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Characterization of the complex morphinan derivative BU72 as a high efficacy, long-lasting mu-opioid receptor agonist

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Abstract

The development of buprenorphine as a treatment for opiate abuse and dependence has drawn attention to opioid ligands that have agonist actions followed by long-lasting antagonist actions. In a search for alternatives to buprenorphine, we discovered a bridged pyrrolidinomorphinan (BU72). In vitro, BU72 displayed high affinity and efficacy for mu-opioid receptors, but was also a partial delta-opioid receptor agonist and a full kappa-opioid receptor agonist. BU72 was a highly potent and long-lasting antinociceptive agent against both thermal and chemical nociception in the mouse and against thermal nociception in the monkey. These effects were prevented by mu-, but not kappa- or delta-, opioid receptor antagonists. Once the agonist effects of BU72 had subsided, the compound acted to attenuate the antinociceptive action of morphine. BU72 is too efficacious for human use but manipulation to reduce efficacy could provide a lead to the development of a treatment for opioid dependence.

Keywords: mu-opioid receptor; Agonism; Antagonism; Antinociception; (Mouse); (Monkey)

1. Introduction

Buprenorphine (Fig. 1) is an alternative to methadone and levo-alpha acetyl methadol (LAAM) substitution therapy for the treatment of opioid abuse. The mixed agonist/antagonist profile of buprenorphine (Cowan et al., 1977a,b; Bickel and Amass, 1995) provides for efficacy for treatment compliance, together with the ability to attenuate any subsequently self-administered heroin or other opiate. However, the degree of reinforcing effect afforded by buprenorphine is less than obtained with methadone, and may not be sufficient to maintain certain opiate addicts in treatment (Fudala and Johnson, 1995).

We, and others, have sought to develop mu-opioid receptor agonists with subsequent and long-acting, buprenorphine-like ability to block the reinforcing effects of subsequently administered heroin for extended periods of time, as improved treatment drugs. Such compounds include methoclocinnamox (MC-CAM; 2a, and Fig. 1; Woods et al., 1995) and its *p*-nitro analogue N-CPM-CACO (2b, Fig. 1; McLaughlin et al., 1999). These compounds incorporate an α,β-unsaturated amide group for possible Michael addition to suitable nucleophiles in the receptor. However, evidence suggests that the long-lasting action of the compounds is due to interaction of the drugs with lipophilic sites in the receptor, rather than through formation of a covalent bond (Sebastian et al., 1993; Piggot et al., 1995).

Consequently, we have considered candidate compounds that are predicted to have powerful lipophilic binding interactions with the mu-opioid receptor and thus slow

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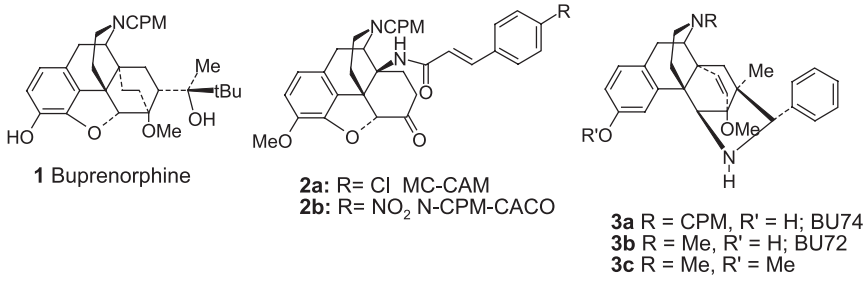

Fig. 1. Structure of BU72 and related compounds.

receptor kinetics that would allow for therapy of long duration. BU72 (3b, Fig. 1) is a bridged pyrrolidinomorphinan with a phenyl group conformationally constrained to interact with a lipophilic binding site in the opioid receptor crucial to the opioid actions of orvinols such as etorphine and buprenorphine (Brandt et al., 1993; Husbands and Lewis, 1995). When compared structurally to buprenorphine, the phenyl ring in BU72 corresponds to the *t*-butyl group of buprenorphine but is attached β- to C_7 whereas the *t*-butyl group of buprenorphine is attached α- to C_7. We have shown in preliminary in vitro studies that BU72 is a high-efficacy mu-opioid receptor agonist (Husbands and Lewis, 1995). Moreover, we have recently reported that its N-CPM derivative (BU74; 3a, Fig. 1) shows wash-resistant binding to opioid receptors (Husbands et al., submitted for publication). Here we report further evaluation of the opioid pharmacology of BU72 in vitro, and in vivo in the mouse, to examine the hypothesis that BU72 has initial agonist action, followed by a long-lasting decrease of morphine-induced antinociception due to its interaction with a lipophilic binding site on the mu-opioid receptor. The results show that in vitro BU72 is a high-efficacy mu- and kappa-opioid receptor agonist, with partial agonist action at the delta-opioid receptor, and that in vivo, the compound is a highly efficacious long-acting mu-opioid receptor agonist, followed by an attenuation of morphine of even longer duration. Experiments in the monkey confirm the extremely high efficacy of the compound.

2. Materials and methods

2.1. Animals

2.1.1. Rodents

The following animals were used: male NIH mice weighing 25–30 g (tail-withdrawal and acetic acid writhing); male ICR mice weighing 20–30 g (phenylquinone writhing, tail-flick and hot-plate); male Swiss Webster mice weighing 30–35 g (vas deferens); male Dunkin–Hartley guinea pigs weighing 350–400 g (ileum). Animals were housed in groups in a temperature-controlled room maintained on a 12-h light/dark cycle. Food and water were available ad libitum until the time of the experiments.

2.1.2. Primates

Two (one male and one female) captive-bred rhesus monkeys (*Macaca mulatta*) were used to evaluate BU72. Monkeys were singly housed in rooms maintained at 20–22 °C with controlled humidity, and a 12-h light/dark cycle. Monkeys were fed standard primate chow biscuits (Purina, Richmond, VA) daily, supplemented by fruit two times per week. Water was freely available.

2.1.3. Animal care and use

Animals were maintained in accordance with the Guidelines of the Institute of Laboratory Animal Resources, National Health Council, U.S.A. (Department of Health, Education and Welfare, Publication ISBN 0-309-05377-3, revised 1996). The experimental protocols were performed in accordance with the Declaration of Helsinki and were approved by the appropriate (University of Michigan or Virginia Commonwealth University) University Committee on the Care and Use of Laboratory Animals.

2.2. Synthesis of BU72

BU72 was prepared by 3-*O*-demethylation of the corresponding methyl ether (3c, Fig. 1; Husbands and Lewis, 1995) by treatment at 110 °C with sodium propane thiolate (six equivalent) in HMPA for 3 h. Addition of NH_4Claq and extraction with diethylether followed by column chromatography yielded BU72 as a white powder in 62% yield. IR 3400 (OH) cm^{-1}; ^{1}Hnmr (270 MHz: $CDCl_3$) δ 1.02 (3 H, s, 7-Me), 2.25 (3 H, s, NMe), 3.43 (3 H, s, 6-OMe), 3.98 (1 H, s, H-5), 4.62 (1 H, s, OH), 5.94 (1 H, d, H-18), 6.04 (1 H, d, H-19), 5.57 (1 H, d, H-1), 6.69 (1 H, s, H-4), 6.95 (1 H, d, H-2), 7.34 (3 H, m, 3′ ,4′ ,5′ –PhC–N), 7.64 (2 H, m, 2′ ,6′ –PhC–N); ^{13}Cnmr (67.5 MHz, $CDCl_3$) δ 15.1, 20.7, 24.6, 32.6, 36.5, 40.3, 43.1, 45.8, 51.2 52.4, 58.9, 59.5, 65.6, 65.8, 91.3, 111.6, 112.4, 125.8, 126.2, 127.0, 127.3, 127.9, 137.6, 142.7, 146.0, 154.7; EIMS 428 (M^+); HRMS ($C_{28}H_{32}N_2O_2$) Calculated 428.246379, Found 428.246208; microanalysis ($C_{28}H_{32}N_2O_2 \cdot 2HCl \cdot 2H_2O$) CHN.

2.3. Other chemicals and drugs

[^{3}H]Diprenorphine (45 and 58 Ci/mmol), [^{3}H]DAMGO ([^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; 54 Ci/mmol), [^{3}H]DPDPE ([^{3}H]-*c*[D-Pen^2, D-Pen^4]enkephalin; 39 Ci/

mmol), [^{3}H]bremazocine (26 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from DuPont NEN (Boston, MA). The following drugs were generous gifts from the National Institute on Drug Abuse (Rockville, MD): CTAP (D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2), fentanyl, buprenorphine, methocinnamox (M-CAM), naloxone, naltrexone, SNC80 ((+)-4-[(*R*)-[(2*S*,5*R*)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-methoxyphenyl)methyl]-*N*,*N*-diethylbenzamide) and U69593 ((5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl) benzeneacetamide). Naltrindole and nor-binaltorphimine (nor-BNI) were a kind gift from National Institutes of Health (Bethesda, MD). BW373U86 ((±)-4-[(*R**)-[(2*S**,5*R**)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-hydroxyphenyl)methyl]-*N*,*N*-diethylbenzamide dihydrochloride) was from Burroughs Wellcome, Research Triangle Park, (NC), and bremazocine was from Sandoz (Basel, Switzerland). Morphine sulfate was purchased from Mallinckrodt (St. Louis, MO). All tissue culture materials were from Invitrogen (Carlsbad, CA, USA). All other biochemicals were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade.

2.4. In vitro studies

2.4.1. Homogenate preparation

2.4.1.1. Cells. C6 glioma cells expressing mu- or delta-opioid receptors (C6μ, C6δ, Lee et al., 1999) and CHO cells expressing human kappa-opioid receptors (CHOκ, Zhu et al., 1997) were cultured under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium without (C6 cells) or with nutrient mix F-12 (CHO cells), supplemented with 10% fetal calf serum, in the presence of 1 mg/ml Geneticin. Once cells were confluent, 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×*g*. The cell pellet was suspended in 50 mM Tris–HCl buffer pH 7.4, and homogenized with a tissue tearor (Biospec Products, Bartlesville, OK). The resultant homogenate was centrifuged for 15 min at 18 000×*g* at 4 °C and the pellet collected, resuspended and recentrifuged. The final pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4 and was stored at −80 °C.

2.4.1.2. Mouse brain. Mice (male NIH Swiss) treated with 0.32 mg/kg BU72 or vehicle subcutaneously (s.c.) were killed by cervical dislocation 24 h later. Brains were rapidly removed and homogenized in Tris–HCl buffer (50 mM, pH 7.4) and were used for ex vivo binding assays without further preparation.

2.4.2. Ligand binding assays

2.4.2.1. Competition assays. To determine the affinity of BU72 for mu-, delta-and kappa-opioid receptors, membrane homogenates from C6μ, C6δ or CHOκ cells (30-60 μg protein, Lowry et al., 1951) were incubated at 25 °C in 50 mM Tris–HCl buffer, pH 7.4 for 1 h with 0.2 nM [^{3}H]diprenorphine and varying concentrations of unlabeled BU72. Nonspecific binding was defined with 10 μM naloxone. Reactions were terminated by filtration through glass fiber filters (Schleicher and Schuell #32, Keene, NH) mounted in a Brandel 24-well harvester. The filters were washed thrice with ice-cold Tris–HCl, pH 7.4, and radioactivity retained determined by scintillation counting after addition of 3 ml of Ultima Gold liquid scintillation fluid. EC_{50} and K_i values were determined using GraphPad Prism (GraphPad, San Diego, CA).

2.4.2.2. Ex vivo binding. Brain homogenates (1 mg protein; Lowry et al., 1951) from mice treated with BU72 (0.32 mg/kg) were incubated with 1.0 nM [^{3}H]DAMGO, [^{3}H]DPDPE, or [^{3}H]bremazocine to label mu-, delta- or kappa-opioid receptors, respectively. Experiments using [^{3}H]bremazocine included 1 μM DAMGO and 1 μM DPDPE to block mu- and delta-opioid binding sites. Assays were terminated and counted as above. Data are presented as percentage of the binding in brain homogenates from vehicle-treated controls.

2.4.3. [^{35}S]GTPγ1S binding assays

Agonist stimulation of [^{35}S]GTPγS binding was measured as described by Traynor and Nahorski, 1995. Cell membranes (30–60 μg protein) prepared as above were incubated for 1 h at 30 °C in GTPγS binding buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). [^{35}S]GTPγS (0.1 nM), GDP (10 μM), and varying concentrations of unlabeled ligand were added to a final volume of 1 ml. The reaction was terminated by rapid filtration as above, samples were washed with GTPγS binding buffer and radioactivity retained on filters analyzed by scintillation counting as above. Basal binding was determined in the absence of unlabeled ligand, and maximal stimulation of binding was defined using the full agonists fentanyl (mu), SNC80 (delta) and U69,593 (kappa), all at a concentration of 10 μM. EC_{50} values were calculated using GraphPad Prism (San Diego, CA).

2.4.4. Isolated tissue bioassays

2.4.4.1. Guinea-pig ileum. The longitudinal muscle from male Dunkin–Hartley guinea pigs, with the myenteric plexus attached, was prepared according to Paton and Vizi (1969). The muscle strip was mounted in Krebs–bicarbonate solution at 37 °C of the following composition (in mM): NaCl 118, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 11.5 and bubbled with 5% CO_2 in oxygen. Field electrical stimulation (supramaximal voltage, 1-ms impulse duration at 0.1 Hz; Grass S-88 stimulator, West Warwick, RI, USA) was delivered through platinum wire electrodes positioned at the top and bottom of the organ bath. Muscle contractions were recorded by isometric

transducer (Metrigram, Cleveland, OH, USA) coupled to a multichannel polygraph (Grass 7D, West Warwick, RI, USA). BU72 concentration–response curves were generated using cumulative additions. Where applicable, the mu-opioid receptor antagonist CTAP or the kappa-opioid receptor antagonist nor-BNI was added to the organ bath 30 min before construction of the BU72 concentration–response curve.

2.4.4.2. Mouse vas deferens. Vasa deferentia were prepared from Swiss–Webster mice according to Hughes et al. (1975), bathed at 31 °C in Mg^{2+}-free Krebs solution (see above), and bubbled with a mixture of oxygen and 5% CO_2. Field stimulation (paired shocks of 100-ms delay between supramaximal rectangular pulses of 1-ms duration, delivered at a rate of 0.1 Hz) and recordings were performed as for the guinea-pig ileum. BU72 concentration–response curves were generated using cumulative additions. Where applicable, the delta opioid receptor antagonist naltrindole was added to the organ bath 30 min before construction of the BU72 concentration–response curve.

2.5. In vivo studies

2.5.1. Mouse

2.5.1.1. Antiwrithing assay. Mice received agonist or vehicle subcutaneously (s.c.) and, except for the time course study, this was followed 20 min later with 0.4 ml of 0.6% acetic acid given intraperitoneally (i.p.; Koster et al., 1959) or 10 min later by 2 mg/kg (i.p.) of a freshly prepared *p*-phenylquinone solution (Pearl and Harris, 1996). Mice were placed in Plexiglas cages and were observed for stretches characterized as an elongation of the mouse's body, development of tension in the abdominal muscles and extension of the hindlimbs. The total number of stretches during a 5-min period was counted. Antinociception was measured as a reduction in the number of stretches in drug-treated as compared to vehicle-treated mice. Where indicated, antagonists were administered s.c. at the following concentrations and at times prior to agonist: M-CAM, 1.8 and 3.2 mg/kg, 1 h; naltrindole, 10 mg/kg, 15 min; nor-BNI, 32 mg/kg, 24 h. These have been shown to be conditions for selective antagonism of mu-, delta- and kappa-opioid receptor-mediated effects, respectively (Broadbear et al., 1994, 2000; Broom et al., 2000).

2.5.1.2. Tail-flick assay. Mice (6–10 per group) were injected with a single dose of test drug or vehicle (s.c.) and, 20 min later (or the times indicated), the tail was placed in a groove containing a slit, under which is a photoelectric cell that focuses heat on the tail causing the mouse to flick its tail (Aceto et al., 1997). The heat source was adjusted to produce tail-flick latencies of 2–4 s under control conditions.

2.5.1.3. Warm water tail-withdrawal assay. Each mouse (5 per group) was placed in a cylindrical restraint (Harvard Apparatus, South Natick, MA, USA) with the tail fully exposed. Approximately one-third of the tail was immersed in water at 50 °C, and latency to complete tail-withdrawal was measured (Houshyar et al., 2000). Baseline latencies were typically 2–4 s. Agonists and vehicle were administered i.p. and tail-withdrawal latencies were measured 25 min later or as specified. A cut-off latency of 20 s was used to prevent injury to the tail. Mice that did not respond within this time were removed and assigned a score of 20 s. Where indicated, antagonists were administered i.p. at the following doses and at times prior to agonist: M-CAM, 1.8 mg/kg, 1 h; naltrindole, 10 mg/kg,15 min; nor-BNI, 32 mg/kg, 24 h.

2.5.1.4. Hot-plate assay. The method described by Eddy and Leimbach (1953) was used. A modified 1000-ml Pyrex beaker (bottom removed) was placed on the hot-plate maintained at 56 °C. Each mouse was exposed to the hot-plate for two trials spaced 5 min apart. Only mice that gave control latency in the range of 6–10 s on both trials served as subjects. Each subject received a single s.c. dose of test drug (6–10 mice per dose), and 30 min later, was tested on the hot plate. Activity was scored as positive if the mouse jumped, licked or shook its paws at least 5 s beyond its average control latency. Cut-off time was 15 s to prevent tissue damage.

2.5.2. Monkey warm water tail-withdrawal assay

The procedure has been described in detail previously (Dykstra and Woods, 1986). Monkeys were seated in primate restraint chairs, and the lower 15 cm of the shaved tail was immersed in water at either 50 or 55 °C. Monkeys were tested at the two water temperatures in varying order, with tests in the same monkey separated from each other by approximately 2 min. Tail-withdrawal latencies were timed on a stopwatch in 0.1 s increments. To prevent tissue damage, tails were removed from the water if they remained immersed for 20 s (cut-off latency). Sessions began with control determinations at each water temperature. The antinociceptive effects of BU72 and morphine were determined using a cumulative dosing procedure with a 30-min inter-injection interval. Experimental sessions were carried out no more frequently than once per week.

2.5.3. Analysis of in vivo data

Assay results were plotted as raw data or converted to percent maximum possible effect (%MPE) as: %MPE=[(response in drug treated mice/response in control mice)]×100%; or percent inhibition of the maximal response as: %inhibition=100−[(response in the drug treated mice)/(response in control mice)]×100. In the heat antinociceptive assays, baseline latencies were subtracted before calculating %MPE or %inhibition. Effective $dose_{50}$ for agonists (ED_{50}) and antagonists (AD_{50}) and 95% confidence limits (CL; given in parentheses) were determined according to the

method of Tallarida and Murray (1987, procedure 8). Dose–effect curves were considered to be significantly different when the range of values contained within the 95% confidence limits did not overlap.

3. Results

3.1. In vitro assays

3.1.1. Ligand binding and [^{35}S]GTPγS assays

BU72 displayed very high affinity binding to mu-, delta- and kappa-opioid receptors as indicated by the K_i values derived from the inhibition of [^{3}H]diprenorphine binding (Table 1). The compound showed no selectivity and Hill coefficients for the binding were all close to unity. The affinity of BU72 for the mu-opioid receptor was an order of magnitude higher than buprenorphine and 100-fold higher than morphine.

BU72 was found to be a potent, highly efficacious agonist at mu- and kappa-opioid receptors. BU72 caused a greater maximal stimulation of [^{35}S]GTPγS binding than the mu-opioid receptor agonist fentanyl and an equivalent maximal effect to the kappa-opioid receptor agonist U69593; at the delta-opioid receptor, BU72 was a partial agonist (Table 2). This contrasts with buprenorphine which is a mu-opioid receptor partial agonist, but lacks delta- or kappa-opioid receptor agonism.

3.1.2. Isolated tissue assays

Confirmation of this efficacy profile of BU72 was obtained in isolated tissue assays. In the guinea pig ileum, BU72 was a very potent agonist (IC_{50} 0.14±0.09 nM). This response was not antagonized by the selective mu-opioid receptor antagonist CTAP (200 nM) but the kappa-opioid receptor antagonist norBNI (5 nM) which caused a small (2.65±0.09 fold) rightward shift in the BU72 concentration effect curve. This shift is much less than predicted for kappa-opioid receptor involvement which should be approximately 50-fold based on the affinity of the antagonist (Traynor et al., 1999), and is likely due to an action of BU72 at mu-opioid receptors. In the mouse vas deferens assay, BU72 was also a potent agonist (IC_{50} 1.31±0.67 nM) and this effect was antagonised by pretreatment with selective antagonist naltrindole (1 nM) which shifted the concentration–effect curve for BU72 by 5.5±0.9-fold, suggesting a delta-opioid receptor-mediated effect in this tissue. In the isolated tissue assays, the agonist effects of BU72 could not be reversed by repeated washing.

3.2. In vivo assays

3.2.1. Mouse antinociception assays for agonist properties of BU72

BU72 (s.c.) produced potent, dose-dependent, antinociception in the mouse writhing assay using acetic acid as the nociceptive stimulus (Fig. 2A). BU72 was about 20 times more potent than buprenorphine (Fig. 2A) and 100-fold more potent than morphine. All compounds were full agonists in this assay. Similar results were obtained using intraperitoneal phenylquinone as the nociceptive stimulus (data not shown). Pretreatment of mice with the long-lasting mu-opioid receptor antagonist methocinnamox (M-CAM, 1.8 and 3.2 mg/kg, 1 h pretreatment) dose-dependently reduced the antinociceptive effectiveness of BU72. Neither the delta-opioid receptor antagonist naltrindole (10 mg/kg, 15 min pretreatment) nor the kappa-opioid receptor antagonist nor-BNI (32 mg/kg, 24 h pretreatment) altered the antinociceptive effect of BU72 (Fig. 2B).

In assays using heat as the nociceptive stimulus, BU72 was highly potent and efficacious. In the warm water tail-withdrawal assay using 50 °C water, BU72 gave an ED_{50} of 0.07 (0.06–0.08) mg/kg compared to a value of 26.8 (23.4–30.7) mg/kg for morphine and 0.12 (0.02–0.84) mg/kg for buprenorphine which was only a partial agonist (Fig. 3A). BU72 was highly potent in the hot-plate assay (ED_{50}=0.01 (0.003–0.029) mg/kg) and in the radiant-heat tail-flick assay (ED_{50}=0.005 (0.003–0.008) mg/kg), and in both assays was considerably more potent than morphine (85 times morphine in the hot-plate and 380 times morphine in the tail-flick). Pretreatment of the mice with M-CAM (1.8 mg/kg, 1 h pretreatment) fully inhibited BU72-mediated antinociception in the tail-withdrawal assay (Fig. 3B), and in the tail-flick assay, naloxone inhibited the response with an AD_{50} of 0.02 (0.01–0.04) mg/kg. In contrast, no antagonism of BU72-mediated antinociception was observed following administration of the naltrindole (10 mg/kg, 15 min pretreatment) or nor-BNI (32 mg/kg, 24 h pretreatment, Fig. 3B).

The maximally effective dose of BU72 (0.32 mg/kg) was antinociceptive for at least 8 h. This was comparable to the time-course for the maximally effective dose of buprenorphine (3.2 mg/kg), but longer than the maximally effective dose of morphine (100 mg/kg; Fig. 3C). A higher dose of BU72 (10 mg/kg) maintained maximal antinociception for at least 12 h after administration, but was ineffective 24 h after administration. Similarly, 8 h after an ED_{80} dose (0.02 mg/kg), the antinociceptive action of BU72 in the tail-flick assay was still at 30% of

Table 1
Affinity values (K_i, nM) for the binding of BU72 to mu-, delta- and kappa-opioid receptors

Compound	mu	delta	kappa
BU72	0.06±0.02	0.12±0.01	0.03±0.01
Buprenorphine	0.74±0.11	1.1±0.3	0.14±0.03
Morphine	6.3±2.5	171±18.9	60.9±17.3

Membranes from C6μ, C6δ and CHOκ cells were incubated with varying concentrations of compound in the presence of 0.2 nM [^{3}H]diprenorphine, as described under Materials and Methods. Data are expressed as the mean K_i±S.E.M. for three determinations performed in duplicate.

Table 2
Stimulation of [^{35}S]GTPγS binding by BU72 acting at mu-, delta- or kappa-opioid receptors

	mu		delta		kappa	
	EC_{50} (nM)	Maximum (%)	EC_{50} (nM)	Maximum (%)	EC_{50} (nM)	Maximum (%)
BU72	0.054±0.009	116±4.3	0.58±0.20	62±6.5	0.033±0.009	98±6.5
Buprenorphine	0.18±0.07	33±4.5	NS		NS	
Morphine	41.1±6.3	86±6	1414±620	37±9	347±142	34±8

Assays were performed in membranes from C6μ, C6δ and CHOκ cells as described in Materials and Methods. Data are expressed as the mean±S.E.M. for three determinations, each performed in triplicate. Percent (%) maximum values represent comparison of stimulation by fentanyl (mu), SNC80 (delta) and U69593 (kappa). NS=less than 10% stimulation.

maximum, but no antinociceptive activity was evident at 18 h postinjection (data not shown).

Because BU72 was designed to have strong lipophilic binding to the mu-opioid receptor, the ability of naloxone and naltrexone to reverse, rather than prevent, the antinociceptive effect of BU72 was investigated in the tail-withdrawal assay. When administered 1 h after, BU72 (0.32 mg/kg) both antagonists at 10 mg/kg reversed the antinociceptive effect of BU72 (Fig. 4). However, the antinociceptive action of BU72 returned once the naloxone was no longer effective, approximately 2–3 h after administration of the antagonist. Naltrexone was longer lasting than naloxone and blocked the antinociceptive effect of BU72 for at least 7 h.

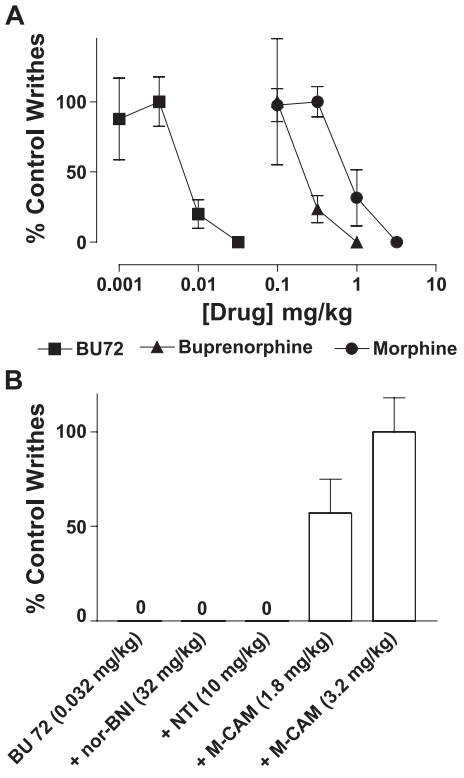

Fig. 2. (A) Antinociceptive effect of s.c. administered BU72 buprenorphine and morphine in the mouse acetic acid-induced writhing assay. (B) Antagonism of BU72 (0.32 mg/kg) by M-CAM (1.8 or 3.2 mg/kg, s.c., 1 h pretreatment), naltrindole (NTI, 10 mg/kg, s.c., 15 min pretreatment) or norBNI (32 mg/kg, s.c., 24 h pretreatment). Values represent the meanšS.E.M. for 56 mice at each data point.

3.2.2. Attenuation of morphine-induced antinociception by BU72

In naïve animals, morphine (3.2 mg/kg) completely blocked the nociceptive response to acetic acid in the writhing assay. About 24 to 72 h after pretreatment with

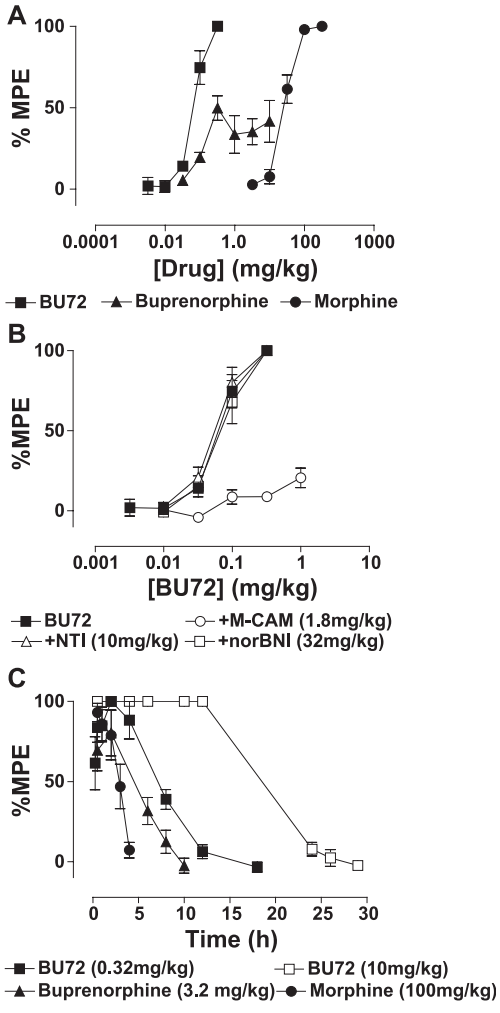

Fig. 3. (A) Antinociceptive effect of i.p. BU72 buprenorphine and morphine in the mousewarm-water tail-withdrawal assay at 50 °C. (B) Antinociceptive effects of BU72 in the absence or presence ofpretreatment with single doses of selective opioid antagonists M-CAM (1.8 mg/kg, 1 h), naltrindole (NTI; 10 mg/kg, 15 min), or nor-BNI (32 mg/kg, 24 h). (C) Time course of the antinociceptive effect of BU72[0.32 and 10 mg/kg], buprenorphine (3.2 mg/kg) and morphine (100 mg/kg).%MPE=percent maximumpossible effect. Values represent the meanšS.E.M. for five mice at each data point.

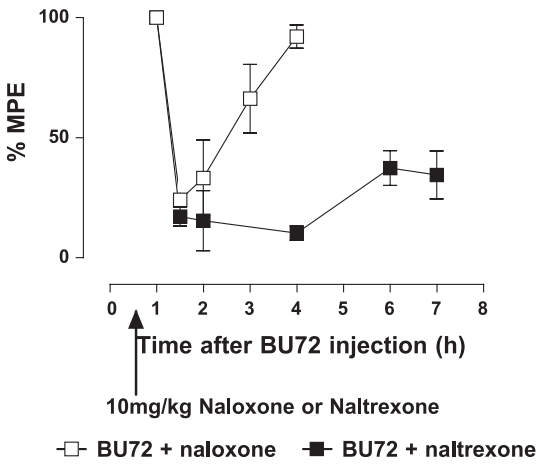

Fig. 4. Antinociceptive effect produced by BU72 (0.32 mg/kg, i.p.) in the mouse warm-water tail withdrawal assay at 50 °C and its reversal by naloxone (□) or naltrexone (■) both 10 mg/kg, i.p. %MPE=percent maximum possible effect. Values represent the means±S.E.M. for five mice at each data point.

BU72 at a maximally effective antinociceptive dose (0.32 mg/kg), the effect of morphine was partially prevented but returned to control values after 1 week (Fig. 5A). A more pronounced and longer lasting attenuation was seen following 10 mg/kg BU72 (Fig. 5B). With this higher dose, there was also partial attenuation of the antinociceptive effect of the delta-opioid receptor agonist BW373U86 and the kappa-opioid receptor agonist bremazocine (Fig. 5C).

The ability of BU72 to attenuate morphine using heat as the nociceptive stimulus was also examined. BU72 reduced the antinociceptive effect of an ED_{80} dose of morphine (6 mg/kg) in the tail-flick test (Table 3). Complete blockade of the morphine response was observed between 48 and 96 h after administration of doses of 1, 10 and 30 mg/kg BU72, but evidence of attenuation was still present after 168 h. Similarly, in the tail-withdrawal assay in 50 °C water 24 h after administration of 0.32 mg/kg BU72, there was threefold shift in the dose–response curve to morphine from a control ED_{50} of 27.1 (18.0–40.9) mg/kg, to an ED_{50} at 24 h of 72.4 (41.8–125.4) mg/kg, but no shift in the dose–response curve for BU72 itself (ED_{50} in naïve animals=0.07 (0.05–0.09) mg/kg, 24 h after BU72 administration ED_{50}=0.07 (0.04–0.12) mg/kg).

3.2.3. In vivo studies in the monkey

BU72 was a highly potent antinociceptive agent in the tail-withdrawal assay in the monkey at water temperatures of 50 °C (ED_{50}=0.006 (0.005–0.009) mg/kg) and 55 °C (ED_{50}=0.018 (0.012–0.027) mg/kg). The maximal effect lasted at least 6 h, but after 24 h, no antinociception was evident. The dose–effect curve for morphine (ED_{50}=11.2 (6.5–19.2) mg/kg) was unaltered 24 h after a maximum dose of BU72 (0.032 mg/kg). BU72 caused severe respiratory depression such that one monkey stopped breathing at 0.01 mg/kg BU72 and the other at 0.1 mg/kg. The monkeys were rescued with naltrexone.

3.3. Ex vivo binding studies in the mouse

24 h after administration of 0.32 mg/kg BU72, there was a significant (P≤0.001) 64±8% reduction in specific binding of the mu-opioid receptor ligand [^{3}H]DAMGO to mouse brain homogenates compared with homogenates from

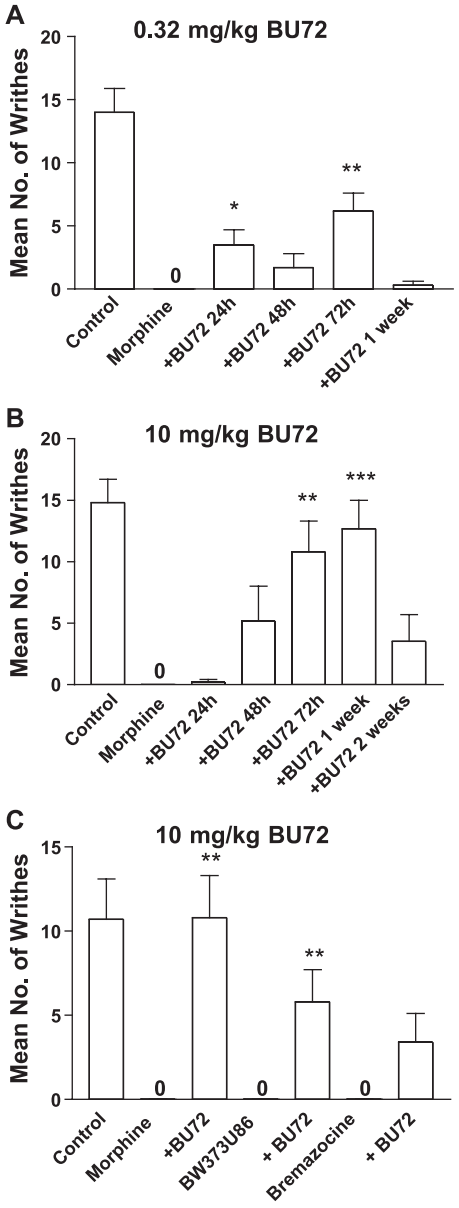

Fig. 5. Antinociceptive effect of morphine (3.2 mg/kg) in the writhing assay before and after pretreatment with: (A) BU72 0.32 mg/kg given as a single dose i.p. 24, 48 and 72 h and 1 week before morphine and (B) BU72 10 mg/kg given as a single dose i.p. 24, 48 and 72 h and 1 and 2 weeks before morphine. (C) Antinociceptive effect of maximally effective doses of morphine (3.2 mg/kg), BW 373U86 (10 mg/kg) and bremazocine (0.1 mg/kg), with or without pretreatment with BU72 10 mg/kg, s.c, for 72 h. Values represent the means±S.E.M. for six mice at each data point. ***P<0.005, **P<0.01, *P<0.05 versus vehicle pretreatment (Students' t-test).

Table 3
Attenuation of the antinociceptive activity of an ED_{80} dose of morphine by BU72 in the tail-flick test in the mouse

Pretreatment time (h)	AD_{50} (mg/kg)	Percentage loss (%) of morphine effect at BU72 dose of		
		1 mg/kg	10 mg/kg	30 mg/kg
24	–	26	51	0
48	2.9 (1.0–8.4)			
72	1.4 (0.5–4.3)			
96	3.6 (0.8–10.7)			
120	–	64	83	52
144	–	51	45	69
168	–	0	61	30

vehicle treated mice. In contrast, binding of the delta-opioid receptor ligand [^{3}H]DPDPE and the kappa-opioid receptor ligand [^{3}H]bremazocine was not altered.

4. Discussion

The present study indicates that the pyrrolidinomorphinan BU72 is a highly efficacious, long-lasting mu-opioid receptor agonist in the mouse and the monkey. The agonist actions of BU72 are followed, at least in the mouse, by delayed and persistent reduction of mu-opioid receptor mediated antinociception, although this was not evident with the lower doses used in the monkey.

The very high efficacy of BU72 is evident from its ability to cause severe respiratory depression in the monkey and its equivalent potency in all antinociceptive assays in the mouse whether the nociceptive stimulus is chemical (antiwrithing assays) or thermal. Confirmation that the antinociception was mediated through the mu-opioid receptor was obtained by a loss of effect on treatment with the long-lasting mu-opioid receptor-selective antagonist M-CAM. Neither naltrindole nor norBNI, selective antagonists for delta- and kappa-opioid receptors, respectively, had any effect on the antinociception produced by BU72. In contrast to the in vivo findings, BU72 has high affinity and agonist activity at all three opioid receptors in vitro. Presumably, the mu-opioid receptor effects predominate in the in vivo antinociceptive assays because delta- and kappa-opioid receptor agonists are less-effective antinociceptive agents (Tyers, 1980; Traynor et al., 1999; Broom et al., 2002).

The antinociceptive effect of BU72 was completely reversed by either naloxone or naltrexone. However, BU72 had access to the mu-opioid receptor several hours after administration of the reversible antagonists since antinociception was reestablished when the effect of the shorter-acting antagonists had dissipated. It is thought that buprenorphine is long-lasting because the C_7 *t*-butyl group forms a tight lipophilic interaction with the receptor, thus slowing down its dissociation kinetics (Hambrook and Rance, 1976). Certainly, it is difficult to reverse the mu-opioid receptor agonist action of buprenorphine once it is established (Cowan et al., 1977a; France et al., 1984). On the other hand, the data presented here would suggest that the mechanism of the long duration of action of BU72 may be different from that of buprenorphine.

One possible explanation involves the continued presence of BU72 in central tissues rather than persistent binding to the recognition site of the receptor. However, ex vivo binding studies 24 h after 0.32 mg/kg BU72 showed a reduction in specific mu-opioid receptor binding, but not delta- or kappa-opioid receptor binding, suggesting that long-lasting antagonist effect of BU72 is mostly confined to the mu-opioid receptor. Indeed, attenuation of kappa- and delta-opioid receptors was seen only after administration of higher doses. An alternative possibility is that BU72 can be displaced from the pharmacophore recognition site of the mu-opioid receptor by antagonist, but remains tethered to the receptor at a secondary binding site. This secondary site could be in the receptor, as is thought to be case with long-lasting β-adrenergic agonist salmeterol (Johnson et al., 1993) or may be due to anchoring to the lipid membrane as has been suggested for the persistent opioid activity of etorphine derivatives containing large hydrophobic groups (Bell and Schaeffer, 1997). The ability of antagonists to reverse the mu-opioid receptor activity of BU72 in vivo contrasts with the guinea pig ileum studies where the mu-opioid receptor antagonist CTAP was ineffective. This could be due to the peptidic nature of CTAP compared with the alkaloids naltrexone or naloxone. Certainly, the alkaloid nor-BNI did reverse BU72 in a manner suggestive of an action at mu-, rather than kappa-opioid receptors.

It is possible that persistent occupation of the mu-opioid receptor by BU72 leads to tolerance, either by desensitization or a combination of desensitization and down-regulation and this is the mechanism underlying the delayed attenuation of morphine's effects. In vivo down-regulation of the mu-opioid receptor by agonists has yielded variable results, some reporting up-regulation and some reporting down-regulation (Tempel et al., 1988; Brady et al., 1989, Yoburn et al., 1993), but it has been shown that chronic treatment for longer periods of time with large doses of high efficacy agonists such as etorphine and fentanyl can produce down-regulation (Tao et al., 1987, Yoburn et al., 1993). The findings with BU72 are reminiscent of data obtained with dihydroetorphine, an oripavine–thebaine derivative 12 000 times more potent than morphine (Bentley and Hardy, 1967). This compound possesses antagonist properties at mu-opioid receptors for up to 6 h following its agonist action (Kamei et al., 1996). The authors conclude that either acute tolerance to mu-opioid receptor agonist-induced antinociception occurs, or that dihydroetorphine acts at a separate 'antagonist' recognition site which is allosterically coupled to the 'agonist' site on the mu-opioid receptor (Portoghese and Takemori, 1983). The fact that BU72 does not antagonize its own actions is probably because, as a high-efficacy compound, it can work in a system with less-functional receptors. The lack of delayed attenuation of

morphine by BU72 in the monkey may be because BU72 is so highly potent and efficacious in this species that very few receptors are occupied and therefore very few are desensitized or down-regulated.

In conclusion, the bridged pyrrolidinomorphinan BU72 represents a novel structure that provides a highly efficacious and long-lasting mu-opioid receptor agonist. The conformationally constrained phenyl group does not confer receptor binding selectivity for the mu-opioid receptor, but in vivo results show long-lasting actions at mu-opioid receptors, including a degree of delayed attenuation of morphine, similar to the clocinnamox family of compounds (Broadbear et al., 2000). BU72, therefore, has the profile that we sought as a potential treatment medication. Unfortunately, the efficacy of BU72 is too high, and the strong respiratory depression seen in the monkey suggests this compound would be unsafe in humans. Nonetheless, an understanding of the basic pharmacology of the compound may provide insights into the design of a treatment for opioid dependence based on this structure.

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