Characterization of a Novel Bivalent Morphinan Possessing κ Agonist and μ Agonist/Antagonist Properties

Jennifer L. Mathews, Xuemei Peng, Wennan Xiong, Ao Zhang, S. Stevens Negus, John L. Neumeyer, and Jean M. Bidlack

Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York (J.L.N., J.M.B.); and Alcohol and Drug Abuse Research Center, McLean Hospital, Harvard Medical School, Belmont, Massachusetts (X.P., W.X., A.Z., S.S.N., J.L.N.)

Received March 1, 2005; accepted July 28, 2005

ABSTRACT

Previous research has shown that compounds with mixed κ and μ activity may have utility for the treatment of cocaine abuse and dependence. The present study characterizes the pharmacological profile of a bivalent morphinan that was shown to be a κ opioid receptor agonist and a μ opioid receptor agonist/antagonist. MCL-145 [bis(N-cyclobutylmethylmorphinan) fumarate] is related to the morphinan cyclophoran and its N-cyclobutylmethyl derivative MCL-101 [3-hydroxy-N-cyclobutylmethyl morphinan S(+)-mandelate]. MCL-145 consists of two morphinans connected by a spacer at the 3-hydroxy position. This compound had Kᵦ values of 0.078 and 0.20 nM for the κ and μ opioid receptors, respectively, using radioligand binding assays as shown by Neumeyer et al. in 2003. In the guanosine 5’-O-(3-[35S]thiotriphosphate) binding assay, MCL-145 produced an Eᵦ₅₀ value of 80% for the κ opioid receptor and 42% for the μ opioid receptor. The EC₅₀ values obtained for this compound were 4.3 and 3.1 nM for the κ and μ opioid receptors, respectively. In vivo MCL-145 produced a full dose-response curve in the 55°C warm water tail-flick test and was equipotent to morphine. The agonist properties of MCL-145 were antagonized by the μ-selective antagonist β-funaltrexamine and the κ-selective antagonist nor-binaltorphimine. MCL-145 also acted as a μ antagonist, as measured by the inhibition of morphine-induced antinociception.

Research over the past several years has demonstrated the utility of using κ opioid receptor agonists as part of the continuing search for viable treatment options for cocaine abuse. κ Agonists and μ antagonists are known to inhibit dopamine release in the nucleus accumbens (Maisonneuve et al., 1994), a region of the brain that has high levels of κ opioid receptors (Mansour et al., 1987, 1988, 1994) and the endogenous opioid peptide dynorphin (Chavkin et al., 1982; Hokfelt et al., 1984). κ Agonists have also been shown to decrease striatal dopamine levels in rats, whereas cocaine and μ opioid receptor agonists increase dopamine levels (DiChiara and Imperato, 1988; Spanagel et al., 1992; Devine et al., 1993). Based on the knowledge that κ agonists and μ antagonists modulate dopamine levels, Archer et al. (1996) postulated that compounds with mixed κ and μ opioid activity may have particular utility in the development of potential pharmaco-therapeutic options for the treatment of cocaine abuse.

Early research in both nonhuman primates and rats showed that κ agonists could functionally antagonize many cocaine-induced behaviors, including hyperactivity (Ukai et al., 1994; Crawford et al., 1995), place preference (Suzuki et al., 1992; Crawford et al., 1995; Shippenberg et al., 1996), self-administration (Glick et al., 1995; Negus et al., 1997; Mello and Negus, 1998), and sensitization to hyperactivity and stereotypes (Shippenberg et al., 1996). Administration of κ agonists also attenuated the reinstatement of extinguished drug-taking behavior, in an animal model of relapse.
(Schenk et al., 1999, 2000). Although highly selective $\kappa$ agonists did have utility for attenuating many cocaine-induced behaviors, these selective agonists produced many severe undesirable side effects. The highly efficacious and selective $\kappa$ agonists enadoline, U50,488, and spiradoline produced salivation, emesis, and sedation in nonhuman primates (Negus et al., 1997; Mello and Negus 1998).

Bowen et al. (2003) recently reported on a series of compounds with mixed $\kappa$ and $\mu$ activity. Included in the report are the morphinans MCL-101 and cyclophan and the benzomorphan Mr2034. These compounds decreased cocaine self-administration in rhesus monkeys and produced fewer side effects than the $\kappa$-selective agonist enadoline (Bowen et al., 2003). It was also noted that compounds with mixed $\kappa$ agonist and $\mu$ agonist activity produced a more sustained decrease in cocaine self-administration than compounds with $\kappa$ agonist and $\mu$ antagonist properties (Bowen et al., 2003).

Our current data focused on a new compound, MCL-145, which was a mixed $\kappa$ agonist and $\mu$ agonist/antagonist. MCL-145 is a novel bivalent ligand related to the morphinan (-)-cyclophan and its N-cyclobutylmethyl derivative MCL-101. This compound had high affinity for both the $\kappa$ and $\mu$ opioid receptors. The present study characterizes the pharmacological properties of MCL-145 in the $[^{35}S]$GTP$\gamma$S binding assay and in mouse antinociceptive tests.

Materials and Methods

In Vitro Studies

$[^{35}S]$GTP$\gamma$S Binding Studies to Measure Opioid Receptor Coupling to G Proteins. Membranes from Chinese hamster ovary cells stably expressing either the human $\kappa$ (L. Toll, Stanford Research Institute, Palo Alto, CA) or $\mu$ (G. Uhl, National Institute on Drug Abuse, Baltimore, MD) opioid receptor were used in the experiments. Native CHO membranes were not transfected with any of the human opioid receptors, but membrane preparations were prepared in the same manner. Cells were scraped from tissue culture plates and were centrifuged at 200g for 10 min at 4°C. The cells were resuspended in phosphate-buffered saline, pH 7.4, containing 0.04% EDTA. After centrifugation at 200g for 10 min at 4°C, the pellet was resuspended in membrane buffer, which consisted of 50 mM Tris-HCl, 3 mM MgCl$_2$, and 1 mM EGTA, pH 7.4. The membranes were homogenized with a Dounce homogenizer, followed by centrifugation at 39,000g for 20 min at 4°C. The membrane pellet was resuspended in membrane buffer, and the centrifugation step was repeated. The membranes were resuspended in assay buffer, which consisted of 50 mM Tris-HCl, 3 mM MgCl$_2$, 100 mM NaCl, and 0.2 mM EGTA, pH 7.4.

Either the hKOR-CHO (15 $\mu$g of protein/tube), hMOR-CHO (10 $\mu$g of protein/tube), or native CHO (15 $\mu$g of protein/tube) membranes were incubated with 12 different concentrations of the agonist in assay buffer for 60 min at 30°C in a final volume of 0.5 ml. The reaction mixture contained 3 $\mu$M GDP and 0.080 nM $[^{35}S]$GTP$\gamma$S. Basal activity was determined in the presence of 3 $\mu$M GDP and in the absence of agonist, and nonspecific binding was determined in the presence of 10 $\mu$M unlabeled GTP$\gamma$S. After the 60-min incubation, the membranes were filtered onto 32 glass fiber filters (Whatman Schleicher and Schuell, Keene, NH) by vacuum filtration, followed by three washes with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5. Samples were counted in 2 ml of Ecoscint A scintillation fluid (Natl Diagnostics, Atlanta, GA). Data are presented as the percentage of agonist stimulation of $[^{35}S]$GTP$\gamma$S binding over the basal activity, defined as [(specific binding/basal binding) $\times$ 100] 100. All experiments were repeated three times and performed in triplicate.

In Vivo Studies

Animals. Male ICR mice (20–30 g) (Harlan, Indianapolis, IN) were housed in groups of five with food and water available ad libitum before any procedures. Animals were maintained on a 12-h light/dark cycle in a temperature-controlled animal colony. Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Chemicals. MCL-145 and MCL-101 were synthesized as described previously (Neumeyer et al., 2000, 2003). MCL-145 and MCL-101 were initially solubilized in dimethyl sulfoxide, and all subsequent dilutions were performed in distilled water. Morphine sulfate was purchased from Mallinckrodt (St. Louis, MO). ICI 174,864, $\beta$-FNA, and nor-BNI were purchased from Sigma-Aldrich (St. Louis, MO).

Injections. Intracerebroventricular (i.c.v.) injections were performed as described previously (Porreca et al., 1984). Briefly, mice were lightly anesthetized with ether, and an incision was made in the scalp. An injection was made with a 10-$\mu$l Hamilton syringe at a point 2 mm caudal and 2 mm lateral from bregma. Compounds were injected at a depth of 3 mm in a volume of 5 $\mu$l.

Antinociceptive Testing. Antinociception was assessed using the 55°C warm-water tail-flick test or the acetic acid writhing test. For the tail-flick test, the latency to the first sign of a rapid tail-flick was taken as the behavioral endpoint (Jannsen et al., 1983). Each mouse was first tested for baseline latency by immersing its tail in the water and recording the time to response. Mice not responding within 15 s were excluded from further testing. Mice were then administered the test compound and tested for antinociception 20 min after the injection. A maximum score was assigned (100%) to animals not responding within 15 s to avoid tissue damage. Antinociception was calculated by the following formula: % antinociception = 100 $\times$ [test latency − control latency]/(15 − control latency). For the acetic acid writhing test, mice were injected i.p. with 0.6% acetic acid in a volume of 10 ml/kg body weight. They were then placed in a clear Plexiglas observation jar and the number of abdominal writhes recorded for 15 min (Mogil et al., 1999). Percentage of antinociception was calculated using the formula % antinociception = 100 − [(no. writhes individual mouse/mean no. writhes control group) $\times$ 100].

Agonist Effects of MCL-145. To further determine the in vivo opioid receptor profile of MCL-145, mice were pretreated with a $\mu$- ($\beta$-FNA, 20 nmol i.c.v., −24 h), $\delta$- (ICI-174,864, 4 nmol i.c.v., −20 min), or $\kappa$-selective antagonist. Control mice received a vehicle injection (5 $\mu$l of distilled water, i.c.v., −24 h or −20 min). Then, mice received MCL-145 (5 nmol i.c.v.). Antinociception was assessed 20 min after agonist injection.

Antagonist Effects of MCL-145. Mice were treated concomitantly with morphine sulfate (3 nmol i.c.v.) and varying doses of MCL-145 i.c.v. Control mice received a vehicle injection (5 $\mu$l of distilled water i.c.v., −20 min). Antinociception was assessed 20 min after agonist injection.

Statistical Analysis. IC$_{50}$ values were calculated by least-squares fit to a logarithm-probit analysis. All dose-response lines were analyzed using the regression methods described by Tallarida and Murray (1986). Regression lines, ED$_{50}$ (dose producing 50% antinociception) values, and 95% CL were determined (Tallarida and Murray, 1986). All data points shown are the mean of seven to 10 mice, with standard error of the mean represented by error bars. Statistical analysis of the $[^{35}S]$GTP$\gamma$S binding data and the antinociceptive data used the Student’s $t$ test.

Results

In Vitro Studies

Affinity, Selectivity, and Efficacy of MCL-145. The binding of the novel bivalent morphinan MCL-145 (Fig. 1) to all the opioid receptors was measured. MCL-145 had a $K_i$
value of less than 0.08 nM for inhibiting the binding of [3H]U69,593 to the κ receptor in CHO membranes stably transfected with the human opioid receptor. MCL-145 had a 2- and 120-fold lower affinity for the μ and δ receptors, respectively (Table 1) (Neumeyer et al., 2003).

To characterize the efficacy of MCL-145, the [35S]GTPγS binding assay was used. CHO membranes stably transfected with the human κ and μ opioid receptors were used for this assay. The native CHO membranes were not transfected with any of the human opioid receptors. MCL-145 produced an E_{max} value, percentage of maximal stimulation, of 80 ± 2% for the κ opioid receptor (Fig. 2) and 42 ± 14% for the μ opioid receptor (Fig. 2). The EC_{50} values obtained for this compound were 4.3 ± 0.5 and 3.1 ± 0.3 nM for the κ and μ opioid receptors, respectively. The well characterized κ agonist U69,593 produced an E_{max} value of 77 ± 11% with an EC_{50} value of 36 ± 5 nM. The μ-selective agonist [d-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin produced an E_{max} value of 116 ± 4% with an EC_{50} value of 55 ± 7 nM. MCL-145 produced an unusual inverted U-shaped curve in the [35S]GTPγS binding assay. At low concentrations, less than 100 nM, MCL-145 produced a dose-dependent increase in stimulation at both the μ and κ receptors. An apparent antagonism was observed at concentrations greater than 100 nM, MCL-145 produced a dose-dependent increase in stimulation at both the μ and κ receptors, respectively (Table 1) (Neumeyer et al., 2003).

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MCL-101 (Fig. 1) is the monomeric parent compound of MCL-145 and produced similar maximal stimulation of [35S]GTPγS binding. Interestingly, MCL-101 showed no antagonism, even at higher concentrations. MCL-101 produced E_{max} values of 64 ± 13 and 102 ± 8% for the μ and κ receptors (Fig. 3). The EC_{50} value for the stimulation of [35S]GTPγS binding mediated by the κ and μ receptor was 10 ± 2.7 and 31 ± 8.2 nM, respectively.

### TABLE 1

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<tr>
<th>Compound</th>
<th>K_i (nM)</th>
<th>Selectivity</th>
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<tr>
<td>[3H]DAMGO (μ)</td>
<td>0.20 ± 0.032</td>
<td>9.4 ± 0.54</td>
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<tr>
<td>[3H]Naltrindole (δ)</td>
<td>2.7 ± 0.11</td>
<td>5.9 ± 0.55</td>
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<tr>
<td>[3H]U69,593 (κ)</td>
<td>8.2 ± 0.7</td>
<td>3 70</td>
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MCL-145 had high af-
finity for the \( \mu \) receptor, as demonstrated by the receptor binding assays. Therefore, the antinociceptive properties of MCL-145 were analyzed in the 55°C warm-water tail-flick test. MCL-145 produced a full dose-response curve in this assay, with an ED\(_{50}\) value and 95% CL of 0.27 nmol (0.13–0.58 nmol) as seen in Fig. 4. Also, MCL-145 was equipotent with morphine in the tail-flick assay (Fig. 4). The monomeric ligand MCL-101 had an ED\(_{50}\) value and 95% CL of 7.3 nmol (5.7–9.4 nmol) (Fig. 4). The duration of action for MCL-145 was very short with most of the antinociceptive activity being lost by 30 min when a 5-nmol dose was administered i.c.v. (Fig. 5). The parent compound MCL-101 had a slightly longer duration of action (Fig. 5). The antinociception produced by MCL-145 was significantly inhibited by 24-h pretreatment with both the \( \mu \)-selective antagonist \( \beta \)-FNA and the \( \kappa \)-selective antagonist nor-BNI (Fig. 6). The \( \delta \)-selective agonist ICI 174,864 had no effect on the antinociceptive properties of MCL-145. The dose of ICI 174,864 used in these experiments had been shown previously to inhibit antinociception produced by a \( \delta \)-selective agonist (Bilsky et al., 1995). The antagonist results observed with MCL-145 are similar to those seen with MCL-101, which was also antagonized by both \( \beta \)-FNA and nor-BNI (Neumeyer et al., 2000).

**Antagonist Properties of MCL-145.** To assess the antagonist activity of MCL-145 at the \( \mu \) receptor, morphine and MCL-145 were administered concomitantly, and antinociception was assessed 20 min after the injection. Figure 7 shows that a low dose, 0.05 nmol, of MCL-145 antagonized morphine-induced antinociception. These data demonstrated that MCL-145 acted as a \( \mu \) antagonist at low doses. In vivo, MCL-101 demonstrated no antagonist activity when administered with morphine (Neumeyer et al., 2000). Likewise, MCL-101 did not exhibit antagonism in the \[^{35}S\]GTP\(\gamma\)S binding assay.

MCL-145 was also tested for antagonist properties when administered with the \( \kappa \)-selective agonist U50,488 (Fig. 8). The acetic acid writhing assay was used in this set of experiments as \( \kappa \)-selective agonists are more sensitive to chemically induced nociception compared with thermal nociception. In vivo, 0.05 nmol of MCL-145 did not antagonize U50,488-induced antinociception. However, this dose of MCL-145 was sufficient to antagonize all morphine doses tested. Additionally, doses of MCL-145 up to 5 nmol had no antagonistic effect on U50,488-induced antinociception (data not shown).

**Discussion**

Several studies using nonhuman primates have demonstrated the efficacy of using a \( \kappa \) agonist and a \( \mu \) antagonist to attenuate many cocaine-induced behaviors. The advantage of using a compound with activity at both receptors is that a reduction in side effects is seen, compared with a highly selective \( \kappa \) agonist (Negus et al., 1997; Mello and Negus, 1998; Bowen et al., 2003). Of particular interest was the previous data obtained with MCL-101. The study demonstrated that MCL-101 dose dependently produced a sustained decrease in cocaine self-administration with minimal side effects (Bowen et al., 2003). The promising results obtained in vivo with MCL-101 prompted continuing development of novel ligands which would bind the \( \mu \) and \( \kappa \) receptors (Neumeyer et al., 2003).

MCL-145 is related to the morphinan cyclorphan and its N-cyclobutylmethy derivative MCL-101. The novel bivalent ligand MCL-145 was synthesized by coupling two identical pharmacophores of MCL-101 with a connecting spacer at the 3-hydroxy position (Neumeyer et al., 2003). Previous data obtained both in vitro and in vivo with MCL-101 demonstrated that this compound had high affinity at both \( \mu \) and \( \kappa \) receptors (Neumeyer et al., 2000). One question of interest in synthesizing MCL-145 was whether the bivalent ligand would have altered pharmacology or advantages compared with the monovalent parent compound.

MCL-145 demonstrated interesting pharmacological properties. In the \[^{35}S\]GTP\(\gamma\)S binding assay, which is a measure

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**Fig. 4.** Dose-response curves for i.c.v. MCL-145, morphine, and MCL-101 in the 55°C warm-water tail-flick test. Antinociception was assessed 20 min after agonist injection.

**Fig. 5.** Time-response curves for i.c.v. (▼) MCL-145 and (●) MCL-101. Antinociception was tested in the 55°C tail-flick test 20 min after agonist injection.
of G protein activation, the compound produced dose-dependent agonist and antagonist stimulation for the μ and κ receptors. However, high concentrations of MCL-145 also inhibited [35S]GTPγS binding below basal in native CHO membranes. This would indicate that much of the apparent antagonism and basal inhibition of [35S]GTPγS binding was not receptor mediated and was perhaps a direct effect on the G protein, or may be a result of nonspecific tissue binding. In vivo, MCL-145 acted as a full agonist at the κ receptor, whereas it had mixed agonist/antagonist activity at the μ receptor. The bivalent structure of MCL-145 significantly changed its pharmacological characteristics in comparison with the monovalent parent compound MCL-101. MCL-101 showed no antagonism in the [35S]GTPγS binding assay, but it did produce similar $E_{\text{max}}$ values. In vivo, MCL-101 acted as an agonist at both the μ and κ receptors, but it showed no antagonist properties (Neumeyer et al., 2000). It seems that the bivalent nature of MCL-145 allows it to interact in a different manner with both the μ and κ receptors.

There are no data to indicate the exact mechanism of how MCL-145 is interacting with the receptor. It is possible that one pharmacophore of the ligand is binding the receptor and that the spacer and adjacent pharmacophore are left free extracellularly. Alternatively, the compound may be metabolized to monomers, each with the ability to bind receptor. However, it seems unlikely that these monomers are MCL-101, based on the in vitro and in vivo data obtained with MCL-145. If MCL-145 is being metabolized to monomers, it is

![Graph showing agonist properties of MCL-145 at the μ, δ, and κ receptors. Antinociceptive responses in the 55°C tail-flick test in mice pretreated with i.c.v. μ-, δ-, or κ-selective opioid antagonists. Pretreatment time for nor-BNI and β-FNA was -24 h. ICI 174,864 pretreatment time was -20 min before agonist injection. Antinociceptive testing was done 20 min after MCL-145 injection. **, $P < 0.01$ in comparison with MCL-145 alone.](image1)

![Graph showing MCL-145 antagonism of morphine-induced antinociception. Mice were injected concomitantly with varying doses of morphine i.c.v. and 0.05 nmol of MCL-145. Antinociception was assessed 20 min after agonist injection in the 55°C tail-flick test. *, $P < 0.05$ and **, $P < 0.01$ in comparison with morphine dose alone.](image2)
also possible that one of the monomeric pharmacophores is left with a portion of the spacer region. In this configuration, it is plausible that the activity of the ligand would be different from that of MCL-101. It is also not unreasonable to postulate that MCL-145 is capable of binding and bridging two receptors. There is evidence to suggest that G protein-coupled receptors, and in particular opioid receptors, are capable of both homo- and heterodimerization. In the endogenous state, the \( \mu \) and \( \kappa \) receptors may not be complexed, but with the addition of a bivalent ligand, such as MCL-145, they could be capable of forming dimers. The concept of G protein-coupled receptor dimerization is still a relatively new field of study with many interesting implications. The role of opioid receptors may not be complexed, but \( \kappa \)-opioid receptors in modulation of basal mesolimbic dopamine release: in vivo microdialysis studies. J Pharmacol Exp Ther 296: 1236–1246.


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Address correspondence to: Dr. Jean M. Bidlack, Department of Pharmacology and Physiology, Box 711, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642-8711. E-mail: jean_bidlack@urmc.rochester.edu