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In vivo and *in vitro* evaluation of novel μ-opioid receptor agonist compounds

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ABSTRACT

Opioids are the most effective and widely used drugs for pain treatment. Morphine is an archetypal opioid and is an opioid receptor agonist. Unfortunately, the clinical usefulness of morphine is limited by adverse effects such as analgesic tolerance and addiction. Therefore, it is important to study the development of novel opioid agonists as part of pain control. The analgesic effects of opioids are mediated by three opioid receptors, namely opioid μ-, δ-, and κ-receptors. They belong to the G protein-coupled receptor superfamily and are coupled to G_i proteins. In the present study, we developed a ligand screening system to identify novel opioid μ-receptor agonists that measures [^{35}S]GTPγS binding to cell membrane fractions prepared from the fat body of transgenic silkworms expressing μ-receptor-G_{i1}α fusion protein. We screened the RIKEN Natural Products Depository (NPDepo) chemical library, which contains 5848 compounds, and analogs of hit compounds. We successfully identified a novel, structurally unique compound, that we named GUM1, with agonist activity for the opioid μ-receptor (EC_{50} of 1.2 μM). The Plantar Test (Hargreaves' Method) demonstrated that subcutaneous injection of 3 mg/kg of GUM1 into wild-type rats significantly extended latency time. This extension was also observed in a rat model of morphine tolerance and was inhibited by pre-treatment of naloxone. The unique molecular skeleton of GUM1 makes it an attractive molecule for further ligand–opioid receptor binding studies.

1. Introduction

Pain is definitely an unpleasant sensory and emotional experience (Merskey et al., 1994). It is associated with actual or potential tissue damage and is also a serious side effect of tumors (Spetea et al., 2013). Morphine is the most effective analgesic and is administered to relieve post-operative and cancer pain (Nersesyan and Slavin, 2007). However, morphine also produces adverse effects, including tolerance, physical dependence, constipation, respiratory depression, and nausea. To suppress the side-effects of morphine, other artificial opioid analogs, such as oxycodone and fentanyl, have been developed and used in a clinical context (Spetea et al., 2013). Opioid rotation is one strategy applied

Abbreviations: DAMGO, (2S)-2-[[2-[[(2R)-2-[[(2S)-2-amino-3-(4-hydroxyphenyl) propanoyl] amino]propanoyl]amino]acetyl]-methylamino]-N-(2-hydroxyethyl)-3-phenylpropanamide; DMSO, Dimethyl sulfoxide; DsRed, *Discosoma sp.* red fluorescent protein; EGFP, enhanced green fluorescent protein; Gal4, the yeast transcription activator protein; GPCRs, G protein-coupled receptors; GUM1, 2-[({2-[2-ethyl-4-(2-methoxyphenyl)-2-methyl-2H-3,4,5,6- tetrahydro pyran-4-yl]ethyl} amino)methyl]phenol hydrochloride; PWL, paw withdrawal latency; TRV130, ([(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro[4.5]decan-9-yl]ethyl})amine); UAS, upstream activating sequence for Gal4

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during opioid therapy and refers to a switch from one opioid to another to improve clinical outcomes (Fine and Portenoy, 2009). It is employed to improve analgesic control or to minimize opioid-related adverse events. In this strategy, incomplete cross-tolerance between the opioids being used is an important property (Skaer, 2014). Although there is a long history of opioid research and development, there are no opioid compounds that completely eliminate the unfavorable side-effects observed after chronic administration. Therefore, there remains an unmet need to continue studying and developing opioid analgesics.

Morphine exerts its analgesic effects by stimulating opioid receptors, which belong to the family of G protein-coupled receptors (GPCRs), expressed in the central nervous system. Three subtypes of opioid receptors, μ-, δ-, and κ-receptor, mediate analgesia with distinct pharmacological profiles (Pasternak, 2001). The μ-receptor is primarily responsible for mediating morphine's analgesic and rewarding properties (Kieffer, 1999). It is one of the most well-studied peptide GPCRs and several pharmacological and biochemical studies have provided information regarding its anatomic distribution, molecular mechanism, and three-dimensional structure (Manglik et al., 2012). Approximately 850 distinct GPCRs have been identified from human genome analysis, of which 350 have endogenous ligands (Fredriksson et al., 2003; Takeda et al., 2002; Vassilatis et al., 2003). Because of their importance in cell biology, many GPCRs have been strategic targets for novel drug discovery. This prompted the development of high-throughput screening systems to identify new therapeutic compounds that target GPCRs. Assays utilizing a fusion protein consisting of a GPCR and its partner G protein α subunit have been useful for studies of GPCR-G protein interactions because of their efficient coupling (Bertin et al., 1994; Milligan, 2000, 2002). Moreover, GPCR-Gα fusion proteins enabled a [^{35}S]GTPγS binding assay for an insect high-yield expression system, although recombinant mammalian GPCRs did not couple with endogenous insect G proteins (Tateno et al., 2009; Zhang et al., 2004). These assays directly measure G protein activation by a target GPCR *in vitro* and are effective as a high-throughput screening method because of the low false-positive reactions (Sasaki et al., 2008; Takeda et al., 2004).

In our studies, we performed a screen for novel human opioid μ-receptor ligands that had unique scaffolds by using cell membranes isolated from transgenic silkworms expressing a μ-receptor-G_{i1}α fusion protein. The most potent new opioid we discovered, termed GUM1, had not only *in vitro* activity as measured by a [^{35}S]GTPγS binding assay but also showed activity in an *in vivo* Plantar Test in rats. Our results indicated that the GPCR-Gα fusion proteins are beneficial for ligand identification of clinically important chemical compounds and that the transgenic silkworm was useful for mass production of GPCRs.

2. Materials and methods

2.1. Chemicals

The chemical library containing 5848 compounds used for ligand screening was provided by RIKEN Natural Product Depository (NPDepo). A novel opioid (GUM1), 2-[({2-[2-ethyl-4-(2-methoxyphenyl)-2-methyl-2H-3,4,5,6- tetrahydro pyran-4-yl]ethyl}amino)methyl]phenol hydrochloride, was purchased from Pharmeks Ltd. (catalog# P2000N-14927, Russia) and InterBioScreen Ltd. (catalog# STCK1N-26179, Russia). Morphine was purchased from Shionogi, Japan. We also obtained the opioid-related compounds (2S)-2-[[2-[[(2R)-2-[[(2S)-2-amino-3-(4-hydroxyphenyl)propanoyl] amino]propanoyl]amino]acetyl]-methylamino]-N-(2-hydroxyethyl)-3-phenylpropanamide (DAMGO), leucine–encephalin, dynorphin A, and naloxone from Sigma-Aldrich, U.S.A.

2.2. Expression of opioid receptor-Gα_{i1} fusion proteins

The cDNAs encoding human μ-, δ-, and κ-receptor, and bovine G_{i1}α subunit were used to generate GPCR-G protein fusion proteins by using a two-step PCR described previously (Takeda et al., 2004; Toyooka et al., 2009). The opioid receptor-G_{i1}α fusion genes were ligated into the baculovirus transfer vector pFASTBacI (Invitrogen, U.S.A.). The *Escherichia coli* DH10Bac carrying baculovirus bacmid was transformed using the pFASTBacI constructs described above and propagated on LB medium containing antibiotics (50 mg/ml kanamycin, 7 mg/ml gentamicin, and 10 mg/ml tetracycline), 200 μg/ml Bluo-Gal (Invitrogen, U.S.A.), and 40 μg/ml isopropylthio-β-D-galactoside. The recombinant bacmid was isolated from white colonies and transfected into Sf9 cells using Cellfectin (Invitrogen, U.S.A.) to amplify recombinant baculovirus. Sf9 cells were grown at 28 °C to a density of approximately 2–3 × 10^9 cells/l of culture medium, and then infected with the recombinant virus. Sf9 cells were harvested 48 h after infection and used for membrane preparation.

2.3. Construction of transgenic silkworms

All silkworms were reared on an artificial diet (Nihon Nosanko, Japan) at 25 °C. Expression of the μ-receptor-G_{i1}α fusion gene, described above, in transgenic silkworms was carried out using a yeast transcription activator protein (Gal4) and upstream activating sequence for Gal4 (UAS) system, a powerful technique for transgenic gene expression. To construct the transgenic vector for the acceptor strain, the μ-receptor-G_{i1}α fusion gene and SV40 polyA signal sequence were inserted downstream of a UAS cis-acting element in the *piggyBac* transposon vector carrying a neuron-specific 3xP3 promoter and an enhanced green fluorescent protein (EGFP) gene as a screening marker (Tateno et al., 2009). This transgenic vector was mixed with a helper plasmid carrying the *piggyBac* transposase gene, and injected into the embryos of *w1-pnd* strain (non-diapausing and white egg strain) at the preblastodermal stage. Hatched silkworms (G0) were mated within the same batch. The resulting G1 embryos were screened 6–7 days after oviposition for EGFP expression in their stemmata (Matsumoto et al., 2014). A donor strain expressing the yeast transcription activator protein Gal4 in the fat body and *Discosoma sp.* red fluorescent protein (DsRed) in the eyes as a selection marker was recently established in our laboratory. The DsRed-expressing Gal4 donor strain and the EGFP and μ-receptor-G_{i1}α fusion gene expressing the acceptor strain were mated and screened for co-expression of DsRed and EGFP. The fat body of fifth instar larvae was eviscerated and used for membrane preparations. DsRed and EGFP fluorescence was observed using fluorescent microscopy with DsRed and GFP2 filter sets (Olympus, Japan), respectively.

2.4. In vitro opioid receptor assay

Sf9 cells and the fat body of transgenic silkworms expressing the recombinant opioid receptor-G_{i1}α fusion proteins were homogenized with a Potter-type glass homogenizer in a buffer containing 50 mM HEPES–KOH (pH 8.0), 10 mM $MgCl_2$, and protease inhibitor cocktail (Sigma-Aldrich, U.S.A.). The cell membrane fraction was isolated by centrifugation at 25,000 rpm (83,000 × *g*) for 2 h (CP80WX ultracentrifuge with the SW28 rotor; Hitachi) and re-suspended in the homogenizing buffer above. Protein concentration was determined using a protein assay kit (Bio-Rad). [^{35}S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer, U.S.A. For measurements of agonist-dependent enhancement of [^{35}S]GTPγS binding and ligand screening for the opioid receptors, the membrane preparation expressing the fusion proteins (20 μg total protein) was incubated in 100 μl of 20 mM HEPES–KOH (pH 8.0),