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In vitro and in vivo functional profile characterization of 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6α-(isoquinoline-3-carboxamido)morphinan (NAQ) as a low efficacy mu opioid receptor modulator

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ABSTRACT

Evidence has shown that downstream signaling by mu opioid receptor (MOR) agonists that recruit β-arrestin2 may lead to the development of tolerance. Also, it has been suggested that opioid receptor desensitization and cyclic AMP overshoot contributes to the development of tolerance and occurrence of withdrawal, respectively. Therefore, studies were conducted with 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6α-(isoquinoline-3-carboxamido)morphinan (NAQ), a MOR selective partial agonist discovered in our laboratory, to characterize its effect on β-arrestin2 recruitment and precipitation of a cyclic AMP overshoot. DAMGO, a MOR full agonist dose-dependently increased β-arrestin2 association with the MOR, whereas NAQ did not. Moreover, NAQ displayed significant, concentration-dependent antagonism of DAMGO-induced β-arrestin2 recruitment. After prolonged morphine treatment of mMOR-CHO cells, there was a significant overshoot of cAMP upon exposure to naloxone, but not NAQ. Moreover, prolonged incubation of mMOR-CHO cells with NAQ did not result in desensitization nor downregulation of the MOR. In functional studies comparing NAQ with nalbuphine in the cAMP inhibition, Ca^{2+} flux and [^{35}S]GTPγS binding assays, NAQ did not show agonism in the Ca^{2+} flux assay but showed partial agonism in the cAMP and [^{35}S]GTPγS assays. Also, NAQ significantly antagonized DAMGO-induced intracellular Ca^{2+} increase. In conclusion, NAQ is a low efficacy MOR modulator that lacks β-arrestin2 recruitment function and does not induce cellular hallmarks of MOR adaptation and fails to precipitate a cellular manifestation of withdrawal in cells pretreated with morphine. These characteristics are desirable if NAQ is pursued for opioid abuse treatment development.

1. Introduction

It is estimated that a total of 246 million people illegally use drugs worldwide, of this number, 32.4 million abuse opioids (United Nations Office on Drugs and Crime, 2015). In the United States, an estimated 4.4 million people aged 12 or older suffer from substance use disorders related to prescription opioids and an estimated 435,000 are addicted to heroin (Center for Behavioral Health Statistics and Quality, 2015). Opioid addiction has devastating effects on societies and an alarming observation is that opioid misuse has been on the rise recently; the number of unintentional overdose deaths from opioid prescription analgesics has soared in the United States, more than quadrupling since 1999 (Muhuri et al., 2013).

Currently, drugs used to treat opioid addiction include opioid agonists methadone and buprenorphine. However, 40–60% of patients on these drugs relapse (National Institute on Drug abuse, 2012). Interestingly, opioid receptor antagonists such as naltrexone and naloxone have been shown to block relapse and curb drug craving in opiate addicts (Chen et al., 2010; George and Ekhtiari, 2010; Minozzi et al., 2011). On the other hand, some severe side effects have been reported with these drugs. For example, patients receiving naltrexone for opioid dependence reported depression, dysphoria and showed high rates of overdose and suicide (Miotto et al., 2002; Ritter, 2002). In addition, naloxone at high doses has been found to cause pulmonary edema and cardiac arrhythmias (van Dorp et al., 2007). Studies have indicated that the observed side effects may be due to the lack of selectivity for the mu opioid receptor (MOR) over other opioid receptors, particularly the delta and kappa opioid receptors (DOR and KOR)

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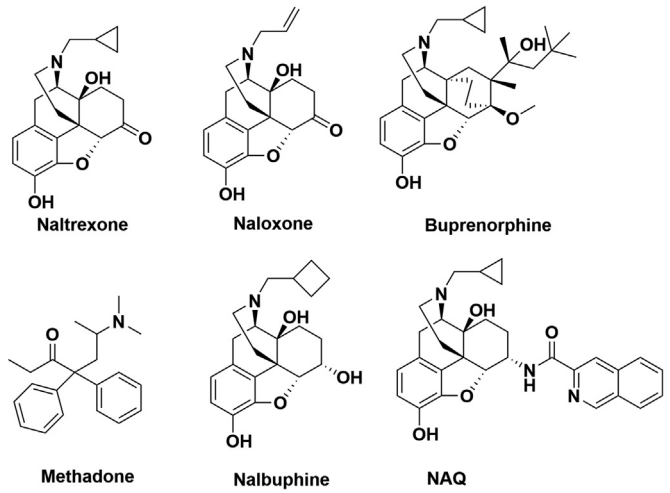

Fig. 1. NAQ together with drugs used to treat opioid addiction.

(Miotto et al., 2002; Ritter, 2002; van Dorp et al., 2007). Moreover, studies using MOR knock-out mice have shown that the dependence and abuse liability, respiratory depression, and constipation associated with opioids were abolished, indicating that the addiction and abuse liability of opioids are mainly mediated through the MOR (Gavériaux-Ruff and Kieffer, 2002; Matthes et al., 1996; Skoubis et al., 2001). Therefore, a highly selective MOR antagonist would be an advantageous agent to treat opioid addiction with fewer side effects than naltrexone and naloxone.

A highly selective MOR ligand 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6α-(isoquinoline-3-carboxamido)morphinan (NAQ), was recently identified in our laboratory (Fig. 1). NAQ showed an affinity of 0.55 nM to MOR with over 200-fold selectivity for the MOR over the DOR and 50-fold selectivity over the KOR (Yuan et al., 2011). NAQ acted as a low-efficacy MOR partial agonist in the [^{35}S]GTPγS binding assay, but antagonized the effects of DAMGO (a MOR full agonist) and morphine in the [^{35}S]GTPγS binding assay and warm-water tail immersion assay (Cornelissen et al., 2018; Li et al., 2009; Siemian et al., 2016; Yuan et al., 2011, 2013, 2015). Further pharmacological characterization showed that NAQ significantly reversed morphine withdrawal-associated depression of intracranial self-stimulation (ICSS) in rats (Altarifi et al., 2015). We herein report further characterization of NAQ to obtain a more comprehensive pharmacological profile, which indicated that NAQ does not produce significant cellular responses associated with opioid withdrawal, and make it a promising candidate for further development to treat opioid abuse and addiction.

2. Materials and methods

2.1. [^{35}S]GTPγS binding assay

Membranes were prepared from human MOR-CHO (hMOR-CHO) cells and mouse MOR-CHO cells (mMOR-CHO). Ligand-stimulated [^{35}S]GTPγS binding was performed as described previously (Selley et al., 1998, 1997). Briefly, membranes (10 μg of protein) were incubated with 0.1 nM [^{35}S]GTPγS (specific radioactivity was 1250 Ci/mmol) and 10 μM GDP for 90 min at 30 °C with or without varying concentrations of indicated ligand in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA, pH 7.4). Nonspecific binding was determined with 20 μM unlabeled GTPγS and basal binding was determined in the absence of MOR ligand. A sample containing 3 μM DAMGO was included in each assay to determine maximal stimulation by a full agonist at the MOR. The incubation was terminated by rapid filtration through GF/B glass fiber filters and rinsed three times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.2). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S. Net-stimulated [^{35}S]GTPγS binding was defined as ligand-stimulated minus basal binding. Percent stimulation was defined as (net-stimulated/basal [^{35}S]GTPγS binding) × 100%. Percent DAMGO-stimulated [^{35}S]GTPγS binding was defined as (net-stimulated binding by ligand/net-stimulated binding by 3 μM DAMGO) × 100%.

2.2. Calcium flux assay

hMOR-CHO cells were maintained as described previously (Zhang and Xie, 2012). Four h after $G\alpha_{qi5}$ transfection, cells were plated at 30,000 cells per well into a clear bottom black walled 96-well plate (Greiner Bio-one) and incubated for 24 h. The growth media was then decanted, and the wells were washed with 50:1 HBSS: HEPES assay buffer. Cells were then incubated with either 80 μl (agonism study) or 55 μl (antagonism study) of Fluo4 loading buffer (40 μl, 2 μM Fluo4-AM (Invitrogen), 84 μl of 2.5 mM probenecid, in 8 or 5.5 ml of assay buffer) for 30 min. For antagonism studies, 25 μl of varying concentrations of test compounds were added in triplicate and the plate was incubated for an additional 15 min. Plates were then read on a FlexStation3 microplate reader (Molecular Devices) at 494/516 ex/em for a total of 120 s. After 15 s of reading, 20 μl of varying concentrations of test compounds in triplicate (agonism study) or 500 nM of DAMGO (NIDA, antagonism study) in assay buffer, or assay buffer alone (control), were added. Changes in Ca^{2+} flux were monitored and peak height values were recorded. The obtained values were then subjected to nonlinear regression analysis to determine EC_{50} or IC_{50} values using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

2.3. cAMP accumulation assays

2.3.1. Inhibition of adenylyl cyclase (AC)

Cells were plated at a density of 3 × 10^5 cells per well in 24-well plates and allowed to double overnight. For assay, the media was replaced by serum-free DMEM/F12 containing 20 mM HEPES, pH 7.3, the phosphodiesterase inhibitors, RO-20-1724 (0.1 mM) and isobutylmethylxanthine (0.1 mM), and 2% BSA (reaction buffer), and cells were incubated for 30 min at 37 °C. Forskolin was then added to a final concentration of 10 μM to all samples (except the basal and blank conditions) with or without varying concentrations of the indicated opioid ligand or an equivalent volume of reaction buffer to a final volume of 0.2 ml, and the reaction was initiated by transferring the plate to 37 °C water bath. The incubation was conducted for 8 min at 37 °C, and the reaction terminated by transferring the plate to an ice bath. Cells were immediately lysed by replacing the incubation solution with 3% perchloric acid. Samples were incubated on ice for 30 min, neutralized with 15% potassium bicarbonate and subjected to centrifugation at 1000 × *g* for 10 min to isolate the precipitate.

cAMP levels were determined in aliquots of the supernatant by competitive binding of unlabeled cAMP from samples with [^{3}H]cAMP (specific radioactivity was 26.4 Ci/mmol) to a cAMP binding protein (PKA regulatory subunit). Briefly, cell supernatant or cAMP standards were combined with [^{3}H]cAMP and cAMP binding protein in a TRIS-EDTA buffer and incubated on ice for 90 min. A blank (no cells), cell blank (with cells), and total binding were assayed in the absence of cAMP binding protein. Unbound cAMP was removed through the addition and subsequent centrifugation (15,000 × *g*, 10 min, 4 °C) of 100 ml of a charcoal/dextran suspension. Radioactivity of the supernatant was determined using liquid scintillation spectrophotometry at 45% efficiency for [^{3}H]. A log transformation calibration curve of radioactivity versus standards was generated on Microsoft Excel.

2.3.2. AC sensitization assay

mMOR-CHO cells were incubated in serum-free DMEM/F12 for 4 h with 5 μM morphine, and the pretreatment was terminated by removal