously [25]. EC_{50} and E_{max} were calculated using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

2.3. β-arrestin recruitment assay

U2OS cells expressing either $\mu^{\beta gal}$OR or $\mu^{\beta gal}$OR-δOR were plated in each well (5000 cells) of a 96-well white clear bottom plate in 100 μl of media. Next day, cells were treated with either DAMGO, loperamide, eluxadoline (0–10 μM final concentration) in the absence or presence of the δOR antagonist, TIPPψ (10 nM final concentration) (a gift from Dr. Peter Schiller) or in the absence or presence of antibodies (1 μg/well) to either μOR, μOR-δOR (generated as reported in [26]) or cannabinoid receptor type1-angiotensin II receptor type 1 heteromer (CB1R-AT1R) (generated as reported in [27]) for 60 min at 37 °C. β-arrestin recruitment was measured using the PathHunter Chemiluminescence detection kit as described in the manufacturer's protocol (DiscoveRx, Fremont, CA, USA). EC_{50} and E_{max} were calculated using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

2.4. Animals

Male C57BL/6 WT and $\delta OR^{-/-}$ mice (25–35 g; 6–12 weeks old) were obtained from either Jackson Laboratories (Sacramento, CA, USA; WT mice) or Charles River Laboratories (Kingston, NY, USA; $\delta OR^{-/-}$ mice). All mice were maintained on a 12-h light:12-h dark cycle with rodent chow and water available *ad libitum*, and housed in groups of five until testing. Animal studies were carried out according to protocols approved by the Icahn School of Medicine at Mount Sinai Animal Care and Use Committee.

2.5. Drug administration

Loperamide (Toronto Research Chemicals, Inc., Ontario, Canada) and eluxadoline (Furiex, Morrisville, NC, USA) were dissolved in 0.5% methylcellulose and 2% DMSO in water. Corresponding vehicle was used for control group. Mice were administered these drugs orally (p.o.). Naltrexone (R&D Systems, Minneapolis, USA) was dissolved in saline and administered intraperitoneally (i.p.). For chronic treatment with eluxadoline and