DiPOA ([8-(3,3-Diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic Acid), a Novel, Systemically Available, and Peripherally Restricted Mu Opioid Agonist with Antihyperalgesic Activity: I. In Vitro Pharmacological Characterization and Pharmacokinetic Properties

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Received December 15, 2003; accepted March 29, 2004

ABSTRACT

Mu opioid receptors are present throughout the central and peripheral nervous systems. Peripheral inflammation causes an increase in mu receptor levels on peripheral terminals of primary afferent neurons. Recent studies indicate that activation of peripheral mu receptors produces antihyperalgesic effects in animals and humans. Here, we describe the in vitro pharmacological and in vivo pharmacokinetic properties of a novel, highly potent, and peripherally restricted mu opioid agonist, [8-(3,3-diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic acid (DiPOA). In a radioligand binding assay, DiPOA inhibited [3H]-diprenorphine binding to recombinant human mu receptors with a $K_{i}$ value of 0.8 nM. The rank order of affinity for DiPOA binding to recombinant human opioid receptors was mu > kappa > ORL-1 > delta. DiPOA showed potent agonist effects in a human mu receptor guanosine 5'-O-(3-[35S]thio)-triphosphate functional assay, with an EC$_{50}$ value of ~33 nM and efficacy of ~85% (normalized to the mu agonist, [D-Ala$_2$, MePhe$_4$, Gly(ol)$_5$]-enkephalin). Low potency agonist activity was also seen at ORL-1 and kappa receptors. DiPOA bound competitively to the opioid binding site of human mu receptors as demonstrated by a parallel rightward shift in its concentration-response curve in the presence of increasing concentrations of naltrexone. High and sustained (>5 h) plasma levels for DiPOA were achieved following intraperitoneal administration at 3 and 10 mg/kg; central nervous system penetration, however, was ~4% of the plasma concentration, even at levels exceeding 1500 ng/ml. As such, DiPOA represents a systemically available, peripherally restricted small molecule mu opioid agonist that will aid in understanding the role played by mu opioid receptors in the periphery.

Opioid receptors belong to the superfamily of G-protein-coupled receptors. To date, four members have been cloned and characterized: the mu, kappa, delta, and opioid receptor-like 1 (ORL-1) receptors (for review, see Pleuvry, 2003). All signal through activation of pertussis toxin-sensitive G proteins to mediate inhibition of adenylate cyclase (Herz, 1993). In addition, receptor activation results in suppression of voltage-gated calcium currents and the opening of receptor-op-
Basbaum, 1999; Yaksh, 1999). Although activation of central opioid receptors provides potent analgesia, this is associated with a variety of adverse effects, including respiratory depression and reinforcing behavior. Mechanistically, these adverse effects involve diminished responsiveness of the brainstem respiratory centers to carbon dioxide (Gustein and Akil, 2001) and stimulation of dopaminergic pathways, particularly the nucleus accumbens (Koob and Bloom, 1988), respectively.

The expression of opioid receptor mRNA has also been shown peripherally in the cell bodies of primary afferent neurons (Wang and Wessendorf, 2001). Immunohistochemical and competition binding studies demonstrate the presence of opioid receptors both on the peripheral terminals and presynaptic central terminals of these neurons (Machelska et al., 1999a; Gustein and Akil, 2001). Interestingly, inflammation may play multiple roles in modulating peripheral opioid receptor function. First, inflammatory conditions increase the axonal transport of mu opioid receptors, resulting in an increased receptor density at peripheral sites (Zöllner et al., 2003). Second, inflammatory conditions may sensitize peripheral opioid receptors. To this end, it has been demonstrated that reduced pH and increased cAMP, two hallmarks of the inflammatory process, can increase opioid agonist efficacy by altering receptor-G protein interactions (Selley et al., 1993) and can increase opioid-induced attenuation of neuronal excitability (Ingram and Williams, 1994), respectively. Third, immune cells such as monocytes, leukocytes, and macrophages are commonly found at sites of inflammation and contain stores of endorphins and enkephalins (for review, see Mousa, 2003). Full-length mRNA for opioid precursor proteins and the enzymes critical for their processing have also been detected in these cells. Importantly, release of opioid peptides from leukocytes can be stimulated by inflammatory mediators, including corticotrophin releasing factor and interleukin-1, the result of which may be endogenous antihyperalgesic and anti-inflammatory effects.

Indeed, peripheral administration of exogenous opioids at the site of inflammation has shown therapeutic utility in animals and humans (for review, see Stein et al., 2003). Local injection of the potent mu receptor agonist fentanyl into the inflamed hind paw of rats dose dependently reversed mechanical hyperalgesia, an effect that was blocked stereospecifically by (+)-naloxone (Stein et al., 1988). Similarly, the inflammation and hyperalgesia associated with acute chemical injury to the rat cornea were effectively reversed by direct application of morphine to the eye (Wenk et al., 2003). In humans, intra-articular administration of morphine has been shown to be effective in relieving pain in patients with osteoarthritis (Stein et al., 1999) or resulting from arthroscopic knee surgery (Kalso et al., 1997). Similar results are seen after subcutaneous administration of morphine to patients with acute inflammatory tooth pain (Likar et al., 1998). These data suggest that an exogenous, peripherally restricted opioid agonist may provide effective pain relief in inflammation-mediated hyperalgesia without centrally mediated adverse effects.

Here, we report the characterization of a high-affinity, high-efficacy mu receptor agonist, [8-(3,3-diphenyl-propyl)-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl]-acetic acid (DiPOA) (Fig. 1), that is systemically available and restricted to the periphery. We show that DiPOA has in vitro pharmacological and in vivo pharmacokinetic properties that should make it a useful tool for exploring the role of peripheral mu opioid receptors in models of chronic pain and other disease states.

**Materials and Methods**

Materials. HEK-293 cells expressing recombinant human ORL-1 were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Membranes prepared from Chinese hamster ovary-K1 cells expressing recombinant human mu or delta opioid receptors and HEK-293 cells expressing recombinant human kappa opioid receptor were purchased from PerkinElmer Life and Analytical Sciences. Radioisotopes were purchased from PerkinElmer Life and Analytical Sciences. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. The synthetic route for DiPOA (free base) has been recently described in patent application WO 2003101953 (Victory and Chen, 2003).

**Generation of HEK-293 Cells Stably Expressing Human Mu Receptor.** First strand cDNA was synthesized from 9 µg of fetal human brain total RNA (BD Biosciences Clontech, Palo Alto, CA) in a final volume of 50 µl using Thermoscript reverse transcriptase (Invitrogen) and oligo(dT)20 at 55°C for 1 h. Human mu opioid receptor (NCBI accession no. L29301.1; Meete et al., 1995) was amplified from the first strand cDNA (2 µl) by polymerase chain reaction (Saiki et al., 1988) using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and the following oligonucleotides: sense strand, 5’-CCGAAGCTTACCACTATGGACGCGCGTGTGGCCCGCAACGAC’; and antisense strand, 5’-TGCGTCTGAGACCTGTTAGGGCACGAGCAGCTGGTTTCTG’.

The oligonucleotides were designed to encode the restriction sites HindIII and XhoI, respectively, immediately outside the mu receptor coding region for oriented, forced cloning. The 1233-base pair cDNA product was digested with the restriction endonucleases HindIII and XhoI and inserted into the expression vector pcDNA3.1(+). Clones encoding wild-type mu receptor were identified by dioxygen chain termination DNA sequencing using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

The human mu opioid receptor cDNA expression construct was transfected into HEK-293 cells by LipofectAMINE Plus reagent (In-
vitrogen), as described by the supplier. Briefly, 5.0 μg of cDNA construct was incubated for 15 min with 60 μl of Plus reagent in 0.2 ml of OptiMEM I. A 40-μl aliquot of LipofectAMINE in an additional 0.2 ml of OptiMEM I was then added to the mixture, and complexes were allowed to form for 15 min. Complexes were then diluted with 0.8 ml of OptiMEM I and layered onto a 80% confluent monolayer of HEK-293 cells in a 10-cm plastic dish, and the cells were incubated at 37°C, 5% CO₂ for 3 h. The media were then replaced with normal growth media composed of Dulbecco’s modified Eagle’s medium with 4.5 g/l glucose, 2 mM L-glutamine, 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Following an additional 20 h of incubation, media were aspirated and cells treated with 3 ml of 0.05% Trypsin and 0.053 mM EDTA for 10 min and gently resuspended in 50 ml of growth media containing 500 μg/ml G418 (gentamicin). Cells were split in limiting dilution into multiple 96-well plates in the selective media and incubated for 3 weeks at 37°C, 5% CO₂, 95% humidity. Whole plates containing approximately one clone per well were replicated into five plates each by trypsinization and the cells incubated further until the majority of wells were confluent. Replicate plates were then transiently transfected with a commercial CRE-luciferase reporter plasmid (pCRE-luc; Stratagene, La Jolla, CA). In this procedure, the LipofectAMINE/DNA transfection complexes were prepared in Optimem I as described above and distributed across the 96-well plates, 100 μl/well. Cells were incubated for another 40 h, then treated with 10 μM forskolin or 10 μM forskolin plus 10 μM DAMGO (Sigma-Aldrich), a specific mu agonist, and stimulated for 6 h. The media were then aspirated and the cells treated with 75 μl/well lysis buffer (Promega, Madison, WI) for 15 min followed by 75 μl/well luciferase assay reagent-containing substrate. Plates were immediately read in a luminescence protocol on a Wallac Microbeta counter (PerkinElmer Life and Analytical Sciences) at 1 s/well. Two identical plates were used for forskolin stimulation and two plates for forskolin plus DAMGO. Individual clones exhibiting >60% DAMGO-stimulated inhibition of luciferase production were chosen for further analysis, and the best clones were further characterized in the radioligand binding GTPγS and CAMP assays (see below). The cell line was reisolated by limiting dilution cloning to assure clonality.

**Membrane Preparation.** Membranes from HEK-293 cells expressing ORL-1 were grown to confluency (20–40 × 10⁶ cells) in 10-cm dishes. Cells were lysed by adding 10 ml/dish ice-cold hypotonic buffer (2.5 mM MgCl₂ and 50 mM HEPES, pH 7.4). Cell lysates were removed using a cell scraper and homogenized for 30 s with a BioSpec Tissue Tearer (Racine, WI). Membranes were collected by centrifugation at 30,000 g for 15 min at 4°C, and pellets were resuspended in hypotonic buffer to a final concentration of 1 to 3 mg/ml. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. Aliquots of the membranes were immediately frozen and stored at −80°C.

**Competition Binding Studies.** Radioligand competition binding assay conditions are outlined in Table 1. All reactions were conducted in the presence of 5% DMSO in 96-well polystyrene plates at room temperature. Binding reactions were terminated by rapid filtration onto Unifilter-96 GF/C plates (PerkinElmer Life and Analytical Sciences), presoaked in 0.5% polyethylenimine using a Brandel 96-well tissue harvester (Brandel Inc., Gaithersburg, MD), followed by three filtration washes with ice-cold binding buffer. Filter plates were subsequently dried at 50°C for 2 to 3 h. Fifty microliters per well Betaplate Scint scintillation cocktail (PerkinElmer Life and Analytical Sciences) was added, and plates were counted in a TopCount (PerkinElmer Life and Analytical Sciences) for 1 min/well. Data were analyzed using GraphPad PRISM version 3.0 (GraphPad Software Inc., San Diego, CA). The concentrations of unlabeled competitors that yielded 50% displacement (IC₅₀ values) were calculated from each curve by a nonlinear regression analysis fitted to a one-site model. In separate experiments, equilibrium dissociation constants (Kₐ) were determined for each receptor-radioligand construct was incubated for 15 min with 60 μl of Plus reagent in 0.2 ml of OptiMEM I. A 40-μl aliquot of LipofectAMINE in an additional 0.2 ml of OptiMEM I was then added to the mixture, and complexes were allowed to form for 15 min. Complexes were then diluted with 0.8 ml of OptiMEM I and layered onto a 80% confluent monolayer of HEK-293 cells in a 10-cm plastic dish, and the cells were incubated at 37°C, 5% CO₂ for 3 h. The media were then replaced with normal growth media composed of Dulbecco’s modified Eagle’s medium with 4.5 g/l glucose, 2 mM L-glutamine, 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Following an additional 20 h of incubation, media were aspirated and cells treated with 3 ml of 0.05% Trypsin and 0.053 mM EDTA for 10 min and gently resuspended in 50 ml of growth media containing 500 μg/ml G418 (gentamicin). Cells were split in limiting dilution into multiple 96-well plates in the selective media and incubated for 3 weeks at 37°C, 5% CO₂, 95% humidity. Whole plates containing approximately one clone per well were replicated into five plates each by trypsinization and the cells incubated further until the majority of wells were confluent. Replicate plates were then transiently transfected with a commercial CRE-luciferase reporter plasmid (pCRE-luc; Stratagene, La Jolla, CA). In this procedure, the LipofectAMINE/DNA transfection complexes were prepared in Optimem I as described above and distributed across the 96-well plates, 100 μl/well. Cells were incubated for another 40 h, then treated with 10 μM forskolin or 10 μM forskolin plus 10 μM DAMGO (Sigma-Aldrich), a specific mu agonist, and stimulated for 6 h. The media were then aspirated and the cells treated with 75 μl/well lysis buffer (Promega, Madison, WI) for 15 min followed by 75 μl/well luciferase assay reagent-containing substrate. Plates were immediately read in a luminescence protocol on a Wallac Microbeta counter (PerkinElmer Life and Analytical Sciences) at 1 s/well. Two identical plates were used for forskolin stimulation and two plates for forskolin plus DAMGO. Individual clones exhibiting >60% DAMGO-stimulated inhibition of luciferase production were chosen for further analysis, and the best clones were further characterized in the radioligand binding GTPγS and CAMP assays (see below). The cell line was reisolated by limiting dilution cloning to assure clonality.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Membrane</th>
<th>Radioligand</th>
<th>Unlabeled Competitor</th>
<th>Binding Buffer</th>
<th>Assay Time</th>
<th>Incubation Volume</th>
<th>Binding Buffer</th>
<th>IC₅₀ Values</th>
<th>Equilibrium Dissociation Constant, Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mu</td>
<td>HEK-293</td>
<td>[3H]Diprenorphine (0.2)</td>
<td>Naltrexone (0.2)</td>
<td>10 mM MgCl₂, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4</td>
<td>100 μl</td>
<td>500 ml</td>
<td>100 μl</td>
<td>200</td>
<td>0.5</td>
</tr>
<tr>
<td>Kappa</td>
<td>HEK-293</td>
<td>[3H]U69,593, U69,593 (0.4)</td>
<td>Naloxone (0.2)</td>
<td>50 mM Tris-HCl, 50 mM HEPES, pH 7.4</td>
<td>100 μl</td>
<td>300 ml</td>
<td>200 μl</td>
<td>300</td>
<td>0.2</td>
</tr>
<tr>
<td>Delta</td>
<td>HEK-293</td>
<td>[3H]U50484, U50484 (0.2)</td>
<td>Naloxone (0.2)</td>
<td>50 mM Tris-HCl, 50 mM HEPES, pH 7.4</td>
<td>100 μl</td>
<td>500 ml</td>
<td>200 μl</td>
<td>500</td>
<td>0.25</td>
</tr>
<tr>
<td>ORL-1</td>
<td>HEK-293</td>
<td>[3H]Nociceptin, Nociceptin (0.1)</td>
<td>Naloxone (0.2)</td>
<td>10 mM MgCl₂, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4</td>
<td>100 μl</td>
<td>300 ml</td>
<td>200 μl</td>
<td>300</td>
<td>0.15</td>
</tr>
</tbody>
</table>
oligand pair (data not shown; $K_i$ values in Table 1); these values were subsequently used to calculate $K_i$ values (Cheng and Prusoff, 1973; see Table 2).

**GTP$\gamma$S Binding Studies.** Functional GTP$\gamma$S binding assays were conducted by sequentially mixing (on ice) the following reagents in the order shown to yield the indicated final concentrations: membrane protein (0.026 μg/μl mu, 0.020 μg/μl kappa, 0.012 μg/μl delta, or 0.066 μg/μl ORL-1), 10 μg/ml saponin, 3 μM GDP, and 0.20 nM GTP$\gamma$S to binding buffer (100 mM NaCl, 10 mM MgCl2, and 20 mM HEPES, pH 7.4). The prepared membrane samples (190 μg/well) were transferred to 96-well polystyrene plates containing 10 μl of 20× concentrated serial dilutions of test compounds prepared in DMSO. For the Schild analysis shown in Fig. 5, 20× dilutions were prepared that contained increasing concentrations of DiPOA in the absence or presence of a single concentration of the antagonist, naltrexone. Plates were incubated for 30 min at room temperature with shaking. Reactions were terminated by rapid filtration onto Unifilter-96 GF/B filter plates (PerkinElmer Life and Analytical Sciences) using a Brandel 96-well tissue harvester, followed by three filtration washes with 200 μl of ice-cold wash buffer (10 mM NaH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$, pH 7.4). Filter plates were subsequently dried at 50°C for 2 to 3 h. Fifty microliters per well Betaplate Scint scintillation cocktail was added and plates counted in a TopCount for 1 min/well. Data were analyzed using GraphPad PRISM version 3.0. The concentrations of agonists that yielded 50% of their maximal inhibition ($IC_{50}$ values) were calculated from each curve by a nonlinear regression analysis fitted to a one-site model.

**Measurement of cAMP in HEK-293 Cells Expressing Human Mu Receptor.** HEK-293 cells expressing human mu receptor were seeded at 100,000 cells/well in growth media (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.4 mg/ml Geneticin, and 1 mM sodium pyruvate) 24 h prior to assay, using BioCoat 96-well collagen-coated plates (BD Biosciences, San Jose, CA). Cells were maintained at 37°C with 5% CO$_2$. The growth media were replaced with OptiMEM media containing 1 mM 3-isobutyl-1-methylyxanthine, 100 μM forskolin, and increasing concentrations of agonist. The final DMSO concentration was 2%. Cells were stimulated at 37°C for 30 min and cAMP levels measured in cell lysates using the Tropix cAMP immunoassay kit (Applied Biosystems). Data were analyzed using GraphPad PRISM version 3.0. The concentrations of DiPOA or DAMGO that yielded 50% of their maximal inhibition ($IC_{50}$ values) were calculated from each curve by a nonlinear regression analysis fitted to a one-site model.

**In Vivo Pharmacokinetic Studies and CNS Permeability of DiPOA.** Commercially available male Sprague-Dawley rats (200–300 g) preimplanted with a jugular cannula were purchased from Taconic Farms (Germantown, NY) and fasted overnight prior to dosing. Five groups of rats (three per group) were administered a single 10 mg/kg i.p. dose of DiPOA or morphine suspended in 25% β-cyclodextran. Blood and brains were taken 0.5, 1, 3, and 5 h (DiPOA) or 1 h (morphine) after drug administration and analyzed by separate bioanalytical methods. DiPOA was separated from the brain tissue via liquid-liquid extraction and reconstituted with 100 μl of acetonitrile. The plasma and brain samples were analyzed by high-performance liquid chromatography on a Waters Alliance 2795 (Milford, MA), coupled with positive electrospray tandem mass spectrometry (MicroMass Quattro II) using a fast liquid chromatography gradient on a Phenomenex 30-mm Synergi Polar RP column (Torrance, CA). Multiple reaction monitoring mode was used to achieve selectivity and sensitivity, with limits of quantitation of 5 ng/ml and 50 ng/g for plasma and brain, respectively. DiPOA standard curves were fit to a 1/X weighted quadratic regression and showed linearity in the range of 5 to 1000 ng/ml ($R^2 = 0.99$). The ratio of drug concentration in brain (nanograms per gram of tissue) to plasma (nanograms per milliliter) was then calculated for each individual rat. All pharmacokinetic parameters were calculated using the NCA algorithm of Pharsight’s WinNonlin Professional version 3.0 software (Pharsight, Mountain View, CA).

**Results**

**Affinity for Human Opioid Receptors.** The affinity of DiPOA for human opioid receptors was measured using competition radioligand binding assays on cell membranes expressing the recombinant receptors (Fig. 2). DiPOA showed high binding affinity to the human mu receptor with a concentration-dependent displacement of [3H]-diprenorphine (Fig. 2A). The affinity of DiPOA was comparable with that of the mu agonist morphine and mu antagonist naltrexone, with all three compounds having $K_i$ values in the low nanomolar range (Table 2). These data indicate that DiPOA binds to recombinant human mu receptors with high affinity.

To assess selectivity of DiPOA across the different opioid
receptors, its binding affinity was also measured at recombinant human kappa, delta, and ORL-1 opioid receptors (Fig. 2). In the kappa binding assay, DiPOA showed a concentration-dependent displacement of the high-affinity kappa radioligand \([3H]\)-U69,593 (Fig. 2B). DiPOA had moderate affinity for the kappa receptor, with a \(K_i\) value of \(\sim 240\) nM (Table 2). In addition, morphine and U69,593 had \(-2\)-fold and \(-66\)-fold higher affinities for the kappa receptor compared with DiPOA in this assay, respectively (Table 2). In the ORL-1 receptor binding assay, DiPOA showed a concentration-dependent displacement of \([3H]\)-nociceptin, with a \(K_i\) value of \(\sim 290\) nM (Fig. 2D; Table 2). DiPOA had a \(-1500\)-fold lower affinity than the native ligand nociceptin. In comparison, morphine showed weak binding activity at ORL-1, with a \(K_i\) value \(>10,000\) nM. Finally, neither DiPOA nor morphine showed appreciable binding affinity at the human delta receptor, with each having \(K_i\) values \(>10,000\) nM, compared with the moderate affinity of the nonselective opioid antagonist naloxone (Fig. 2C; Table 2).

**Functional Activity at Human Opioid Receptors.** The ability of DiPOA to activate the human mu receptor was first assessed in a functional GTP\(\gamma\)\(^{35}\)S binding assay using membranes prepared from recombinant cells. Its activity was compared with that of the mu agonists DAMGO and morphine as well as the antagonist naltrexone (Fig. 3A). DiPOA caused a concentration-dependent increase in GTP\(\gamma\)\(^{35}\)S binding to mu membranes, with an \(EC_{50}\) value of \(\sim 33\) nM (Table 3). As such, DiPOA was 3.6- and 6-fold more potent than morphine and DAMGO in this assay, respectively. In addition, DiPOA showed \(\sim 85\%\) efficacy (\(E_{\text{max}}\)) compared with the full mu agonist DAMGO. In contrast, morphine had an \(E_{\text{max}}\) value of \(\sim 56\%\) in this assay. Naltrexone showed no stimulation of GTP\(\gamma\)\(^{35}\)S binding at doses up to \(1 \mu\)M, indicating a lack of agonist activity at the mu receptor.

To confirm the mu agonist activity seen in the GTP\(\gamma\)\(^{35}\)S assay, the ability of DiPOA to inhibit forskolin-mediated cAMP production in the human mu HEK-293 cell line was investigated (Fig. 4). In this assay, DiPOA caused a concentration-dependent inhibition of forskolin-mediated cAMP accumulation, with an \(EC_{50}\) value of \(7.4 \pm 2.4\) nM (mean \(\pm\) S.E.M.; \(n = 4\)). Similarly, DAMGO was a potent agonist in this assay, showing an \(EC_{50}\) value of \(17.2 \pm 6.8\) nM (mean \(\pm\) S.E.M.; \(n = 4\)). In this assay system, both agonists showed robust efficacy, inhibiting forskolin-mediated cAMP produc-
kappa and ORL-1 receptors, its functional activity was also determined at these targets (Fig. 3). At kappa receptors, DiPOA caused a concentration-dependent increase in GTP\(^{\gamma}\)[\(35S\)] binding (Fig. 3B) with an EC\(_{50}\) value of \(~550\) nM (Table 3). Both morphine and U69,593 were more potent than DiPOA in this assay and showed the same rank order of potency as in the kappa binding assay. DiPOA had an \(E_{\text{max}}\) value of \(~67\%) compared with the full kappa agonist U69,593, whereas morphine showed full agonist activity. DiPOA also showed agonist activity at the ORL-1 receptor (Fig. 3C), with an EC\(_{50}\) value of \(~3\) μM (Table 3). In addition, DiPOA had an \(E_{\text{max}}\) value of \(~73\%) compared with the full ORL-1 agonist, nociceptin. Morphine was not tested in the ORL-1 functional assay due to low binding affinity.

**Schild Analysis.** To determine whether DiPOA competitively interacts at the opioid binding site of the mu receptor, concentration-response curves in the GTP\(^{\gamma}\)[\(35S\)] binding assay were generated for DiPOA in the presence of increasing concentrations of the mu receptor antagonist naltrexone (0, 1, 3, 10, and 30 nM). Incubation of human mu receptors with escalating concentrations of naltrexone caused progressive rightward shifts in the DiPOA concentration-response curves, with no inhibition of maximal efficacy (Fig. 5A). Analysis of these data by Schild regression estimated a \(K_{i}\) of 1.10 ± 0.37 nM with a slope factor of 0.93 ± 0.09 (Fig. 5B). These data argue that DiPOA competitively interacts with naltrexone for binding to the opioid binding site of the human mu receptor.

**In Vivo Pharmacokinetic Profile of DiPOA.** DiPOA was administered to rats to examine its pharmacokinetic profile (Fig. 6). Mean DiPOA plasma concentrations were determined following a single 3 mg/kg i.v. dose (Fig. 6A). The following pharmacokinetic parameters were calculated for DiPOA after i.v. administration using a noncompartmental model: plasma half-life, 0.44 ± 0.02 h; clearance rate, 4.68 ± 0.47 l/h/kg; volume of distribution, 3.00 ± 0.46 l/kg (values given are mean ± S.E.M.; \(n = 3\) rats). DiPOA plasma concentrations were also measured following a single-dose i.p. administration at 0.1, 0.3, 1, 3, or 10 mg/kg (Fig. 6B). Working with a detection limit of 5 ng/ml, measurable, but low, plasma concentrations of DiPOA were seen at doses of 0.1 and 0.3 mg/kg. Higher plasma levels (>100 ng/ml) were measured after dosing larger quantities at 1, 3, and 10 mg/kg. In addition, sustained plasma levels were evident for up to 5 h at the 3 and 10 mg/kg doses (>30 ng/ml remaining at 5 h).

Penetration into the CNS was determined 1 h after a single 10 mg/kg i.p. dose of DiPOA (plasma concentration, 18,579 ± 11,680 ng/ml; brain concentration, 161 ± 50 ng/g tissue) or morphine (plasma concentration, 368 ± 137 ng/ml; brain concentration, 173 ± 37 ng/g tissue). The mean brain-to-plasma ratios for DiPOA and morphine were calculated at 0.019 ± 0.014 and 0.42 ± 0.13 (\(n = 3\) rats each), respectively, indicating that DiPOA, unlike morphine, does not significantly penetrate into the CNS. Finally, the penetration of DiPOA into brain tissue was also determined over a 5-h time course. Rats (two–three per group) were administered a single 10 mg/kg i.p. dose of DiPOA and brain-to-plasma ratios measured at 0.5, 1, 3, and 5 h. The ratios for individual rats were as follows: 0.5 h, 0.07 and 0.01; 1 h, 0.03 and 0.05; 3 h, 0.02, 0.02, and 0.06; and 5 h, below quantitative levels (\(n = 3\)). Ratios were low at all time points (brain levels ≤ 7% of
plasma levels), indicating that DiPOA does not accumulate in brain tissue after a single dose, even over an extended period of time.

Discussion

In this paper, we present the in vitro pharmacological and in vivo pharmacokinetic properties of the novel, systemically available, and peripherally restricted opioid receptor agonist, DiPOA. In addition, we compare these properties with the classic, clinically relevant opioid agonist, morphine. Although morphine serves as a potent and highly efficacious analgesic drug, its central activity is associated with undesirable side effects. Improved pharmacological tools are required to distinguish and further our understanding of the central and peripheral mechanisms of opioid-induced pain modulation. The pharmacological and pharmacokinetic profiles of DiPOA underscore its potential as a tool for further exploring the biological role of peripheral opioid receptors.

In the first set of experiments, we determined DiPOA’s activity at the mu opioid receptor. DiPOA showed high affinity in a radioligand binding assay, with a $K_i$ value similar to that of morphine ($\sim 0.8$ and $\sim 2.1$ nM, respectively) (Table 2). The $K_i$ value obtained for morphine in this assay was comparable with previously reported values (Raynor et al., 1995). Additionally, using a functional GTP$\gamma$S binding assay, we showed that DiPOA is a potent full mu agonist ($EC_{50}$ value $\sim 30$ nM), with 3.6-fold higher potency and appreciably higher efficacy than morphine (Table 3). Compared with the GTP$\gamma$S assay, DiPOA had similar potency and efficacy in a cAMP inhibition assay (Fig. 4). In numerical terms, DiPOA has a 43-fold lower potency in the GTP$\gamma$S functional assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (nM)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>n</th>
<th>EC$_{50}$ (nM)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>n</th>
<th>EC$_{50}$ (nM)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiPOA</td>
<td>32.7 ± 8.1</td>
<td>85.1 ± 0.7</td>
<td>3</td>
<td>554 ± 100</td>
<td>67.0 ± 4.0</td>
<td>3</td>
<td>2940 ± 1229</td>
<td>72.9 ± 8.3</td>
<td>5</td>
</tr>
<tr>
<td>Morphine</td>
<td>119 ± 23</td>
<td>55.9 ± 3.5</td>
<td>3</td>
<td>350 ± 112</td>
<td>96.7 ± 5.7</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Naltrexone</td>
<td>Inactive</td>
<td>2</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>DAMGO</td>
<td>202 ± 52</td>
<td>95.4 ± 0.5</td>
<td>8</td>
<td>27.3 ± 12.9</td>
<td>96.3 ± 1.7</td>
<td>3</td>
<td>0.09 ± 0.06</td>
<td>98 ± 0.6</td>
<td>3</td>
</tr>
<tr>
<td>U69,593</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Nociceptin</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
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<td>ND</td>
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</tbody>
</table>

ND, not determined.

![Fig. 4](https://example.com/fig4.png)

Fig. 4. Functional activity of DiPOA at recombinant human mu opioid receptors as measured in a cAMP accumulation assay. The assay was conducted as described under Materials and Methods. The points on each graph represent the mean ± S.E.M. of four independent experiments with duplicate wells on each plate. The data were normalized to the signal in the absence of forskolin (defined as 0%) and in the presence of 100 µM forskolin (defined as 100%).

![Fig. 5](https://example.com/fig5.png)

Fig. 5. DiPOA binds competitively to the human mu opioid receptor. A, concentration-response curves for DiPOA in the absence or presence of 1, 3, 10, or 30 nM naltrexone (NTX) were conducted using a functional GTP$\gamma$S binding assay as described under Materials and Methods. The points on each graph represent the mean ± S.E.M. of three independent experiments with duplicate wells on each plate. B, Schild regression analysis of the concentration-response curves from A (Arunlakshana and Schild, 1959).
compared with its \( K_i \) value measured in the radioligand binding assay. The discrepancy between apparent affinity and potency probably results from the inclusion of NaCl in the GTP\( ^{[\text{35S}]i \rightleftharpoons K \rightleftharpoons \text{M}) \) and cAMP assays. It is well established that sodium ions decrease agonist affinity for the mu opioid receptor, an effect that is generally more pronounced for higher efficacy agonists (Emmerson et al., 1996). Indeed, in our assays, morphine showed 58-fold reduced potency in the GTP\( ^{[\text{35S}]i \rightleftharpoons K \rightleftharpoons \text{M}) \) assay compared with its \( K_i \) value in the radioligand binding assay.

Although structurally divergent from morphine, the Schild analysis clearly argues that DiPOA interacts competitively at the opioid binding site of the mu receptor. The calculated \( K_i \) value from Schild regression of these curves was \(~1.1\) nM. This value is consistent with the \( K_i \) value obtained from the concentration inhibition binding assays described above \( (~0.8\) nM). Thus, DiPOA is a competitive high-affinity agonist for the mu opioid receptor. The ability of DiPOA to competitively interact with the mu receptor is not unexpected based on structural similarities to other competitive \( \mu \)-selective agonists; for example, carfentanil and loperamide (Fig. 1). All three compounds have a phenyl group in their upper portion and a mono- or diphenyl group in their lower portion. These two hydrophobic groups are linked by a piperidine ring, which possesses a critical basic nitrogen atom, and a two to three-carbon chain with or without a nitrogen atom. It has been previously postulated that the 3,3-diphenyl propyl group of loperamide serves as an anchor to selectively interact with a hydrophobic pocket in the mu receptor (Cometta-Morini et al., 1992); a similar interaction could be envisioned for DiPOA. Furthermore, the five-membered spiro ring system of DiPOA may serve as a conformationally constrained analog of the two side chains in carfentanil and related molecules, also allowing the phenyl group in their top portions to reside in a similar orientation. Finally, the amide carbonyl group of DiPOA is a mimic of the hydroxyl group of loperamide and the ester group of carfentanil, which may participate in hydrogen bond interactions with the mu receptor (Cometta-Morini et al., 1992).

In the second set of experiments, we determined the selectivity profile of DiPOA across the four opioid receptors (Table 2). Compared with its affinity for mu, DiPOA showed good selectivity against kappa, delta, and ORL-1 receptors, with ratios of 320-, >13,000-, and 376-fold, respectively. In comparison, morphine showed only 65-fold selectivity over the kappa receptor versus mu but approximately 5000-fold selectivity over delta and ORL-1 receptors. Again, the \( K_i \) values obtained for morphine at kappa, delta, and ORL-1 receptors were comparable with previously published values (Raynor et al., 1994; Fawzi et al., 1997). DiPOA also showed agonist activity at the kappa receptor with potency similar to morphine (EC\( _{50} \) values \(~550\) and \(~350\) nM, respectively); however, morphine was \(~44\%\) more efficacious than DiPOA (Table 3). Although morphine was inactive at the ORL-1 receptor, DiPOA showed low potency (EC\( _{50} \) value \(~3\) \( \mu \)M) and moderate efficacy (\(~73\%\)) in the functional assay.

Taken together, the opioid receptor selectivity and functional properties of DiPOA suggest a pharmacological profile unique from other opioids commonly used to study peripheral mechanisms of pain modulation. Compared with morphine, DiPOA has higher affinity, potency, and efficacy at the mu receptor. The antihyperalgesic activity of peripherally administered morphine in a number of chronic pain models (for review, see Stein et al., 2003) argues that DiPOA will have a similar, if not more robust, profile in vivo. In addition, DiPOA shows low ORL-1 agonist activity, whereas morphine is inactive. Recent studies indicate that activation of peripheral ORL-1 receptors on primary afferent neurons can reverse both axotomy-induced excitation of dorsal root ganglia neurons in vitro (Abdulla and Smith, 1998) and capsaicin-induced thermal hyperalgesia in vivo (Ko et al., 2002). The combined effects of potent and efficacious mu receptor agonism and moderate ORL-1 receptor agonism on pain modulation are currently unknown but warrant further investigations in vivo.

Peripheral kappa opioid receptor activation has also been associated with pain modulation in vivo. Using the peripheralized kappa receptor agonist, asimadoline (EMD 61753), antihyperalgesic (Barber et al., 1994) and analgesic (Machelska et al., 1999b) activities were shown in rodent pain models. In these same studies, however, lower doses and later time points in asimadoline-treated rats were characterized by hyperalgesic activity. Similarly, patients who had undergone knee surgery showed no clinical improvement in pain scores in response to asimadoline treatment (Machelska et al., 1999b). Although DiPOA does have moderate agonist
activity at the kappa receptor in vitro, the implications for this property in vivo are questionable given the ambiguous preclinical and clinical data associated with peripheral kappa agonism.

Investigations into the physiological role(s) played by peripheral opioid receptors in vivo have also been hampered by a limited number of agonists with suitable pharmacokinetic properties. One such example, loperamide, has an in vitro pharmacological profile very similar to that of morphine (Dehavan-Hudkins et al., 1999), although it is “gut restricted” upon oral administration. Although a small number of studies using intraperitoneal and intracutaneous administration of loperamide have been reported in the literature (Yoshida et al., 1979; Shannon and Lutz, 2002), a single comprehensive and correlative investigation into both the pharmacological and pharmacokinetic properties has not been described. For DiPOA, the pharmacokinetic data presented here indicate that sustained, high plasma levels can be achieved in rats after a single and relatively low systemic dose. Importantly, even at plasma levels exceeding 1500 ng/ml (>3.5 μM), on average ≤4% of this quantity was measured in the CNS. Moreover, these rats revealed no aberrant clinical signs, suggesting a general lack of opioid-like side effects, even at high plasma levels. Although other classic opioids such as morphine, fentanyl, and methadone have pharmacokinetic properties similar to that of DiPOA, they also show significant penetration into the CNS (Stanski, 1987; Abbruscato et al., 1997; Garrido et al., 1999), thus setting DiPOA and loperamide (Heykants et al., 1974) into a distinct class of peripherally restricted mu opioid receptor agonists.

Several reasons may account for the poor CNS permeability of DiPOA. First, its physical-chemical properties may limit CNS exposure. In particular, the carboxylic acid group on the five-membered spiro ring system may form zwitterions with the basic tertiary amine of the piperidine ring (Fig. 1). The charged nature of these zwitterions could prevent DiPOA from efficiently penetrating the blood-brain barrier (Abraham et al., 1997). Second, DiPOA may be a substrate for drug transporters of the P-glycoprotein (P-gp) family that reside in brain. In this case, DiPOA would be actively pumped out of the CNS, effectively reducing its brain concentrations. Indeed, loperamide is a P-gp substrate that is actively pumped out of the CNS (Schnikel et al., 1996) and shows very low brain levels upon systemic administration (Heykants et al., 1974). Again, structural similarities between DiPOA and loperamide could argue for a P-gp interaction.

Regardless of the mechanism responsible for the peripheralization, DiPOA offers a useful pharmacokinetic and pharmacological profile, demonstrating good systemic availability and tissue distribution. Because DiPOA does not cross the blood-brain barrier, it should be devoid of central mu opioid receptor-mediated analgesia and adverse effects. In addition, the volume of distribution of 3 μg/kg suggests that unbound DiPOA could be available to interact with mu receptors in peripheral tissues. This property is important to further understand the role of peripheral mu receptors in animal models with pathological conditions. To this end, Whiteside et al. (2004) show that DiPOA has robust antihyperalgesic properties in rat models of inflammatory pain.

Acknowledgments

We thank Richard Woodward and Garth Whiteside for critically reviewing this manuscript.

References


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