0022-3565/00/2943-1195$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 294:1195–1200, 2000

Vol. 294, No. 3
2493/846872
Printed in U.S.A.

BU48: A Novel Buprenorphine Analog That Exhibits δ-Opioid-Mediated Convulsions but Not δ-Opioid-Mediated Antinociception in Mice[1]

DANIEL C. BROOM, LI GUO, ANDREW COOP,[2] STEPHEN M. HUSBANDS, JOHN W. LEWIS, JAMES H. WOODS, and JOHN R. TRAYNOR

Departments of Pharmacology (D.C.B., J.H.W., J.R.T.) and Psychology (J.H.W.), University of Michigan Medical School, Ann Arbor, Michigan; Department of Chemistry, University of Bristol, Bristol, United Kingdom (A.C., S.M.H., J.W.L.); and Department of Chemistry, Loughborough University, Loughborough, United Kingdom (L.G., J.R.T.)

Accepted for publication May 25, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

N-Cyclopropylmethyl-[7α,8α,2′,3′]-cyclohexano-1′[*S*]-hydroxy-6,14-*endo*-ethenotetrahydronororipavine (BU48) is a novel, ring-constrained analog of buprenorphine. In vivo, BU48 (0.1–10 mg/kg s.c.) produced brief, nonlethal convulsions in mice followed by brief Straub tail and a short period of catalepsy characteristic of BW373U86 and other nonpeptidic δ-receptor agonists. BU48-induced convulsions were sensitive to antagonism by naltrindole (10 mg/kg s.c.) and were also prevented by administration of the putative δ_1 antagonist 7-benzylidenenaltrexone and the putative δ_2 antagonist naltriben, with the latter being more potent. In the abdominal stretch assay in the mouse, only low-efficacy antinociceptive activity of BU48 (0.1–10 mg/kg) was seen. This was reversed by the κ-opioid antagonist norbinaltorphimine (32 mg/kg s.c.) but not by the δ-opioid antagonist naltrindole (10 mg/kg s.c.). BU48 (10 mg/kg s.c.) acted as a δ-antagonist in this assay. In mouse brain homogenates, BU48 had high (nanomolar) binding affinity for all three opioid receptors in the order $\mu > \delta = \kappa$. In vitro, the compound acted as a potent (EC_{50} = 1.4 nM) κ-opioid agonist in the guinea pig ileum and a potent (EC_{50} = 0.2 nM) δ-opioid agonist in the mouse vas deferens but showed partial agonist activity at the rat cloned δ-opioid (40%) and human cloned κ-opioid (59%) receptors with very low efficacy at the rat cloned μ-opioid receptor (10%); findings consistent with its in vivo profile. BU48 is the first described compound that produces δ-opioid-mediated convulsions without any evidence of δ-opioid-mediated antinociception and will be a useful tool in investigations of the δ-opioid receptor.

In the search for narcotic analgesic agents without the unwanted complications of μ-opioid receptor agonists such as morphine, research has focused on the δ-opioid receptor as a target for antinociceptive agents. This has culminated in the suggestion of δ-opioid receptor subtypes (for review, see Traynor and Elliot, 1993) and the discovery of the nonpeptide δ-opioid agonist BW373U86 and related compounds (e.g., Chang et al., 1993). BW373U86 produces antinociception without any apparent respiratory complications (Negus et al., 1994), although studies in mice showed a consistent and reproducible dose-dependent convulsive effect associated with BW373U86 administration (Comer et al., 1993). The convulsive activity of this compound has also been noted in squirrel monkeys (Dykstra et al., 1993; Pakarinen et al., 1995) and rhesus monkeys (Hong et al., 1998).

The synthesis of SNC80, the methyl ether of the (+)-isomer of BW373U86 (Calderon et al., 1994), produced a compound more potent than BW373U86 as an antinociceptive agent.

Received for publication January 14, 2000.
[1] This work was supported by U.S. Public Health Service Grants DA00254, DA07315, and GM07767. Portions of this work were presented in abstract form at the 1999 meeting of the College on Problems of Drug Dependence, Acapulco, Mexico, June 12–17 (Broom et al., 2000).
[2] Present address: Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 N. Pine St., Baltimore, MD 21201.

ABBREVIATIONS: BW373U86, (±)-[1(*S**),2α,5β]-4-[[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl](3-hydroxyphenyl)methyl]-*N*,*N*-diethylbenzamide hydrochloride; BNTX, 7-benzylidenenaltrexone; BU48, *N*-cyclopropylmethyl-[7α,8α,2′,3′]cyclohexano-1′[S]-hydroxy-6,14-*endo*-ethenotetrahydronororipavine; CI977, 5*R*-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl-1-oxaspiro[4,5]dec-8-yl]-4-benzofuranacetamide; C6δ, C6 glioma cells transfected with the rat δ-receptor; CHO, Chinese hamster ovary; EEG, electroencephalographic; CNS, central nervous system; CHO-hkor, Chinese hamster ovary cells transfected with the human κ-receptor; C6μ, C6 glioma cells transfected with the rat μ-receptor; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; GPI, myenteric plexus-longitudinal muscle of the guinea pig ileum; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; MVD, mouse vas deferens; norBNI, norbinaltorphimine; NTB, naltriben; NTI, naltrindole; SNC80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; (−)-TAN-67, 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydroquinolino[2,3,3-*g*]isoquinoline; U69593, 5α,7α,8β-(+)-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide; DMSO, dimethyl sulfoxide.

Although initially observed to be a weaker proconvulsant than its parent compound (Bilsky et al., 1995), SNC80 has since been shown to have significant convulsive properties, including a lethal convulsion that becomes apparent at higher doses (Hong et al., 1998). To be of therapeutic benefit, the antinociceptive properties of such δ-opioid agonists need to be separated from their convulsive activity.

In this regard, the octahydroquinolinoisoquinoline derivative (−)-TAN-67 (Nagase et al., 1994) has been reported to produce antinociception that is reversed by the putative δ_1-selective antagonist benzylidene naltrexone (BNTX) but not the putative δ_2-selective antagonist naltriben (NTB) (Nagase et al., 1994; Kamei et al., 1995; Suzuki et al., 1995; Tseng et al., 1997), whereas its (+)-isomer produces hyperalgesia in some situations and convulsions (Tseng et al., 1997). Identification of compounds that show a separation of δ-mediated convulsions and antinociception is an important step in the elucidation and understanding of δ-opioid receptor pharmacology.

This study examined BU48 (Fig. 1), a novel, ring-constrained, buprenorphine analog synthesized as part of a structure-activity relationship study of buprenorphine (Traynor et al., 1999). In vivo BU48 produced naltrindole (NTI)-reversible convulsions in mice, without NTI-reversible antinociception. Indeed, BU48 acted as an antagonist of the highly efficacious δ-agonist SNC80. A small degree of antinociception was observed in the acetic acid-induced abdominal stretch assay, but this was blocked by pretreatment with the κ-receptor antagonist norbinaltorphimine (norBNI). In vitro, ligand binding assays showed the compound to be nonselective, but the δ- and κ-agonist activity of the compound was confirmed. Thus, BU48 was a δ-opioid receptor agonist in the mouse vas deferens (MVD) and a κ-opioid receptor agonist in the guinea pig ileum. Guanosine-5′-*O*-(3-[^{35}S]thiotriphosphate ([^{35}S]GTPγS) assays at recombinant opioid receptors showed BU48 to be a partial δ- and κ-opioid agonist but an extremely low-efficacy, μ-opioid receptor agonist. BU48 is the first compound described that produces δ-opioid-mediated convulsions in the absence of δ-opioid-mediated antinociceptive activity.

Materials and Methods

Chemicals. [^{35}S]GTPγS was obtained from New England Nuclear (Boston, MA). SNC80 and BNTX were gifts from Dr. K. C. Rice (National Institutes of Health, Bethesda, MD), and norBNI was synthesized by Dr. H. Mosberg (School of Pharmacy, University of Michigan). NTI, BW373U86, and naltriben (NTB) were from Research Biochemicals International (Natick, MA). Fentanyl, GDP, and U69593 were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were from Sigma Chemical Co. and were of analytical grade. NTB and BNTX were dissolved in 10% dimethyl sulfoxide (DMSO). SNC80 base was dissolved in sterile water with a little 1.13 N hydrochloric acid. All other drugs listed above were dissolved in sterile water.

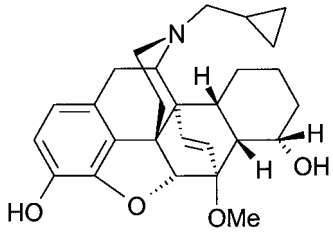

Fig. 1. Structure of BU48.

Synthesis of BU48. BU48 was synthesized by lithium aluminum hydride reduction of *N*-cyclopropylcarbonyl-[7α,8α,2′,3′]-1′-oxo-cyclohexano-6,14-*endo*-ethenotetrahydronorthebaine (Barton et al., 1993) to give the *N*-cyclopropylmethyl-1′[*S*]-secondary alcohol exclusively, which was 3-*O*-demethylated with sodium propane thiolate and converted to the hydrochloride salt. The stereochemistry of the 1′-secondary alcohol was assigned through comparison with the previously reported cyclopentano analogs BU46 and BU47 (Traynor et al., 1999; NMR: 1′β-H 4.2 ppm; IR: 1′α-OH 3563 cm^{-1}). BU48 was dissolved in 10% DMSO.

Animals. For isolated tissue preparations, 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 in vivo assays, male NIH Swiss mice (20–35 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) All animals were fed a standard laboratory diet and kept on a 12-h light/dark cycle at a temperature of 20°C. Studies were performed 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 protocols were approved by the University of Michigan University Committee on the Use and Care of Animals.

In Vivo Assays. The measurement of convulsant activity was performed as previously described (Comer et al., 1993). Male NIH Swiss mice were injected s.c. with test drug and placed in individual Plexiglas boxes (18 × 28 × 13 cm) for the duration of the observation period. Mice were observed for convulsant activity for 20 min after drug injection. Postconvulsion catalepsy was assessed by placing the forepaws of a mouse on a horizontal rod; a positive catalepsy score was assigned if the mouse had not removed its paws within 15 s. For a positive convulsion score, a mouse had to exhibit a convulsive episode and a subsequent period of catalepsy. All antagonists were administered s.c. 20 min before test drug administration except norBNI, which was administered s.c. 24 h before the start of the assay. Data are expressed as percentage of the number of animals convulsing.

Antinociception was performed immediately after the convulsion assay in the same mice according to previously described mouse acetic acid-induced abdominal stretch methods (Hong et al., 1998). Immediately after the conclusion of the convulsion assay (i.e., 20 min after injection of the test drug), 0.4 ml of 0.6% acetic acid was injected via the i.p. route. At 5 min after administration, the animals were observed for abdominal stretches for 5 min. Abdominal stretches were characterized by a wave of contraction of the abdominal musculature followed by extension of the hind legs. Data were expressed as mean number of abdominal stretches performed per treatment group.

Isolated Tissue Preparations. Segments of ileum were removed from male Duncan-Hartley guinea-pigs and placed in Krebs' solution containing 118 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, and 11 mM glucose. Vas deferens from male CSI mice were placed in Krebs' solution minus $MgSO_4 \cdot 7H_2O$. 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. EC_{50} and maximal values were computed using Prism (GraphPad Software, San Diego, CA). Antagonist equilibrium dissociation constants (K_e, nM) were determined from the ratios of EC_{50} values for agonists in the absence or presence of antagonist (added 15 min before the redetermination of agonist concentration-response curves) using the formula: K_e = [antagonist]/dose ratio − 1 (Kosterlitz and Watt, 1968).

Cell Culture and Membrane Preparation. C6 glioma cells transfected with either the cloned rat μ- (C6μ) and δ- (C6δ) receptors

and Chinese hamster ovary (CHO-hkor) cells transfected with the human κ-receptor were cultured under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (C6 cells) or Dulbecco's modified Eagle's medium/F-12 (CHO cells) supplemented with 10% fetal calf serum. For subculture, one flask from each passage was grown in the presence of 1 mg/ml geneticin. Cells used for experiments were grown in either the absence (C6 cells) or presence (CHO cells) of 1 mg/ml geneticin. This did not change the level of receptors. Once cells had reached confluency, they were harvested in HEPES (20 mM, pH 7.4)-buffered saline containing 1 mM EDTA, dispersed by agitation, and collected by centrifugation at 1600 rpm. The cell pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, separated into aliquots (0.75–1.0 mg protein), and frozen at −80°C.

Ligand Binding Assay. Brains from CSI mice were homogenized in Tris-HCl buffer (pH 7.4, 50 mM) using a Polytron at setting 7. After centrifugation at 25,000*g* for 15 min, the pellet was resuspended in 10 volumes of buffer and incubated at 37°C for 30 min to remove endogenous opioid ligands. The homogenates were then recentrifuged, and the pellets were resuspended in Tris-HCl buffer at a final protein concentration of 500 μg/ml (Lowry et al., 1951).

Membrane protein (approximately 450 μg) was incubated in Tris-HCl (pH 7.4) with varying concentrations of BU48 and 1 nM [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), 2 nM [^{3}H][D-Pen^2,D-Pen^5]-enkephalin (DPDPE), or 0.5 nM [^{3}H]CI977 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 typically >80% of total binding at the radioligand affinity (K_d). Competition data were analyzed by nonlinear curve fitting (GraphPad, San Diego, CA) to give IC_{50} values that were converted to affinity (K_i) values using the Cheng and Prusoff (1973) equation.

[^{35}S]GTPγS Binding Assay. Freshly prepared membranes (30–50 μg of protein for C6δ, 40–50 μg of protein for C6μ, or 15–20 μg of protein for CHO-hkor) were incubated in GTPγS binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, and 10 mM $MgCl_2 \cdot 6H_2O$) containing [^{35}S]GTPγS (0.1 nM), GDP (30 μM), and varying concentrations of test compound to a final volume of 1 ml for 60 min at 30°C as previously described (Traynor and Nahorski, 1995). Bound and free [^{35}S]GTPγS were separated by vacuum filtration through GF/C glass-fiber filters mounted in a Brandell 24-well harvester. The filters were subsequently washed three times with ice-cold GTPγS binding buffer, and the radioactivity was determined by liquid scintillation counting. The EC_{50} values for stimulation of [^{35}S]GTPγS binding obtained at various drug concentrations were determined from nonlinear curve fitting of the data (Prism; GraphPad Software).

Results

In Vivo Assays. BU48 afforded dose-dependent convulsant activity. Mild convulsions were observed in all mice tested at a dose of 10 mg/kg administered by s.c. injection. BU48 was considerably more potent than BW373U86 (Fig. 2a). The convulsant action of BU48 was completely antagonized by pretreatment of the animals with the δ-selective antagonist NTI (10 mg/kg) administered 20 min before BU48 but not by pretreatment with the κ-selective antagonist norBNI (32 mg/kg) administered approximately 24 h before BU48 (Fig. 2b). These results indicate δ-mediated convulsive activity of BU48 in vivo. The putative δ-subtype-selective antagonists BNTX (δ_1) and NTB (δ_2) both antagonized the convulsive activity of BU48 in a dose-dependent manner, with NTB being approximately 80-fold more potent than BNTX (Fig. 3).

In the acetic acid-induced mouse abdominal stretch assay,

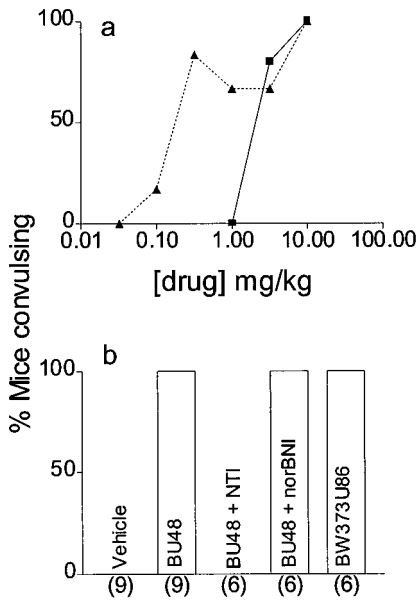

Fig. 2. Convulsant activity of BU48. a, dose-response relationship for BU48 (▲) and BW373U86 (■). All doses were administered via s.c. injection. b, BU48 (10 mg/kg s.c.) or BW373U86 (10 mg/kg s.c.) was administered to NIH Swiss mice, and each mouse was subsequently observed for up to 20 min. NTI (10 mg/kg s.c.) was administered 20 min before BU48. norBNI (32.0 mg/kg s.c.) was administered approximately 24 h before BU48. Numbers in parentheses correspond to *n*.

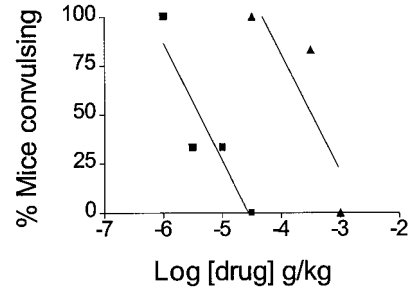

Fig. 3. The effects of BNTX (▲) and NTB (■) on BU48-mediated convulsive activity in mice. BNTX or NTB (s.c.) was administered 20 min before the administration of BU48 (10 mg/kg s.c.). Linear regression analysis was performed by GraphPad Prism software (n = 3–6 animals for each point).

BU48 showed a potent but partial agonist effect on inhibition of stretching compared with vehicle control (Fig. 4a) and BW373U86 (Fig. 4b). This effect was not antagonized by pretreatment of mice with 10 mg/kg NTI (Fig. 4b), indicating that BU48-mediated antinociception is not due to its δ-agonist activity. Indeed, BU48 prevented the antinociceptive action of the potent δ-agonist SNC80, indicating δ-antagonist activity (Fig. 4c). After prior (24 h) pretreatment of the animals with norBNI (32 mg/kg), BU48 administration was not significantly different from vehicle control (Fig. 4b), suggesting BU48 was exerting its partial agonist action via κ-receptors in this preparation.

To characterize the δ- and κ-opioid activities of the compound in vitro, assays were performed in smooth muscle preparations and cloned cells and binding assays in mouse brain homogenates.

Isolated Tissue Assays. In the electrically stimulated MVD, BU48 was a potent agonist, with an IC_{50} value lower than that of DPDPE (Table 1). NTI shifted the concentration-

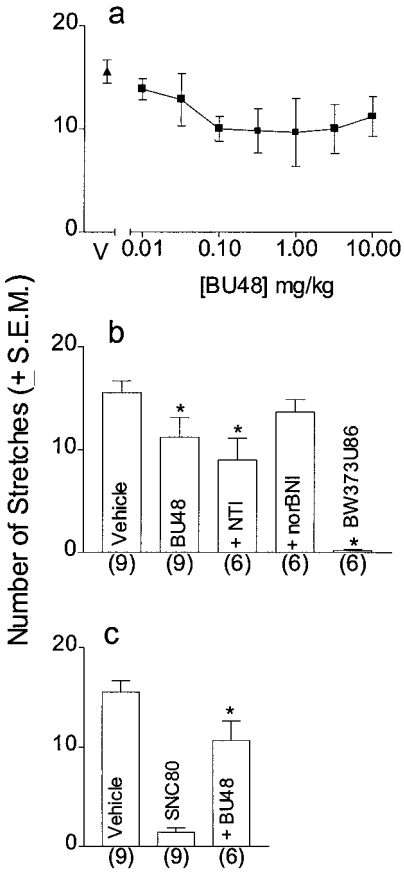

Fig. 4. BU48 (10 mg/kg s.c.)-mediated antinociception in the acetic acid-induced mouse abdominal stretch assay. BU48 was injected 20 min before acetic acid administration. a, dose-response relationship for BU48 (■). All doses were administered by s.c. injection. Number of stretches at dose of 0.1 mg/kg BU48 and above are significantly different from controls (Student's *t* test, $P < .05$). b, effect of NTI and norBNI on BU48 (10 mg/kg s.c.). NTI (10 mg/kg s.c.) was administered 20 min before BU48 administration. norBNI (32 mg/kg s.c.) was administered approximately 24 h before BU48. BW373U86 (10 mg/kg s.c.) and vehicle (10% DMSO in sterile water s.c.) were administered alone 20 min before administration of acetic acid. Numbers in parentheses correspond to *n*. *$P < .05$ compared with vehicle control. c, effect of BU48 (10 mg/kg s.c.) on SNC80 (32 mg/kg s.c.)-mediated antinociception. SNC80 was injected 20 min before acetic acid administration. BU48 was administered 10 min before SNC80 administration. Vehicle (10% DMSO in sterile water s.c.) was injected alone 20 min before the administration of acetic acid. Numbers in parentheses correspond to *n*. *$P < .0001$ compared with SNC80.

TABLE 1

Agonist actions of BU48 and standard opioids on the electrically induced contractions of the mouse vas deferens and their antagonism by NTI

Experiments were performed as described under *Materials and Methods.* Values represent means ± S.E. from three separate tissues and were determined as described under *Materials and Methods.*

	IC_{50}	NTI (K_e)
	nM	
BU48	0.2 ± 0.10	0.11 ± 0.02
DAMGO	17.6 ± 2.0	6.5 ± 1.7
DPDPE	0.93 ± 0.04	0.14 ± 0.03
U69593	28.4 ± 3.6	19.6 ± 2.3

effect curve of BU48 to the right in a parallel fashion. The equilibrium affinity constant (K_e) calculated for NTI was in the range of the affinity values calculated for this antagonist against the δ-selective agonist DPDPE rather than the κ-selective agonist U69593 or the μ-selective agonist DAMGO (Table 1), indicating a δ-mediated mechanism of action in this tissue.

In the myenteric plexus of the GPI, BU48 inhibited the electrically evoked twitch and was more potent than either the μ-selective agonist DAMGO or the κ-selective agonist U69593 (Table 2). The equilibrium affinity constant (K_e) calculated for norBNI was in the range of the affinity values calculated for this antagonist against the κ-selective agonist U69593 rather than the μ-selective agonist DAMGO (Table 2), indicating a κ-mediated effect in this tissue.

Ligand Binding and [^{35}S]GTPγS Binding Assays. In mouse brain homogenates, BU48 had good affinity for all three opioid receptor types in the order μ (K_i = 0.36 ± 0.05 nM) > δ (K_i = 1.41 ± 0.30 nM) = κ (1.56 ± 0.10 nM). The relative efficacy of BU48 at each of the opioid receptors was examined using the [^{35}S]GTPγS binding assay (Table 3). BU48 displayed a dose-dependent, potent partial δ-agonist activity compared with SNC80 in C6δ cell membranes. In this assay, BW373U86 is a full agonist with an EC_{50} value of 1.3 nM (Clarke et al., 1997). Potent partial agonist activity was also seen compared with U69593 in CHO-hkor cell membranes. BU48 had very low efficacy relative to the full agonist fentanyl in C6μ cell membranes, as demonstrated by a small maximal effect, but with high potency (Table 3).

Discussion

The novel ring-constrained buprenorphine analog BU48 showed a very different pharmacological profile from the μ-partial agonist, κ- and δ-antagonist profile of buprenorphine (Cowan, 1995; Lee et al., 1999). In direct contrast, BU48 was both a δ- and a κ-opioid agonist in vivo in the mouse but in different ways. δ-Opioid agonism was demonstrated by the presence of a convulsion, which was reversed by the δ-antagonist NTI but not by the κ-antagonist norBNI. The convulsions seen were similar to those produced by systemic administration of the prototypical nonpeptidic δ-selective agonist BW373U86 (Comer et al., 1993), although BU48 was more potent. However, BU48 did not afford δ-mediated antinociception in the acetic acid-induced mouse abdominal stretch assay; instead, the compound acted as a δ-antagonist in this assay. Additionally, in the tail-flick assay, BU48 has no antinociceptive activity (Aceto et al., 1995). The abdominal stretch assay is extremely sensitive to δ-agonists (Comer et al., 1993; Hong et al., 1998); therefore, it is unlikely that BU48 has any δ-mediated antinociceptive properties. However, it is possible that BU48 might show activity in situations even more sensitive to δ-agonists such as allodynia (Butelman et al., 1995).

The low level of antinociception afforded by BU48 in the

TABLE 2

Agonist activity of BU48 and standard opioids on the electrically induced contractions of the myenteric plexus-longitudinal muscle preparation of the guinea pig ileum and their antagonism by norBNI

Experiments were performed as described under *Materials and Methods.* Values represent means ± S.E. from three separate tissues and were determined as described under *Materials and Methods.*

	IC_{50}	norBNI (K_e)
	nM	
BU48	1.38 ± 0.23	0.10 ± 0.05
DAMGO	5.6 ± 1.8	13.0 ± 1.9
U69593	31.4 ± 9.7	0.07 ± 0.01

TABLE 3

Stimulation of [^{35}S]GTPγS binding by BU48 acting at recombinant δ-, μ-, or κ-opioid receptors

Membranes from C6δ, C6μ, or CHO-hkor cells were incubated with [^{35}S]GTPγS (0.1 nM), GDP (30 μM), and varying concentrations of BU48 for 60 min at 30°C as described under *Materials and Methods.* Data represent means ± S.E. from three determinations.

	EC_{50}	Maximal Effect[a]
	nM	%
C6δ	0.81 ± 0.27	39.7 ± 2.0
C6μ	0.085 ± 0.053	10.0 ± 3.3
CHOκ	0.78 ± 0.22	58.6 ± 6.6

[a] Percentage of response caused by a maximal (10 μM) concentration of SNC80 (C6δ), fentanyl (C6μ), or U69593 (CHOκ).

abdominal stretch assay appeared to be mediated through an action at the κ-opioid receptor. An analog of buprenorphine (BU47) that is constrained through a cyclopentyl as opposed to a cyclohexyl ring, but has a similar stereochemistry in the constrained ring and a β-OH, is also a very weak κ-agonist in the mouse abdominal stretch assay. This compound is able to antagonize the action of more efficacious κ-agonists (Traynor et al., 1999).

BU48 was nonselective in ligand binding assays at μ-, δ-, and κ-opioid receptors. However, in confirmation of its in vivo opioid agonist actions, BU48 was a potent δ-selective agonist in the MVD preparation and a potent κ-selective agonist in the GPI preparation, with no evidence of any μ-receptor-mediated action. Stimulation of [^{35}S]GTPγS binding by BU48 confirmed potent agonist actions of the compound at recombinant δ- and κ-opioid receptors, although only a partial agonist effect was seen. In addition, although BU48 appeared potent at activating [^{35}S]GTPγS binding through the recombinant μ-opioid receptor, the level of maximal effect was extremely low. This low μ-opioid efficacy explains why κ-opioid, not μ-opioid, effects of the drug were seen in the GPI and in vivo. This is supported by a report that BU48 antagonizes morphine in the tail-flick assay (AD_{50} = 0.02 mg/kg) and precipitates withdrawal in morphine-dependent rhesus monkeys with a potency five times that of naloxone (Aceto et al., 1995).

The present results indicate a disconnection of δ-mediated behavioral effects with BU48 in that the compound shows δ-mediated convulsive activity without δ-mediated antinociception. BW373U86 and SNC80, the prototypical nonpeptidic δ-opioid receptor agonists, produce both convulsions (Comer et al., 1993) and antinociception (Wild et al., 1993; Hong et al., 1998), which are antagonized by the δ-selective antagonist NTI.

A plausible explanation for this separation of δ-opioid pharmacological effects with BU48 is the involvement of putative δ-opioid receptor subtypes. Both BNTX, a putative δ_1 receptor-selective antagonist, and NTB, a putative δ_2 receptor-selective antagonist, prevent the convulsive effects of BU48. The more potent antagonism of BU48-mediated convulsions by NTB rather than BNTX suggests that BU48 is acting via δ_2 receptors. However, the relative potency of these two antagonists in inhibiting BU48-mediated convulsions, with NTB being 80-fold more potent than BNTX, corresponds with in vitro data using recombinant rat and human δ-opioid receptors. These data describe a 30-fold higher affinity of NTB for the δ-opioid receptor than BNTX (Toll et al., 1998; Neilan et al., 1999). The slightly higher potency ratio between NTB and BNTX in vivo can be explained by differential access of the antagonists to the central nervous system (CNS); NTB is reported to have a 4-fold higher CNS penetration than BNTX (Lever et al., 1996). Thus, BU48 is acting at the same type of δ-opioid receptor in vivo and in vitro. (−)-TAN-67 is also an agonist at the recombinant human δ-receptor (Quock et al., 1997), but unlike BU48, it is active as an antinociceptive agent through an action at a putative δ_1, BNTX-sensitive, receptor and does not give convulsions, although its (+)-isomer shows hyperalgesia and convulsions (Tseng et al., 1997). Thus, the in vivo and in vitro data for BU48 and TAN-67 are not readily reconciled in terms of receptor identity. Nonetheless, the relationship of the in vitro binding affinities of BNTX and NTB to their in vivo antagonism of BU48 strongly argues that both NTB and BNTX are acting at a single δ-receptor type to antagonize the convulsive activity of BU48, and this is the same as the cloned δ-receptor. Also consistent with this theory are studies showing the putative δ_2-receptor agonist DSLET when administered i.c.v. to rats produces electroencephalographic (EEG) seizures that are blocked by the δ-opioid receptor antagonist ICI 174864 (Haffmans and Dzoljic, 1987). The putative δ_1-opioid agonist DPDPE does not produce EEG seizures or convulsive behavior when given i.c.v. to rats but does afford a complex EEG response that is prevented by high-dose naloxone (Tortella et al., 1984).

If the presence of δ-subtypes is not an adequate explanation for the unusual pharmacology of BU48, two other possibilities exist. Distribution may play an important role. BU48 could only be reaching δ-opioid receptor populations in CNS regions important for mediation of convulsive activity while not reaching regions important for antinociception. However, this hypothesis would require the nonpeptidic δ-ligands, namely BW373U86 and SNC80, to reach areas where δ-opioid receptors can mediate both convulsions and antinociception. Nevertheless, it is interesting to note that BW373U86 has a nonopioid receptor-mediated convulsive action only after i.c.v. administration (Comer et al., 1993), whereas SNC80 also produced a lethal nonopioid convulsion when administered systemically at high doses (Hong et al., 1998). These differences in effect, depending on the route of administration, indicate that distribution plays a role in the observed pharmacological actions of the drugs.

The most plausible explanation for the unusual δ-opioid profile of BU48 is its low level of intrinsic efficacy. δ-Opioid receptor agonists are effective antinociceptive agents in certain situations; however, low efficacy, as indicated by the [^{35}S]GTPγS binding assay, may be the reason for the absence of δ-mediated antinociception with BU48. It is plausible that convulsive activity requires less intrinsic efficacy in a δ-compound than antinociception. This would explain the observation that BU48 is able to antagonize SNC80-mediated antinociception. Both BW373U86 and SNC80 are highly efficacious at the δ-opioid receptor, which lends further weight to this argument (Clark et al., 1997). Unfortunately, data with the δ-selective agonist (−)-TAN-67 are confounding if, as we suspect, convulsive actions of δ-agonists require lower efficacy. In vitro (−)-TAN-67 is a full agonist in the [^{35}S]GTPγS assay, although it does have lower intrinsic efficacy than SNC80 (Quock et al., 1997). This compound has antinociceptive properties when given i.t. or i.c.v. Unfortunately, there are no reports of the systemically administered

(−)-TAN-67, and as discussed above, the route of administration may be important for the induction of δ-mediated convulsive behaviors.

BU48 is the only compound yet described that produces δ-mediated convulsions without any evidence of δ-opioid-mediated antinociception. This is apparently in direct contrast to nonpeptidic δ-opioid-selective agents that produce both δ-opioid-mediated antinociception and convulsive activity. The mechanisms underlying this differential display of δ-opioid-mediated effects could be due to a number of reasons. However, the fact that δ-antagonism in the abdominal stretch assay and δ-agonism in causing convulsions occur at similar doses of BU48 is strong evidence for the compound acting at one type of δ-receptor. Thus, the antinociception and convulsive consequences of δ-receptor activation require differing levels of intrinsic efficacy in the agonist.

Finally, BU48 is very different structurally from other nonpeptide δ-ligands with a δ-opioid agonist profile in vivo. It is not known how its different chemical structure contributes to the unusual actions reported here. Nevertheless, BU48 could be an important tool in further elucidating the antinociceptive and convulsive pharmacology mediated by agonists at the δ-opioid receptor.

Acknowledgments

We thank Hui-Fang Song for expert technical assistance with the [^{35}S]GTPγS binding assay. We thank Dr. H. Akil (Mental Health Research Institute, University of Michigan) for the supply of C6δ and C6μ cells and Dr. Lee-Yuan Lui-Chen (Temple University School of Medicine) for the CHO-hkor cells.

References

Aceto MD, Bowman ER, Harris LS and May EL (1995) Dependence studies of new compounds in the rhesus monkey, rat and mouse. *NIDA Res Monogr* **168:**408–451.

Barton JW, Coop A and Lewis JW (1993) Diels-Alder reactions of thebaines with cyclopentenones: $LiBF_4$ as a novel Diels-Alder catalyst. *Tetrahedron Lett* **34:**6777–6778.

Bilsky EJ, Calderon SN, Wang T, Bernstein RN, Davis P, Hruby VJ, McNutt RW, Rothman RB, Rice KC and Porreca F (1995) SNC80, a selective, nonpeptidic and systemically active opioid delta agonist. *J Pharmacol Exp Ther* **273:**359–366.

Broom DC, Coop A, Lewis JW, Woods JH and Traynor JR (2000) A novel buprenorphine analog, BU48, causes δ-mediated convulsions but not antinociception in mice. *NIDA Res Monogr* **180:**249.

Butelman ER, Negus SS, Gatch MB, Chang KJ and Woods JH (1995) BW373U86, a delta-opioid receptor agonist, reverses bradykinin-induced thermal allodynia in rhesus monkeys. *Eur J Pharmacol* **277:**285–287.

Calderon SN, Rothman RB, Porreca F, Flippen-Anderson JL, McNutt RW, Xu H, Smith LE, Bilsky EJ, Davis P and Rice KC (1994) Probes for narcotic receptor mediated phenomena. 19. Synthesis of (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5 dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide (SNC80): A highly selective, nonpeptide δ opioid receptor agonist. *J Med Chem* **37:**2125–2128.

Chang K-J, Rigdon GC, Howard JL and McNutt RW (1993) A novel, potent and selective nonpeptidic delta opioid receptor agonist BW373U86. *J Pharmacol Exp Ther* **267:**852–857.

Cheng YC and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Clark MJ, Emmerson PJ, Mansour A, Akil H, Woods JH, Portoghese PS, Remmers AE and Medzihradsky F (1997) Opioid efficacy in a C6 glioma cell line stably expressing the *delta* opioid receptor. *J Pharmacol Exp Ther* **283:**501–510.

Comer SD, Hoenicke EM, Sable AI, McNutt RW, Chang K-J, DeCosta BR, Mosberg HI and Woods JH (1993) Convulsive effects of systemic administration of the *delta* opioid agonist BW373U86 in mice. *J Pharmacol Exp Ther* **267:**888–895.

Cowan A (1995) Update on the general pharmacology of buprenorphine, in *Buprenorphine: Combatting Drug Abuse with a Unique Opioid* (Cowan A and Lewis JW eds) pp 31–47, Wiley-Liss, New York.

Dykstra LA, Schoenbaum GM, Yarbrough J, McNutt R and Chang K-J (1993) A novel *delta* opioid agonist, BW373U86, in squirrel monkeys responding under a schedule of shock titration. *J Pharmacol Exp Ther* **267:**875–882.

Haffmans J and Dzoljic MR (1987) Effect of delta opioid antagonists on enkephalin-induced seizures. *Pharmacology* **34:**61–65.

Hong EJ, Rice KC, Calderon SN, Woods JH and Traynor JR (1998) Convulsive behavior of nonpeptide δ-opioid ligands: Comparison of SNC80 and BW373U86 in mice. *Analgesia* **3:**269–276.

Kamei J, Saitoh A, Ohsawa M, Suzuki T, Misawa M, Nagase H and Kasuya Y (1995) Antinociceptive effects of the selective non-peptidic δ-opioid receptor agonist TAN-67 in diabetic mice. *Eur J Pharmacol* **276:**131–135.

Kosterlitz HW and Watt AJ (1968) Kinetic parameters of narcotic agonists and antagonists with particular reference to N-allylnorhydroxymorphone (naloxone). *Br J Pharmacol* **33:**266–270.

Lee KO, Akil H, Woods JH and Traynor JR (1999) Differential binding properties of oripavines at cloned μ- and δ-opioid receptors. *Eur J Pharmacol* **378:**323–330.

Lever JR, Stathis M, Kinter CM and Scheffel U (1996) *In vivo* labeling of delta opioid receptors in mouse brain by [^{3}H]benzylidenenaltrexone, a ligand selective for the delta1 subtype. *Life Sci* **58:**331–336.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Nagase H, Wakita H, Kawai K, Endoh T, Matsuura H, Tanaka C and Takezawa Y (1994) Synthesis of non-peptide delta opioid agonists and their structure activity relationships. *Jpn J Pharmacol* **64Su:**1:35P.

Neilan CL, Akil H, Woods JH and Traynor JR (1999) Constitutive activity of the δ-opioid receptor expressed in C6 glioma cells: Identification of non-peptide δ-inverse agonists. *Br J Pharmacol* **128:**556–562.

Negus SS, Butelman ER, Chang K-J, DeCosta B, Winger G and Woods JH (1994) Behavioral effects of the systemically active *delta* opioid agonist BW373U86 in rhesus monkeys. *J Pharmacol Exp Ther* **270:**1025–1034.

Pakarinen ED, Woods JH and Moerschbaecher JM (1995) Repeated acquisition of behavioral chains in squirrel monkeys: Comparisons of a *mu*, *kappa* and *delta* opioid agonist. *J Pharmacol Exp Ther* **272:**552–559.

Quock RM, Hosohata Y, Knapp RJ, Burkey TH, Hosohata K, Zhang X, Rice KC, Nagase H, Hruby VJ, Porreca F, Roeske WR and Yamamura HI (1997) Relative efficacies of δ-opioid receptor agonists at the cloned human δ-opioid receptor. *Eur J Pharmacol* **326:**101–104.

Suzuki T, Tsuji M, Mori T, Misawa M, Endoh T and Nagase H (1995) Effects of a highly selective nonpeptide δ opioid receptor agonist, TAN-67, on morphine-induced antinociception in mice. *Life Sci* **57:**155–168.

Toll L, Berzetei-Gurske IP, Polgar WE, Brandt SR, Adapa ID, Rodriguez L, Schwartz W, Haggart D, O'Brien A, White A, Kennedy JM, Craymer K, Farrington L and Auh JS (1998) Standard binding and functional assays related to medications development division testing for potential cocaine and opiate narcotic treatment medications. *NIDA Res Monogr* **178:**440–445.

Tortella FC, Robles L, Mosberg HI and Holaday JW (1984) Electroencephalographic assessment of the role of δ receptor in opioid peptide-induced seizures. *Neuropeptides* **5:**213–216.

Traynor JR, Corbett AD and Kosterlitz HW (1987) Diprenorphine has agonist activity at κ-receptors in the myenteric plexus of the guinea pig ileum. *Eur J Pharmacol* **137:**85–89.

Traynor JR and Elliot J (1993) Delta-opioid receptor subtypes and cross-talk with mu receptors. *Trends Pharmacol Sci* **14:**84–86.

Traynor JR, Guo L, Coop A, Lewis JW and Woods JH (1999) Ring constrained orvinols as analogs of buprenorphine. *J Pharmacol Exp Ther* **291:**1093–1099.

Traynor JR and Nahorski SR (1995) Modulation by μ-opioid agonists of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **47:**848–854.

Tseng LF, Narita M, Mizoguchi H, Kawai K, Mizusuna A, Kamei J, Suzuki T and Nagase H (1997) *Delta*-1 opioid receptor-mediated antinociceptive properties of a nonpeptidic *delta* opioid receptor agonist (−)TAN-67, in the mouse spinal cord. *J Pharmacol Exp Ther* **280:**600–605.

Wild KD, Vanderah TW, Bilsky EJ, McNutt R, Chang K-J and Porreca F (1993) Antinociceptive actions of BW373U86 in the mouse. *J Pharmacol Exp Ther* **267:**858–865.

Send reprint requests to: Dr. J. R. Traynor, Department of Pharmacology, University of Michigan Medical School, 1301 MSRBIII, Ann Arbor, MI 48109-0632. E-mail: jtraynor@umich.edu