Ring-Constrained Orvinols as Analogs of Buprenorphine: Differences in Opioid Activity Related to Configuration of C20 Hydroxy Group†

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ABSTRACT

The relative positions of the C20 substituents in buprenorphine, particularly the hydroxyl group, have been implicated in its actions as a partial µ-agonist and a κ-antagonist (for review, see Cowan, 1995). The compound has a unique pharmacology that has led to the serious consideration of buprenorphine as a drug for the treatment of opiate abuse in the United States (Segal and Schuster, 1995) and in France (Henrion, 1997). Particularly relevant properties include a slow rate of dissociation from opioid receptors (Hambrook and Rance, 1976), which could prevent the disruption to homeostasis caused by abrupt withdrawal, and a level of µ-efficacy that provides some morphine-like effects but also a good margin of safety (Cowan, 1995). In addition in several in vivo tests, including antinociception, buprenorphine displays an unusual, but highly characteristic, bell-shaped dose-effect curve in rodents (Cowan et al., 1977a; Tyers, 1980; Bryant et al., 1983; Wheeler-Aceto and Cowan, 1991) and in the monkey (Woods et al., 1992). This effect probably contributes to the safety and reduced abuse liability of buprenorphine although the underlying mechanism has not been resolved. In vitro buprenorphine acts as a partial µ-agonist (Traynor and Nahorski, 1995; Toll et al., 1998), but has little or no agonist properties at κ-receptors (Zhu et al., 1997; Toll et al., 1998) and no agonist actions at δ-receptors (Kajiwara et al., 1986; Toll et al., 1998). Recent ligand-binding assays at cloned opioid receptors confirm that buprenorphine has high affinity for all three opioid receptor types with no selectivity (Toll et al., 1998). This suggests the differential pharmacological actions of buprenorphine at µ-, δ-, and κ-receptors but does influence κ-potency and κ-efficacy, particularly in vivo.

Buprenorphine (Fig. 1) is a potent, clinically useful analgesic agent. In vivo it is a partial µ-agonist and a κ-antagonist (for review, see Cowan, 1995). The compound has a unique pharmacology that has led to the serious consideration of buprenorphine as a drug for the treatment of opiate abuse in the United States (Segal and Schuster, 1995) and in France (Henrion, 1997). Particularly relevant properties include a slow rate of dissociation from opioid receptors (Hambrook and Rance, 1976), which could prevent the disruption to homeostasis caused by abrupt withdrawal, and a level of µ-efficacy that provides some morphine-like effects but also a good margin of safety (Cowan, 1995). In addition in several in vivo tests, including antinociception, buprenorphine displays an unusual, but highly characteristic, bell-shaped dose-effect curve in rodents (Cowan et al., 1977a; Tyers, 1980; Bryant et al., 1983; Wheeler-Aceto and Cowan, 1991) and in the monkey (Woods et al., 1992). This effect probably contributes to the safety and reduced abuse liability of buprenorphine although the underlying mechanism has not been resolved. In vitro buprenorphine acts as a partial µ-agonist (Traynor and Nahorski, 1995; Toll et al., 1998), but has little or no agonist properties at κ-receptors (Zhu et al., 1997; Toll et al., 1998) and no agonist actions at δ-receptors (Kajiwara et al., 1986; Toll et al., 1998). Recent ligand-binding assays at cloned opioid receptors confirm that buprenorphine has high affinity for all three opioid receptor types with no selectivity (Toll et al., 1998). This suggests the differential pharmacological actions of buprenorphine at µ-, δ-, and κ-receptors but does influence κ-potency and κ-efficacy, particularly in vivo.

ABBREVIATIONS: BU46, N-cyclopropylmethyl-[7α,8α,2',3']-cyclopentanolo-1’[R]-hydroxy-6,14-endo-ethenotetrahydronororipavine; BU47, N-cyclopropylmethyl-[7α,8α,2',3']-cyclopentanolo-1’S)-hydroxy-6,14-endo-ethenotetrahydronororipavine; GTP·S, guanosine-5′-O-(3-thiotriphosphate; DPDPE, [d-Pen2, d-Pen5]enkephalin; DAMGO, [(d-Ala2,MePhe4,Gly-ol5]enkephalin; [35S]guanosine-5′-O-(3-thiotriphosphate assay in SH-SY5Y cells. The major difference between the isomers was an 11- to 12-fold higher potency of the β-OH isomer (BU46) compared with the α-OH isomer (BU47) at the κ-receptor in the guinea pig ileum and CHO-hkor cells and a somewhat higher efficacy of BU46 in CHO-hkor cells. BU46 and BU47 were evaluated in vivo. BU46 was a full agonist in the mouse writhing assay and antinociception was prevented by norbinaltorphimine, showing a κ-mechanism of action. In contrast, BU47 acted as an antagonist of µ-, δ-, and κ-mediated antinociception in the writhing assay. The results show that the configuration of the hydroxyl group is not important in binding affinity at µ-, δ-, or κ-receptors but does influence κ-potency and κ-efficacy, particularly in vivo.

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Materials and Methods

Animals. For the binding assays and in vitro assays, male CSI mice (25–30 g; Nottingham University Medical School) and male Duncan-Hartley guinea pigs (250–500 g; David Hall, Burton on Trent, UK) were used. For the in vivo assays male, National Institutes of Health Swiss mice (20–36 g; Harlan Sprague-Dawley, Indianapolis, IN) were used. Animals were fed on standard laboratory diet and kept on a 12 h light/dark cycle at a temperature of 20°C. Studies were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocol was approved by the University of Michigan University Committee on the Use and Care of Animals.

Chemicals and Drugs. [35S]GTPγS (guanosine-5′-O-3-[35S]thio triphosphate) and [3H]DPDPE ([3H]-[D-Pen2,D-Pen5]enkephalin) were from New England Nuclear (Stevenage, UK). [3H]DAMGO ([3H]-[D-Ala2,MePhe4,Gly-ol5]enkephalin) and [3H]IC977 ([3H]-SR-(5a,7a,β)-N-methyl-N-[7-(1-phenyl)oxycyclopentano-6,14-yl]benzofurancetamide-HCl) were from Amersham International (Aylesbury, UK). Fentanyl, DAMGO, DPDPE, naloxone, and U69593 (5a,7a,β)-N-methyl-N-[7-(1-phenyl)oxycyclopentano-6,14-yl]-benzeneacetamide were from Sigma Chemical Co. (Poole, UK). Naltrindole (NTI) and norbinaltorphimine (norBNI) were from Research Biochemicals Inc. (St. Albans, UK) and morphine sulfate was from Mallinckrodt Inc. (St. Louis, MO). The following were gifts as indicated: bremazocine (Sandoz, Basel, Switzerland), methocin-namox (M-CAM; Reckitt and Colman, Hull, UK) and SNC890 ([+H-[(5a,7a,β)-N-cyclopropylcarbonyl-(7S)-7-(1-pyrrolidinyl)-3-methoxybenzyl]-N,N-dimethylbenzamide] (Dr. K. C. Rice, National Institutes of Health, Bethesda, MD). Minimal essential medium, fetal calf serum, fungizone, penicillin/streptomycin, and L-glutamine were from Gibco BRL ( Paisley, Scotland). All other chemicals were from Sigma Chemical Co. and were of analytical grade.

Ring-Constrained Orvinols. BU46 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentane-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronororipavine) and BU47 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentane-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronoripavine) were synthesized by lithium aluminum hydride reduction of N-cy clopropylcarbonyl-[7a,8a,2’,3’]-1’-oxocyclopentano-6,14-endo-ethe notetrahydronorthebaine (Barton et al., 1993), which gave an equal mixture of the corresponding N-cyclopropylmethyl-1’-secondary alcohols. After separation by column chromatography, 3-O-demethylation with sodium propane thiolate gave BU46 and BU47. The 1’-β-H of BU47 gave rise to a signal at 4.5 ppm, whereas the 1’-α-H of BU46 was observed at 3.6 ppm. The 1’-α-β peak in the infrared (IR) was at 3582 cm⁻¹ and the 1’-α-α at 3528 cm⁻¹. BU95 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronororipavine) and BU96 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronoripavine) were synthesized by lithium aluminum hydride reduction of N-cyclopropylcarbonyl-[7a,8a,2’,3’]-1’-oxocyclopentano-6,14-endo-ethenotetrahydronorthebaine (Barton et al., 1993), which gave an equal mixture of the corresponding N-cyclopropylmethyl-1’-secondary alcohols. After separation by column chromatography, 3-O-demethylation with sodium propane thiolate gave BU46 and BU47. The 1’-β-H of BU47 gave rise to a signal at 4.5 ppm, whereas the 1’-α-H of BU46 was observed at 3.6 ppm. The 1’-α-β peak in the infrared (IR) was at 3582 cm⁻¹ and the 1’-α-α at 3528 cm⁻¹. BU95 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronororipavine) and BU96 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronoripavine) were synthesized by lithium aluminum hydride reduction of N-cyclopropylcarbonyl-[7a,8a,2’,3’]-1’-oxocyclopentano-6,14-endo-ethenotetrahydronorthebaine (Barton et al., 1993), which gave an equal mixture of the corresponding N-cyclopropylmethyl-1’-secondary alcohols. After separation by column chromatography, 3-O-demethylation with sodium propane thiolate gave BU46 and BU47. The 1’-β-H of BU47 gave rise to a signal at 4.5 ppm, whereas the 1’-α-H of BU46 was observed at 3.6 ppm. The 1’-α-β peak in the infrared (IR) was at 3582 cm⁻¹ and the 1’-α-α at 3528 cm⁻¹. BU95 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronororipavine) and BU96 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronoripavine) were synthesized by lithium aluminum hydride reduction of N-cyclopropylcarbonyl-[7a,8a,2’,3’]-1’-oxocyclopentano-6,14-endo-ethenotetrahydronorthebaine (Barton et al., 1993), which gave an equal mixture of the corresponding N-cyclopropylmethyl-1’-secondary alcohols. After separation by column chromatography, 3-O-demethylation with sodium propane thiolate gave BU46 and BU47. The 1’-β-H of BU47 gave rise to a signal at 4.5 ppm, whereas the 1’-α-H of BU46 was observed at 3.6 ppm. The 1’-α-β peak in the infrared (IR) was at 3582 cm⁻¹ and the 1’-α-α at 3528 cm⁻¹. BU95 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronororipavine) and BU96 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronoripavine) were synthesized by lithium aluminum hydride reduction of N-cyclopropylcarbonyl-[7a,8a,2’,3’]-1’-oxocyclopentano-6,14-endo-ethenotetrahydronorthebaine (Barton et al., 1993), which gave an equal mixture of the corresponding N-cyclopropylmethyl-1’-secondary alcohols. After separation by column chromatography, 3-O-demethylation with sodium propane thiolate gave BU46 and BU47. The 1’-β-H of BU47 gave rise to a signal at 4.5 ppm, whereas the 1’-α-H of BU46 was observed at 3.6 ppm. The 1’-α-β peak in the infrared (IR) was at 3582 cm⁻¹ and the 1’-α-α at 3528 cm⁻¹. BU95 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronororipavine) and BU96 (N-cyclopropylmethyl-[7a,8a,2’,3’]-cyclopentano-5,5’-dimethyl-1-[1’]-hydroxy-6,14-endo-ethenotetrahydronoripavine) were synthesized by lithium aluminum hydride reduction of N-cyclopropylcarbonyl-[7a,8a,2’,3’]-1’-oxocyclopentano-6,14-endo-ethenotetrahydronorthebaine (Barton et al., 1993), which gave an equal mixture of the corresponding N-cyclopropylmethyl-1’-secondary alcohols. After separation by column chromatography, 3-O-demethylation with sodium propane thiolate gave BU46 and BU47. The 1’-β-H of BU47 gave rise to a signal at 4.5 ppm, whereas the 1’-α-H of BU46 was observed at 3.6 ppm. The 1’-α-β peak in the infrared (IR) was at 3582 cm⁻¹ and the 1’-α-α at 3528 cm⁻¹.
7. After centrifugation at 25,000g for 15 min, the pellet was resuspended in 10 ml of buffer/g of tissue and incubated at 37°C for 30 min to remove endogenous opioid ligands. The homogenates were then recentrifuged and the pellets resuspended in Tris-HCl buffer at a final protein concentration of 500 μg/ml (Lowry et al., 1951).

Membrane protein (~450 μg) was incubated in Tris-HCl (pH 7.4) with 1 nM [3H]DAMGO, 2 nM [3H]DPDPE, or 0.5nM [3H]C1977 in a final volume of 1 ml. After 1 h at 25°C, the mixture was rapidly vacuum-filtered through GF/B filters to separate bound from free ligand and the filters were rinsed three times with 3 ml of ice-cold buffer (Tris-HCl, pH 7.4). Radioactivity retained on the filters was determined by liquid scintillation counting. Nonspecific binding was defined as the binding remaining in the presence of 10 μM naloxone. Specific binding was typically >80% of total binding at the radioligand Kᵦ. Competition data was analyzed by nonlinear curve fitting (GraphPad Software, San Diego, CA) to give IC₅₀ values that were converted to affinity (Kᵦ) values with the Cheng and Prusoff (1973) equation.

**Bioassays.** Segments of ileum were removed from male Dunkin-Hartley guinea pigs and placed in Krebs’ solution containing 118 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl₂ - 2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ - 7H₂O, 25 mM NaHCO₃, and 11 mM glucose. Vasa deferentia from male CSI mice were placed in Krebs’ solution minus MgSO₄ - 7H₂O. Mouse vasa deferentia (MVD) and myenteric plexus-longitudinal muscle preparations of the guinea pig ileum (GPI) were set up for field stimulation as previously described (Traynor et al., 1987). Concentration-effect curves for the inhibition of electrically induced contractions were constructed by cumulative addition of agonists to the bathing fluid. EC₅₀ and maximal values were computed with GraphPad Prism (GraphPad Software). Antagonist equilibrium dissociation constants (Kᵦ, nM) were determined from the ratios of EC₅₀ values for agonists in the absence or presence of antagonist (added 15 min before the redetermination of agonist concentration-response curves) with the formula Kᵦ = [agonist]/dose-ratio-1 (Kosterlitz and Watt, 1968).

[³⁵S]GTP·γ·S Binding Assay. Undifferentiated human neuroblastoma SH-SY5Y cells (passage number 78–100) were cultured in minimum essential medium supplemented with 10% fetal calf serum and antibiotics, as described previously (Traynor and Nahorski, 1995). Chinese hamster ovary (CHO) cells expressing the human α-opioid receptor (CHO-hkor) were cultured in Dulbecco’s modified Eagle’s medium/F-12 with 10% fetal calf serum and 0.2mg/ml geneticin (Zhu et al., 1997). Cells were grown in monolayers to confluency at 37°C in a humidified 5% CO₂ atmosphere. The cells were harvested in HEPES (20 mM, pH 7.4)-buffered saline containing 1 mM ethylenediaminetetraacetic acid, dispersed by agitation, and collected by centrifugation at 500g. The cell pellet was suspended in a buffer of 20 mM HEPES, pH 7.4, 100 mM NaCl, and 10 mM MgCl₂·6H₂O (buffer A) and homogenized with a tissue tearor (BioSpec Products, Inc., Bartlesville, OK). The resultant homogenate was centrifuged at 50,000g and the pellet collected, washed in buffer A, and recentrifuged. The pellet was finally resuspended in buffer A to give a protein concentration of between 100 and 200 μg/ml (Lowry et al., 1951). All procedures were performed at 4°C. Freshly prepared membranes (100–200 μg protein) were incubated in buffer A containing [³⁵S]GTP·γ·S (80 PM), GDP (3 μM), and varying concentrations of test compound in a total volume of 1 ml, for 60 min at 30°C as described previously (Traynor and Nahorski, 1995). Nonspecific binding was defined with unlabeled GTP·γ·S (10 μM). Bound and free [³⁵S]GTP·γ·S were separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting. Specific binding was 90 to 95% of total binding. The EC₅₀ for stimulation of [³⁵S]GTP·γ·S binding obtained at various drug concentrations was determined from nonlinear curve fitting of the data (GraphPad Software) with maximal stimulation of [³⁵S]GTP·γ·S determined with 10 μM fentanyl (SH-SY5Y) or 10 μM U69593 (CHO-hkor cells). Antagonist equilibrium affinity constants (Kᵦ) were calculated from [³⁵S]GTP·γ·S binding assay concentration-effect curves in the absence or presence of a single concentration of antagonist as described for the bioassays.

**Antinociception.** The acetic acid-induced writhing assay was carried out as previously described (Hong et al., 1998) to determine the degree of antinociception. Briefly, mice received an i.p. injection of 0.6% acetic acid and were placed in individual Plexiglas boxes (18 x 28 x 13 cm) for observation. Five minutes after the acetic acid injection, a 5-min observation period was initiated during which time the number of writhes, typically a wave of contraction of the abdominal musculature followed by extension of the hind legs, was recorded. Vehicle or test drugs were administered s.c. 15 min before acetic acid. Antagonists were administered s.c. as follows: norBNI (32 mg/kg, 24-h pretreatment), NTI (10 mg/kg, 15-min pretreatment), and M-CAM (1.8 mg/kg, 1-h pretreatment). Each treatment group consisted of six mice and each mouse was used for only one treatment. The control number of writhes per mouse was defined as the mean number of writhes per mouse when a s.c. injection of sterile water was given 15 min before the acetic acid injection. For treatment groups, the number of writhes for each mouse was expressed as the percentage control number of writhes per mouse.

**Results.** Five ring-constrained analogs of buprenorphine were synthesized and evaluated. These included two pairs of secondary alcohols epimeric at C₂₀ (C₁,) with (BU96, BU95) or without (BU46, BU47) 2’-methyl substitution equivalent to the β-buty group of buprenorphine. These methyl groups in the precursor ketone (Coop et al., 1995) prevented its Grignard addition to give a tertiary alcohol that would have been closer in structure to buprenorphine, but a tertiary alcohol (BU61) was synthesized without 2’-methyl groups. This has the C₂₀α-OH group corresponding to BU47. Stereochemistry was assigned from analysis of the proton NMR and IR spectra (Fulmor et al., 1967). The 1 β-H of BU47 gave rise to a NMR signal at 4.5 ppm, whereas the 1α-H of BU46 is shielded by the 18,19-double bond and was observed at higher field (3.6 ppm). Similarly, the 1α-OH peak in the IR (3552 cm⁻¹) was shifted relative to the 1 β-OH peak (3582 cm⁻¹). Comparable evidence was used to assign structures to BU95 and BU96. In the case of BU61, only one C-’ isomer was formed, and the expected 1α-OH stereochemistry (Bentley et al., 1967) was confirmed by the NMR resonance of H-19 at 5.26 ppm, and the 1’-OH peak in the IR at 3528 cm⁻¹, both of which were consistent with assignments in BU47.

**Ligand-Binding Assays.** Epimeric pairs of secondary alcohols BU 46 and 47 and BU 96 and 95, in addition to the tertiary alcohol BU 61, readily displaced the binding of ligands selective for opioid receptor types [³H]DAMGO (μ), [³H]DPDPE (δ), and [³H]C1977 (κ) from mouse brain membranes. There were no marked differences between the compounds at single receptor types or across receptor types and the compounds showed comparable affinities to the parent buprenorphine (Table 1). Similar results were obtained for μ-opioid binding in SH-SY5Y cell membranes (data not shown).

**Isolated Tissue Assays.** In the electrically stimulated MVD all five compounds were potent agonists affecting concentration-effect curves similar to the δ-peptide DPDPE. The 20R-epimer BU46 (β-OH) was 2- to 3-fold more potent than its corresponding 20S-epimer BU47 (α-OH) (Table 2). This difference was not seen with the C₉ dimethyl-substituted pair BU95 and BU96 where the α-OH isomer (BU95) was the more potent (Table 2). Naloxone (100 nM) and the δ-selective antagonists MOP and ORL-1 are more specific for the δ- and κ-opioid receptors, respectively.
antagonist NTI (1 nM) shifted the concentration-effect curves of all compounds to the right in a parallel fashion. Equilibrium affinity constants ($K_a$) calculated for naloxone and for NTI were in the range of affinity values calculated for these antagonists against the $\delta$-agonist DPDPE, rather than $\mu$-agonist DAMGO or the $\kappa$-agonist U69593 (Table 2), indicating the constrained oripavines were acting through $\delta$-receptors in this tissue.

In the myenteric plexus of the GPI, the compounds inhibited the electrically evoked twitch, with a slightly reduced calculated maximum compared with DAMGO or U69593 (Table 3). BU46 was $\sim 12$ times more potent than its epimer BU47, although this difference was reduced in the tertiary alcohol (BU61), and was not seen with the methyl-substituted epimeric pair BU95 and BU96 (Table 3). The activity of the compounds was antagonized by the $\kappa$-selective norBNI, which caused a parallel rightward shift in the concentration-effect curves. The $K_a$ value obtained for norBNI was the same as that obtained against the standard $\kappa$-agonist U69593, but very different from that obtained with the $\mu$-selective DAMGO as agonist (Table 3).

$[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ Binding Assay. Like buprenorphine, the ring-constrained oripavines were potent partial agonists in stimulating the binding of $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ to membranes of SH-SY5Y cells. All the compounds had similar potency, but with a greatly reduced maximal effect compared with the efficacious $\mu$-agonist fentanyl. Indeed, the compounds had a lower maximum than buprenorphine itself (Table 4). The degree of stimulation shown by the compounds was identical between pairs of epimers, suggesting a similar low level of $\mu$-efficacy. Naloxone shifted the dose-effect curve of BU47 to the right affording a $K_a$ value of $3.3 \pm 0.7$ nM, confirming the response was mediated via $\mu$-opioid receptors.

In CHO-hkor cells, BU46 and BU47 showed $\kappa$-agonist properties. BU46 ($EC_{50} = 0.18 \pm 0.04$ nM) was $\sim 11$ times more potent than BU47 ($EC_{50} = 2.02 \pm 0.52$ nM). Both compounds stimulated binding of $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ to a lesser extent than U69593 (Fig. 2) but BU46 had significantly greater relative efficacy with a maximal stimulation of $77.8 \pm 6.5\%$ compared with $54.2 \pm 3.2\%$ for BU47.

In Vivo Assay. Two of the ring-constrained epimers, BU46 and BU47, were examined for activity in the acetic acid-induced writhing assay in the mouse. BU46 afforded complete blockade of writhing at 0.1 mg/kg s.c. but BU47 only caused a 30% inhibition of writhing even at 32 mg/kg s.c. (Fig. 3, top). BU46 (0.1 mg/kg) was fully effective after 15 min, but its antinociceptive activity was short-lived and lasted <60 min (data not shown). The antinociceptive effect of BU46 was blocked by the $\kappa$-antagonist norBNI (32 mg/kg s.c. given 24 h previously (Fig. 3, bottom). Under these conditions, norBNI is selective for antagonism of $\kappa$-mediated antinociception (Broadbear et al., 1994). The selective $\delta$-antagonist NTI (10 mg/kg given 15-min pretreatment) did not antagonize BU46-mediated antinociception; the irreversible $\mu$-antagonist M-CAM (1.8 mg/kg given 1 h before testing) afforded some degree of reversal of the effect of BU46 (Fig. 3, bottom). The antinociceptive effectiveness of BU46 in the writhing assay was also antagonized by its isomer BU47 at 10 mg/kg s.c. (15-min pretreatment) (Fig. 3, bottom). At this same dose, BU47 also was able to antagonise the $\kappa$-agonist bremazocine, the $\mu$-agonist morphine, and the $\delta$-agonist SNC80 (Table 5).

### Discussion

The constrained oripavines, prepared as analogs of buprenorphine, acted as potent $\delta$-opioid agonists in the MVD preparation with an efficacy at least equal to that of the peptide DPDPE. To examine the relative efficacy of the compounds at $\mu$- and $\kappa$-receptors, the GPI preparation was used. In this preparation, the compounds were efficacious agonists. Studies with norBNI showed that the observed action was due to the $\kappa$-agonist properties of the compounds. The ileum is considered to have a high receptor reserve for $\mu$-agonists such that lower efficacy compounds, for example, morphine, behave as full agonists. This suggests the oripavines must have very low $\mu$-receptor efficacy. The ability of $\mu$-agonists to stimulate the binding of $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ to membranes from SH-SY5Y human neuroblastoma cells has been shown to be indicative of their degree of $\mu$-efficacy (Traynor and Nahor ski, 1995). With this assay, the cyclic analogs were confirmed to be very low efficacy $\mu$-agonists, but to have $\kappa$-agonist activity in CHO-hkor cells supporting their $\kappa$-agonistic activity in the GPI. In confirmation of their low $\mu$-efficacy, BU46 and BU47 do not substitute for morphine in morphine-dependent monkeys and indeed BU46 precipitates withdrawal with a potency similar to that of naloxone (Aceto et al., 1995, 1996).

Ligand-binding assays in mouse brain showed the constrained oripavines to have similar affinity across all three opioid receptor types. Thus, the apparent selectivity of the agonist actions of the compounds for $\kappa$-receptors rather than $\mu$-receptors in the GPI and for $\delta$-receptors rather than $\mu$- or $\kappa$-receptors in the MVD must be governed by efficacy differences. The constrained oripavines differ greatly from buprenorphine, which is considered a $\mu$-partial agonist but a $\kappa$-agonist. The contrast at the $\delta$-receptor was equally striking. In the MVD, the cyclic derivatives were potent full $\delta$-agonists but the action of buprenorphine in this tissue is mediated by $\mu$- and $\kappa$-receptors, not by the $\delta$-receptor (Kajiwara et al., 1986). Indeed at the $\delta$-receptor expressed in CHO or C6 rat glioma cells, buprenorphine has no agonist action (Toll et al., 1998; Lee et al., 1999).

In the mouse acetate-induced abdominal constriction, BU46 showed a potent antinociceptive action, completely preventing writhing at 0.1 mg/kg s.c. This effect was not observed with BU47, which was only weakly active even at a dose of 32 mg/kg s.c. The agonist action of BU46 was blocked.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\mu$</th>
<th>$\delta$</th>
<th>$\kappa$</th>
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<tr>
<td>BU 46</td>
<td>0.60 ± 0.05</td>
<td>0.86 ± 0.08</td>
<td>1.02 ± 0.10</td>
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<td>BU 47</td>
<td>0.88 ± 0.20</td>
<td>1.45 ± 0.19</td>
<td>2.75 ± 0.10</td>
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<td>BU 61</td>
<td>0.55 ± 0.06</td>
<td>1.78 ± 0.11</td>
<td>1.79 ± 0.10</td>
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<tr>
<td>BU 95</td>
<td>0.48 ± 0.19</td>
<td>1.41 ± 0.19</td>
<td>3.20 ± 1.00</td>
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<tr>
<td>BU 96</td>
<td>1.50 ± 0.15</td>
<td>1.31 ± 0.39</td>
<td>2.99 ± 0.50</td>
</tr>
<tr>
<td>Buprenorphine$^a$</td>
<td>0.57 ± 0.05</td>
<td>1.30 ± 0.03</td>
<td>2.00 ± 0.30</td>
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</tbody>
</table>

$^a$ Performed in guinea pig brain homogenates.

[Note: The table content is not transcribed due to the limitations of this format.]

[Note: The table content is not transcribed due to the limitations of this format.]
by the κ-antagonist norBNI and also was partially reversed by the long-lasting μ-antagonist M-CAM, but not at all by the δ-antagonist NTI. Thus, although BU46 behaves as a potent δ-agonist in the MVD it is its κ-oid receptor actions that are mainly responsible for the antinociception in vivo. Aceto et al. (1995) report copious urination in the mouse at a dose of 30 mg/kg, supporting a κ-agonist action of the compound. Thus, full δ-agonism in the MVD does not guarantee an effective antinociceptive agent in vivo through this receptor mechanism even in the sensitive mouse writhing assay. However, this may not be so for assays highly sensitive to δ-agonists, for example, allostynia (Butelman et al., 1995).

The results for BU46 are very different from those observed for buprenorphine, which in vivo acts as a partial μ-agonist. For example, buprenorphine-mediated antinociception in the mouse acetic acid abdominal constriction is antagonized by pretreatment with the irreversible μ-antagonist β-funaltrexamine (Zimmerman et al., 1987) and buprenorphine, like morphine, suppresses urine output (Cowan et al., 1977b). In contrast to BU46, BU47 acts as a κ-agonist in vivo. This is like buprenorphine, which in vivo blocks bremazocine-induced diuresis, U50488H-mediated antinociception, and precipitates a κ-abstinence syndrome (for review, see Cowan, 1995). However, BU47 also blocks μ- and δ-mediated responses.

There are several feasible explanations for the differences in the animal pharmacology of BU46 and BU47. First, they may not equally recognize the κ-receptor in vivo. Both compounds have similar affinity as determined by binding assays at central κ-receptors, labeled with [3H]CI977. In the GI, the compounds showed similar maximal effects, but BU46 was 12-fold more potent. In CHO-hkor cells, BU46 was 11-fold more potent and had somewhat higher efficacy. This efficacy difference appears to be greatly exaggerated in vivo because BU47 acts as an antagonist of BU46 and must therefore be binding to the κ-receptor, but unable to induce a response. It is possible that this is due to metabolism of the α-OH in BU47, but not the β-OH in BU46, to a compound that retains affinity but loses efficacy. However, this is unlikely because the major metabolites of buprenorphine are norbuprenorphine and buprenorphine-3-glucuronide, with no reported metabolites at the C20 hydroxyl position (Walter

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**TABLE 2**

Agonist actions of ring-constrained orvinols and standard opioids on the electrically induced contractions of the MVD and their antagonism by naloxone and naltrindole

<table>
<thead>
<tr>
<th>Agonist Activity</th>
<th>Inhibition of Twitch %</th>
<th>Naloxone M</th>
<th>Naltrindole M</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU46</td>
<td>1.3 ± 0.3</td>
<td>102.4 ± 1.6</td>
<td>50.8 ± 23</td>
</tr>
<tr>
<td>BU47</td>
<td>3.6 ± 0.3</td>
<td>112.4 ± 3.6</td>
<td>28.0 ± 12</td>
</tr>
<tr>
<td>BU61</td>
<td>2.5 ± 0.8</td>
<td>100.5 ± 6.7</td>
<td>24.5 ± 4.5</td>
</tr>
<tr>
<td>BU95</td>
<td>4.1 ± 1.6</td>
<td>91.5 ± 0.7</td>
<td>27.1 ± 6.3</td>
</tr>
<tr>
<td>BU96</td>
<td>7.2 ± 0.2</td>
<td>82.3 ± 14.8</td>
<td>14.8 ± 0.3</td>
</tr>
<tr>
<td>DAMGO</td>
<td>17.6 ± 2.0</td>
<td>100.9 ± 13.3</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>DPDPE</td>
<td>0.93 ± 0.04</td>
<td>108.1 ± 20.4</td>
<td>54.4 ± 2.1</td>
</tr>
<tr>
<td>U69593</td>
<td>28.4 ± 2.6</td>
<td>101.5 ± 2.6</td>
<td>19.6 ± 2.3</td>
</tr>
</tbody>
</table>

Values were determined as described in Materials and Methods and represent means ± S.E. from three separate tissues.

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**TABLE 3**

Agonist actions of ring-constrained orvinols and standard opioids on the electrically induced contractions of the myenteric plexus-longitudinal muscle preparation of the GPI and their antagonism by norBNI

<table>
<thead>
<tr>
<th>Agonist Activity</th>
<th>Inhibition of Twitch %</th>
<th>norBNI M</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU46</td>
<td>0.3 ± 0.2</td>
<td>81.6 ± 0.6</td>
</tr>
<tr>
<td>BU47</td>
<td>3.6 ± 1.3</td>
<td>82.9 ± 3.7</td>
</tr>
<tr>
<td>BU61</td>
<td>1.7 ± 0.5</td>
<td>85.1 ± 7.8</td>
</tr>
<tr>
<td>BU95</td>
<td>2.0 ± 0.2</td>
<td>87.1 ± 6.6</td>
</tr>
<tr>
<td>BU96</td>
<td>3.7 ± 1.2</td>
<td>71.8 ± 4.4</td>
</tr>
<tr>
<td>DAMGO</td>
<td>5.6 ± 1.8</td>
<td>99.0 ± 10.1</td>
</tr>
<tr>
<td>U69593</td>
<td>2.4 ± 0.9</td>
<td>102.6 ± 2.4</td>
</tr>
</tbody>
</table>

Values were determined as described in Materials and Methods and represent means ± S.E. from at least three determinations each performed in triplicate.

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**TABLE 4**

Stimulation of [35S]GTPγS binding to membranes from SH-SY5Y cells by buprenorphine and analogs

Membranes from SH-SY5Y cells were incubated with [35S]GTPγS (80 μM) and varying concentrations of opioid for 60 min at 30°C as described in Materials and Methods. Data represent means ± S.E. from at least three determinations each performed in triplicate.

<table>
<thead>
<tr>
<th>Agonist Activity</th>
<th>Maximal Effect M</th>
<th>EC50 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>BU46</td>
<td>0.21 ± 0.04</td>
<td>49.4 ± 1.5</td>
</tr>
<tr>
<td>BU47</td>
<td>0.40 ± 0.07</td>
<td>51.0 ± 1.6</td>
</tr>
<tr>
<td>BU61</td>
<td>0.23 ± 0.04</td>
<td>45.8 ± 1.6</td>
</tr>
<tr>
<td>BU95</td>
<td>0.33 ± 0.06</td>
<td>53.6 ± 0.7</td>
</tr>
<tr>
<td>BU96</td>
<td>0.56 ± 0.08</td>
<td>51.7 ± 0.8</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.18 ± 0.10</td>
<td>73.4 ± 2.1</td>
</tr>
</tbody>
</table>

Values were determined as described in Materials and Methods and represent means ± S.E. from at least three separate tissues.

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Fig. 2. Stimulation of [35S]GTPγS binding to membranes of CHO-khor cells by BU46 and BU47.
and Inturrisi, 1995). A second explanation involves a role for \( \kappa \)-receptor subtypes. There are many suggestions for \( \kappa \)-receptor heterogeneity with a broad consensus for at least two subtypes of the \( \kappa \)-receptor.

The \( \kappa_1 \)-receptors have high affinity for the benzeneacetamide ligands, such as U50488H and the dynorphins, whereas \( \kappa_2 \)-receptors are not recognized by the benzeneacet- amides and dynorphins, but do bind bremazocine and ethylketocyclazocine (Traynor, 1989). The ligand-binding assays described herein used the benzeneacetamide ligand \(^{3}H\)CI977, which might be expected to label only the \( \kappa_1 \)-subtype (but see Ko et al., 1998). Agonists at \( \kappa_1 \)-receptors are efficacious opioids in the GPI (Dixon and Traynor, 1990) as are the putative \( \kappa_2 \)-ligands bremazocine and ethylketocyclazocine (Toll et al., 1998) and BU46 and BU47. These facts may suggest that the antinociceptive effects of BU46 are mediated through a different \( \kappa \)-receptor, possibly \( \kappa_2 \). In support of this proposal, the structures of BU46 and BU47 are closer to bremazocine and ethylketocyclazocine than U50488H and dynorphin, and there is evidence from studies in both the mouse and the monkey that bremazocine exhibits antinociception through a receptor that is distinct from the \( \kappa_1 \)-receptor (Horan et al., 1993; Ko et al., 1998). This hypothesis would, however, require that BU46 and BU47 have marked differential efficacy at the putative \( \kappa_2 \)-receptor.

The difference in the \( \kappa \)-pharmacology of BU46 and BU47 must be due to the position of the \( C_2 \)-hydroxyl group. In BU46, this group presumably interacts with a suitably located hydrogen bond donor or acceptor on the \( \kappa \)-receptor that is not available to the corresponding hydroxyl in BU47. This could explain the 12-fold difference in potency in the GPI and the difference in efficacy seen in vivo, but does not appear to contribute to differential binding, at least at the \( \kappa_1 \)-receptor in mouse brain. This role for the hydroxyl function supports an earlier model of oripavine binding (Hutchins and Rapoport, 1984) that proposes a hydrophilic site above and away from \( C_7 \) and a lipophilic site below \( C_8 \). The same argument should apply to the isomeric pair BU95 and BU96. However, these are not differentiated in the GPI where they have a similar potency to BU47, indicating that the additional methyl groups at \( C_2 \) are preventing a favorable interaction of the \( \beta \)-OH in BU96 with the receptor.

In conclusion, the findings show that the configuration of the \( -OH \) function at \( C_20 \) is very important in governing the in vivo properties of the ring-constrained orvinols. The corresponding hydroxyl group at \( C_20 \) in buprenorphine can occupy the same position (Fig. 4, c and d) as in either of the con-

![Fig. 3](image)

**Fig. 3.** Top, dose-response curves for BU46 and BU47 in the acetic acid-induced writhing assay. Drugs were administered s.c. 15 min before administration of i.p. acetic acid. Bottom, BU46 (0.1 mg/kg s.c.)-mediated antinociception in the mouse writhing assay and its reversal by BU47 (10 mg/kg, 15-min pretreatment), norBNI (32 mg/kg s.c., 24-h pretreatment), M-CAM (1.8 mg/kg s.c. 1-h pretreatment), and NTI (10 mg/kg s.c., 15-min pretreatment). In both graphs, the ordinate represents the percentage of control number of writhes per mouse and each value represents the mean ± S.E. for six mice.

| Table 5 |

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Saline pretreatment</th>
<th>BU47 pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine (3.2 mg/kg)</td>
<td>0</td>
<td>53.3 ± 12.4</td>
</tr>
<tr>
<td>Bremazocine (0.1 mg/kg)</td>
<td>0</td>
<td>38.5 ± 11.1</td>
</tr>
<tr>
<td>SNC80 (10 mg/kg)</td>
<td>2.5 ± 1.2</td>
<td>58.4 ± 14.3</td>
</tr>
</tbody>
</table>
strained isomers BU46 (Fig. 4a) and BU47 (Fig. 4b). However, neither of the isomers has a similar pharmacology to buprenorphine. This suggests that in the receptor-bound conformation of buprenorphine, the rotationally free C₂₀ hydroxyl is in a position that cannot be taken up by the hydroxyl in either of the isomers. It has been suggested that the hydroxy group in the oripavines forms an intramolecular hydrogen bond with the C₆ methoxy group that may influence receptor binding (Loew and Berkowitz, 1979). Such H-bonding would place the C₂₀ hydroxyl of buprenorphine in a similar position to the C₁ hydroxyl in BU46 (Fig. 4, b and c). The present findings suggest that such H-bonding does not occur in the receptor-bound conformation of buprenorphine, either due to the presence of an alternative hydrogen bonding site in the binding pocket or as a consequence of rotation to allow accommodation of the bulky t-butyl group within the binding pocket. In this regard, it is noteworthy that the dimethyl substituent at C₂₀ in BU95 and BU96 was detrimental to activity compared with the proposed role of the corresponding t-butyl group in buprenorphine (Loew and Berkowitz, 1979).

Finally, the different properties of the compounds in the vivo compared with their activities in vitro suggests that the GPO and CHO-hkr cells may not represent particularly suitable in vivo systems to examine compounds with potential for K-mediated analgesia. This could be due to the existence of different types of K-receptors that may, because the compounds show large in vivo efficacy differences, relate not to primary receptor structure but rather to the receptor environment and coupling systems.

Acknowledgments

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References


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