Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 509 (2005) 117–125

www.elsevier.com/locate/ejphar

BU74, a complex oripavine derivative with potent kappa opioid receptor agonism and delayed opioid antagonism

Stephen M. Husbands[a,*], Claire L. Neilan[b], Jillian Broadbear[b], Peter Grundt[a], Simon Breeden[c], Mario D. Aceto[d], James H. Woods[b], John W. Lewis[a], John R. Traynor[b]

[a]*Department of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, UK*
[b]*Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109, USA*
[c]*School of Chemistry, University of Bristol, Bristol, BS8 1TS, UK*
[d]*Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia 23298, USA*

Received 23 November 2004; received in revised form 21 December 2004; accepted 24 December 2004
Available online 2 February 2005

Abstract

In the search for opioid agonists with delayed antagonist actions as potential treatments for substance abuse, the bridged morphinan BU74 (17-cyclopropylmethyl-3-hydroxy-[5β,7β,3′,5′]-pyrrolidino-2′[*S*]-phenyl-7α-methyl-6,14-*endo*etheno morphinan) (**3f**) was synthesized. In isolated tissue and [^{35}S]GTPγS opioid receptor functional assays BU74 was shown to be a potent long-lasting kappa opioid receptor agonist, delta opioid receptor partial agonist and mu opioid receptor antagonist. In antinociceptive tests in the mouse, BU74 showed high efficacy and potent kappa opioid receptor agonism. When its agonist action had waned BU74 became an antagonist of kappa and mu opioid receptor agonists in the tail flick assay and of delta, kappa and mu opioid receptor agonists in the acetic acid writhing assay. The slow onset, long-duration kappa opioid receptor agonist effects of BU74 suggests that it could be a lead compound for the discovery of a treatment for cocaine abuse.

Keywords: Efficacy; Antinociception; Kappa-opioid; Delayed antagonism

1. Introduction

Buprenorphine (**1**, Fig. 1) is now established as a treatment for opiate abuse and dependence based on its pharmacological profile as a long acting mu opioid receptor partial agonist with even longer duration of action as a mu opioid receptor antagonist (Cowan and Lewis, 1995). Methoclocinnamox (**2**, Fig. 1) has a similar pharmacological profile (Woods et al., 1995); both buprenorphine and methoclocinnamox owe their extended duration of action to very powerful lipophilic interactions with the mu opioid receptor (Cowan and Lewis, 1995; Woods et al., 1995; Hambrook and Rance, 1976; McLaughlin et al., 1999). An example of a structure related to buprenorphine with powerful mu opioid receptor agonist actions followed by antagonism is the bridged morphinan derivative (BU72, 3-hydroxy-[5β,7β,3′,5′]-pyrrolidino-2′[*S*]-phenyl-7α-methyl-6,14-*endo*etheno morphinan, **3a**, Fig. 1) (Husbands and Lewis, 1995; Husbands and Lewis, 2003, Neilan et al., 2004).

There is substantial interest in the use of kappa opioid receptor agonists as potential pharmacotherapies for cocaine and other psychostimulant abuse, particularly as repeated administration of kappa opioid receptor agonists has been shown to prevent, or reduce, many of cocaine's behavioural effects (Crawford et al., 1995; Shippenberg et al., 1996; Mello and Negus, 2000). The kappa opioid receptor agonists used in these studies are not long-acting, nor do they display delayed antagonist properties. The reported

* Corresponding author. Tel.: +44 1225 383103; fax: +44 1225 386114.
E-mail address: s.m.husbands@bath.ac.uk (S.M. Husbands).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.12.035

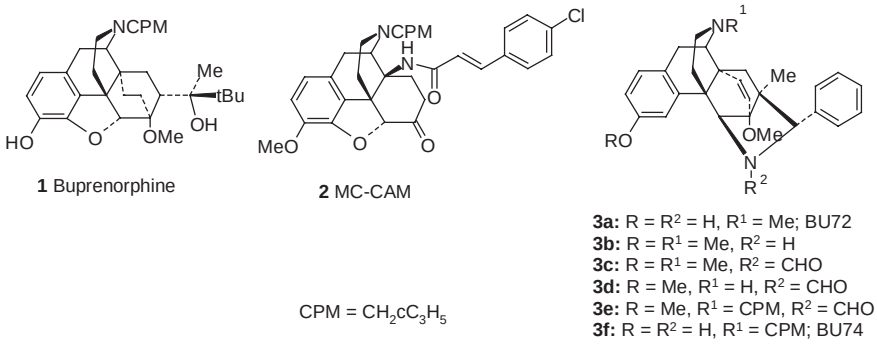

Fig. 1. Structures of BU74 and related compounds.

examples of agonists at kappa opioid receptors with subsequent antagonist actions are β-FNA (β-funaltrexamine) and DIPPA (2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl] acetamide hydrochloride). These compounds have electrophilic groups which can bind covalently to opioid receptors. β-FNA has short duration partial agonism at kappa opioid receptors, followed by selective irreversible mu opioid receptor antagonism (Portoghese et al., 1980; Ward et al., 1982). DIPPA is an agonist in the mouse acetic acid-induced writhing assay and a kappa opioid receptor antagonist in the mouse tail flick assay (Chang et al., 1994a,b).

We here report the synthesis and pharmacological investigation of BU74 (**3f**, Fig. 1), the *N*-cyclopropylmethyl congener of the long-acting mu opioid receptor agonist BU72 (**3a**); BU74 (**3f**) is a long-acting selective kappa opioid receptor agonist with high efficacy in vivo that later displays non-selective opioid receptor antagonism.

2. Materials and methods

2.1. Animals

The following animals were used: NIH mice weighing 25–30 g (tail-withdrawal and acetic acid writhing), ICR male mice weighing 20–30 g (phenylquinone writhing 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. Animals were maintained in accordance with the Guidelines of the Institute of Laboratory Animal Resources, National Health Council, U.S.A. The experimental protocols were performed in accordance with the Declaration of Helsinki.

2.2. Synthesis of BU74

From **3b** (Fig. 1), prepared as previously described in Husbands and Lewis (1995). Protection of the pyrrolidine NH group by formic acid/acetic anhydride induced formylation was followed by *N*-demethylation of the piperidine *N*-methyl group with diethylazodicarboxylate (acetonitrile, reflux, 6 h) affording the nor-compound (**3d**). This was alkylated with cyclopropylmethyl bromide in a sealed tube (CH_3CN, 100 °C, 19 h) in the presence of sodium bicarbonate to give **3e**. **3e** was deformylated using 6 M hydrochloric acid at reflux (16 h) and finally 3-*O*-demethylated using sodium propanethiolate at 120 °C (4 h) to give BU74 (**3f**), 17-cyclopropylmethyl-3-hydroxy-[5β,7β,3′,5′]-pyrrolidino-2′[*S*]-phenyl-7α-methyl-6,14-*endo*etheno morphinan: m.p. (HCl–salt) >220 °C; Rf (CH_2Cl_2/MeOH/NH_4OH, 200:10:1): 0.28; ^{1}H NMR (selected peaks) δ 1.04 (s, 3H), 3.34 (s, 3H), 3.94 (s, 1H), 4.61 (s, 1H), 5.91 (d, 8.9, 1H), 6.05 (d, 8.9, 1H), 6.54 (dd, *J* 8.3, 2.3, 1H), 6.67 (d, *J* 2.3, 1H), 6.91 (d, *J* 8.3, 1H); mass (EI): *m*/*z* 468; HRMS: $C_{31}H_{36}N_2O_2$: calc.: 468.2777, found: 468.2766; anal. ($C_{31}H_{36}N_2O_2 \cdot 2HCl \cdot 3H_2O$); calculated C: 62.51, H: 7.45, N:4.70, found C: 62.18, H: 7.05, N: 4.55.

2.3. Other chemicals and drugs

[^{3}H]Diprenorphine (45 Ci/mmol and 58 Ci/mmol), [^{3}H]DAMGO ([D-Ala, NMe-Phe, Gly-ol]-enkephalin) (54 Ci/mmol), [^{3}H]DPDPE ([D-Pen D-Pen]enkephalin) (39 Ci/mmol), [^{3}H]bremazocine (26 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from DuPont NEN (Boston, MA, USA). The following drugs were generous gifts from the National Institute on Drug Abuse (Rockville, MD, USA): CTAP (D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2, fentanyl hydrochloride, buprenorphine hydrochloride, naltrexone hydrochloride, SNC80 ((+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide), sufentanil citrate, U50,488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate salt), U69,593 ((+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide). Naltrindole and nor-binaltorphimine were a kind gift from National Institutes of Health (Bethesda, MD, USA). BW373U86 ((±)-[1(*S**),2α,5β]-4-[[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl](3-hydroxyphenyl)methyl]-*N*,*N*-diethylbenzamide dihydrobromide) was from Burroughs Wellcome (Research Triangle Park, NC, USA), bremazo-

cine from Sandoz (Basel, Switzerland) and enadoline from Pfizer (Ann Arbor). Morphine sulfate was purchased from Mallinchrodt (St. Louis, MO, USA). Methocinnamox was synthesized as previously described (Broadbear et al., 2000). All tissue culture materials were from Invitrogen (Carlsbad, CA, USA). All other biochemicals were purchased from Sigma Chemical (St. Louis, MO, USA) and were of analytical grade.

2.4. In vitro studies

2.4.1. Cell culture and membrane preparation

C6 glioma cells stably transfected with the mu or delta opioid receptors (C6μ, C6δ; Lee et al., 1999) and Chinese hamster ovary cells expressing the human kappa opioid receptor (CHOκ; Zhu et al., 1997) were cultured under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (C6 cells) or Dulbecco's modified Eagle's medium/nutrient mix F-12 (Chinese hamster ovary 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 the absence of Geneticin with no significant reduction in receptor number. Once cells had reached confluency, they were harvested in HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], 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 and homogenized with a tissue tearor (Biospec Products, Bartlesville, OK, USA). The resultant homogenate was centrifuged for 15 min at 18,000 rpm at 4 °C and the pellet collected, resuspended and recentrifuged. The final pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4, separated into 0.5-ml aliquots (0.75–1.0 mg protein) and frozen at −80 °C. Protein concentration was determined by the method of Lowry et al. (1951), using a bovine serum albumin standard.

2.4.2. Ligand binding assays

Cell membranes (30–60 μg protein) were incubated at 25 °C in 50 mM Tris–HCl buffer, pH 7.4 for 1 h with 0.2 nM [^{3}H]diprenorphine to give a final volume of 1 ml. Non-specific binding was defined with 10 μM naloxone. Reactions were terminated by filtration through glass fiber filters (Schleicher and Schuell #32, Keene, NH, USA) mounted in a Brandel 24 well harvester. The filters were washed 3 times 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.

2.4.3. [^{35}S]GTPγS 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 (guanosine-5′-*O*-(3-thio)triphosphate) (0.1 nM), GDP (guanosine 5′-diphosphate) (10 μM) and varying concentrations of unlabeled ligand were to a final volume of 1 ml. The reaction was terminated by rapid filtration as above, samples were washed with GTPγS binding buffer, pH 7.4 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 opioid receptor agonists fentanyl (mu), SNC80 (delta) and U69593 (kappa), all at a concentration of 10 μM. EC_{50} values were calculated using GraphPad Prism (San Diego, CA, USA).

2.4.4. Electrically stimulated guinea-pig ileum bioassay

Male Hartley guinea-pigs were decapitated and the small intestine removed and 20 cm of the terminal ileum discarded. The longitudinal muscle with the myenteric plexus attached was gently separated from the underlying circular muscle by the method of Paton and Vizi (1969). The muscle strip was mounted in an 8-ml water jacketed organ bath containing Krebs-bicarbonate solution 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. The tissues were kept at 37 °C and bubbled with 5% CO_2 in oxygen. An initial tension of 0.6 g was applied to the strips. The muscle strip was stimulated for 60 min before the start of each experiment. Field electrical stimulation (Grass S-88 stimulator, West Warwick, RI, USA) was delivered through platinum wire electrodes positioned at the top and bottom of the organ bath and kept at a fixed distance of 3.5 cm. The parameters of rectangular stimulation were supramaximal voltage, 1-ms impulse duration at 0.1 Hz. The electrically induced twitches were recorded by using an isometric transducer (Metrigram, Cleveland, OH, USA) coupled to a multichannel polygraph (Grass 7D, West Warwick, RI, USA).

2.4.5. Electrically stimulated isolated vas deferens bioassay

Vasa deferentia were prepared from Swiss Webster mice according to Hughes et al. (1975), bathed at 31 °C in Mg^{2+}-free Krebs solution, and bubbled with a mixture of oxygen and carbon dioxide (95:5). An initial tension of 150–200 mg was used. The experiments were performed in an 8-ml organ bath. The parameters of field stimulation were modified slightly from the original description (Toll et al., 1998); paired shocks of 100-ms delay between supramaximal rectangular pulses of 1-ms duration were delivered at a rate of 0.1 Hz, using a Grass S-88 stimulator. Contractions were recorded with an isometric transducer (Metrigram) connected to a Grass 7D polygraph.

2.5. Mouse behavioural assays

2.5.1. Antiwrithing assays

Mice received agonist or vehicle subcutaneously (s.c.) and, except for the time course study, this was followed 10

min later by 2 mg/kg intraperitoneally (i.p.) of a freshly prepared *p*-phenylquinone solution (Pearl and Harris, 1996) or 20 min later with 0.4 ml of 0.6% acetic acid given i.p. (Koster et al., 1959). Mice were placed in Plexiglass cages and 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. The antinociceptive response was expressed as number of stretches. Where indicated antagonists were administered s.c. at the following times prior to agonist: methocinnamox, 1 h; naltrindole, 15 min; nor-BNI, 24 h.

2.5.2. Hot-plate assay

The method described by Eddy and Leimbach was used (1953). 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 to 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. Percent activity for each dose tested was calculated as (total number of mice scored as positive)/(total number tested)×100.

2.5.3. 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. Where indicated, varying doses of the antagonists naltrindole or nor-binaltorphimine (nor-BNI) were administered s.c. 10 min before BU74 and tail-flick latencies measured 20 min later.

2.5.4. 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 (Janssen et al., 1963). 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 times prior to agonist: methocinnamox, 1 h; naltrindole, 15 min; nor-BNI, 24 h.

2.6. Data analysis

For binding assays, K_i values were determined and for the [^{35}S]GTPγS assays and in vitro bioassays EC_{50} were determined using GraphPad Prism (GraphPad, San Diego, CA). Antagonist affinities were determined as K_e values calculated from a single dose of antagonist according to the formula K_e=[antagonist]/(DR−1).

In vivo assay results were plotted as raw data or converted to % maximum possible effect (% MPE) as % MPE=[(response in drug treated mice/response in control mice response)]×100% or % inhibition of the maximal response as 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 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. Cell assays

In cloned cells, BU74 exhibited very high affinity for mu, delta and kappa opioid receptors with no selectivity. In this respect, it was similar to the prototype kappa opioid receptor agonist ketocyclazocine but with over 100-fold higher affinity for each opioid receptor type (Table 1). In the [^{35}S]GTPγS assay to determine agonist efficacy, BU74 gave almost the same degree of stimulation of [^{35}S]GTPγS binding as the standard kappa opioid receptor agonist U69593 and ketocyclazocine, and was 7000-times more potent than ketocyclazocine (Table 2). In C6δ cells, BU74 was 145-times less potent than in CHOκ cells and was only a partial agonist compared with the full delta opioid receptor

Table 1
Affinity values (K_i) for the binding of BU74 to mu, delta and kappa opioid receptors

Compound	K_i (nM)		
	mu	delta	kappa
BU74	0.05±0.03	0.05±0.01	0.03±0.01
Buprenorphine	0.74±0.11	1.1±0.3	0.14±0.03
Ketocyclazocine[a]	6.0±0.8	6.9±0.6	4.0±0.9

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

[a] Taken from Woods and Traynor, 2001.

Table 2
Agonist activity of BU74 in the [^{35}S]GTPγS assay

	mu		delta		kappa	
	EC_{50}	% stimulation[a]	EC_{50}	% stimulationa	EC_{50}	% stimulation[a]
BU74	ND	<10	0.29 (0.16–0.55)	42 (36–48)	0.002 (0.001–0.004)	84 (74–93)
Ketocyclazocine[b]	273±103	19	122±35	46	14.3±1.6	91

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.
ND=not determinable.
[a] Values are related to the maximal effect of the full agonists DAMGO (mu), SNC80 (delta) and U69593 (kappa).
[b] Taken from Woods and Traynor (2001).

agonist SNC80. No stimulation of [^{35}S]GTPγS binding was observed in C6μ cells expressing the mu opioid receptor but in the presence of 1 nM BU74 the dose–effect curve for the mu opioid receptor agonist fentanyl was shifted 10-fold from an EC_{50} of 10.0 (8.5–11.7) nM to 98.7 (76.5–127) nM (data not shown).

3.1.2. Isolated tissue assays

BU74 had high agonist potency in the guinea-pig ileum preparation, an effect that could not be reversed by selective competitive antagonists for mu (CTAP) or kappa (nor-BNI) opioid receptors (Table 3). However, the action of BU74 could also not be reversed by prolonged washing suggesting BU74 was pseudo-irreversibly bound. In the mouse, vas deferens preparation BU74 was a partial agonist (62% of maximum). This effect was predominantly delta opioid receptor mediated as it was readily reversed by naltrindole with K_e=0.03±0.003 nM (Table 3). Since the maximum effect in the mouse vas deferens was below full efficacy, the antagonist effect of BU74 against selective mu, delta and kappa opioid receptor agonists was determined. BU74 was an extremely potent antagonist for all three types of opioid receptor in the order mu>kappa>delta (Table 4).

Table 3
Agonist activity of BU74 in isolated tissue preparations compared with kappa agonists U69593 and bremazocine

Compound	Tissue	EC_{50} (nM)	K_e (nM) (antagonist[a])
BU74	guinea-pig ileum	0.11±0.15	NR (CTAP; nor-BNI)
BU74	mouse vas deferens	26.5±15.6[b]	0.03±0.003 (NTI)
U69,593	guinea-pig ileum	1.66±0.63	0.06±0.017 (nor-BNI)
U69,593	mouse vas deferens	208.3±139	NR (NTI)
Bremazocine	guinea-pig ileum	0.067±0.015	0.09±0.014 (nor-BNI)

Assays were performed as previously described in the Materials and methods. Data are expressed as the mean±S.E.M. of at least 4 experiments.
NR=failed to antagonise the response.
[a] CTAP=D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2, norBNI=-norbinaltorphimine, NTI=naltrindole.
[b] Partial agonist; peak effect 62% at 100 nM.

3.2. In vivo assays

3.2.1. Antinociceptive activity of BU74 against chemical nociception

In the *p*-phenylquinone-induced writhing assay (Aceto et al., 1997), BU74 was 10-fold more potent than ketocyclazocine and 10-fold less potent than the kappa opioid receptor agonist enadoline (Table 5). BU74 was also a potent antinociceptive agent when acetic acid was employed as the nociceptive stimulus (Fig. 2), an effect that lasted for 8 h (Fig. 3) at a dose of 0.1 mg/kg. The antinociceptive effect of BU74 was antagonized by nor-BNI, but not by naltrindole or the mu opioid receptor selective antagonist methocinnamox (Fig. 2).

3.2.2. Antinociceptive activity of BU74 against heat nociception

In the tail flick and hot-plate assays, BU74 was about equal in potency to ketocyclazocine, but remained an order of magnitude less potent than enadoline (Table 5). The antinociceptive effect of BU74 in the tail-flick test was not prevented by pre-treatment with the mu opioid receptor antagonist β-FNA, or the selective delta opioid receptor antagonist naltrindole, but it was antagonized by pre-treatment with the kappa opioid receptor antagonist nor-BNI with an AD_{50} 8.47 (3.2–22.4) mg/kg. In this animal model, nor-BNI antagonized the standard kappa opioid receptor agonist U50,488H with an AD_{50} 1.45 (0.82–2.58) mg/kg (data not shown).

BU74 was also a potent agonist in the mouse tail-withdrawal assay and was somewhat more potent at a water

Table 4
Antagonist affinity (K_e) of BU74 in the mouse vas deferens preparation

Compound	K_e (nM)		
	mu	delta	kappa
BU74	0.004±0.001	0.165±0.07	0.018±0.008
Naltrexone	0.44±0.09[a]	7.18±0.34	8.04±0.63[a]

Antagonist affinities were obtained as described in Materials and methods using DAMGO (mu), DPDPE (delta) and U69593 (kappa) as agonists. K_e values were calculated from a single dose of antagonist according to the formula K_e=[antagonist]/(DR−1). Data are expressed as the mean of at least 4 experiments.
[a] Determined in the guinea pig ileum.

Table 5
Agonist activity of BU74 in antinociceptive assays in the mouse following s.c. administration

Compound	ED_{50} (mg/kg)		
	p-Phenylquinone writhing	Tail flick	Hot plate
BU74	0.018 (0.011–0.028)	0.37 (0.008–1.62)	0.31 (0.12–0.81)
Enadoline	0.0015 (0.0004–0.006)	0.015 (0.003–0.059)	0.01 (0.004–0.04)
Ketocyclazocine[a]	0.12 (0.05–0.28)	0.69 (0.26–1.84)	0.14 (0.03–0.61)

Assays were performed as described in Materials and methods. Values represent means with 95% confidence interval obtained from at least three doses and 6–10 mice per dose.
[a] Data from Aceto et al. (2001).

temperature of 50 °C than at 55 °C (ED_{50} 0.41 mg/kg compared to 1.49 mg/kg), whereas the morphine dose–response curves for the different temperatures did not change (ED_{50} 15.3 mg/kg and 11.4 mg/kg) (Fig. 4). The duration of action of a 10 mg/kg of BU74 was approximately 10 h (Fig. 5) as was a just-maximally effective dose of 1 mg/kg, whereas a fully effective dose of morphine lasted less than 4 h. Surprisingly in this assay, the agonist effect of BU74 was not prevented by pre-treatment with nor-BNI (32 mg/kg) given 24 h before measurement (data not shown).

3.2.3. Antagonist activity of BU74

Opioid antagonist activity of BU74 in vivo was observed when the agonist effects were no longer evident. In the tail-flick assay, the peak effect for antagonism of the mu opioid receptor agonist sufentanyl and the kappa opioid receptor agonist enadoline was 48 h after administration of BU74 (Table 6). In neither case was there a clear dose–effect relationship though the higher doses of BU74 were most effective. BU74 (10 mg/kg) at the time of its peak antagonist effect (48 h) reduced the antinociceptive action of the selective agonists morphine (mu), BW373U86 (delta) and bremazocine (kappa) in the acetic acid writhing assay (Fig. 6).

4. Discussion

BU74 had equal affinity for mu, delta and kappa opioid receptors, but activity at the different receptors was separable by efficacy. Thus, the profile of BU74, as determined using in vitro opioid functional assays, guinea-pig ileum, mouse vas deferens and [^{35}S]GTPγS, is consistent with a designation as an extremely potent kappa opioid receptor agonist, delta opioid receptor partial agonist and mu opioid receptor antagonist. Since in the [^{35}S]GTPγS assays BU74 was an efficacious kappa opioid receptor agonist but a mu opioid receptor antagonist, it may be deduced that its agonist activity in guinea-pig ileum (a tissue expressing kappa and mu, but not delta opioid receptors) is mediated through the kappa opioid receptor, even though its agonist activity was not reversible by the selective kappa opioid receptor antagonist nor-BNI. Taken together with the finding that the agonist effect of BU74 in guinea-pig ileum could not be reversed by repeated prolonged washing, it can be concluded that binding to kappa opioid receptors in guinea-pig ileum is pseudoirreversible. In contrast, the agonist activity in the mouse vas deferens was reversible by naltrindole, suggesting that BU74 has faster kinetics at the delta opioid receptor. It was somewhat surprising that no

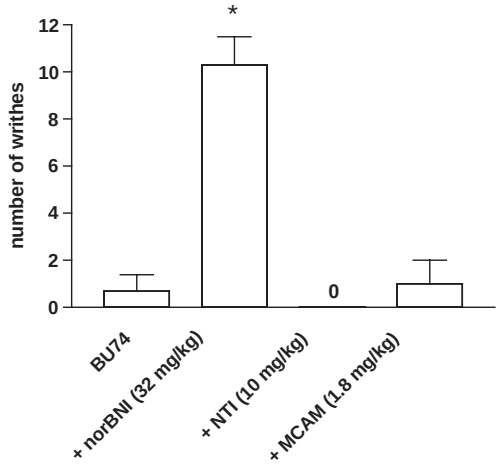

Fig. 2. Agonist action of BU74 (0.1 mg/kg, s.c.) in the acetic acid induced writhing assay and its reversal by nor-BNI, but not naltrindole (NTI) or methocinnamox (MCAM) given s.c. Values are means±S.E.M. from six animals per group.

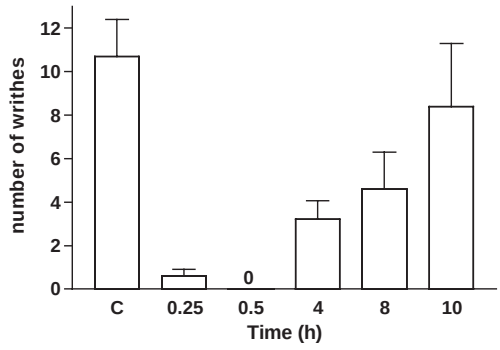

Fig. 3. Time-course of the antinociceptive action of BU74 (0.1 mg/kg, s.c.) in the acetic acid induced writhing assay. Values are means from six animals per time point.

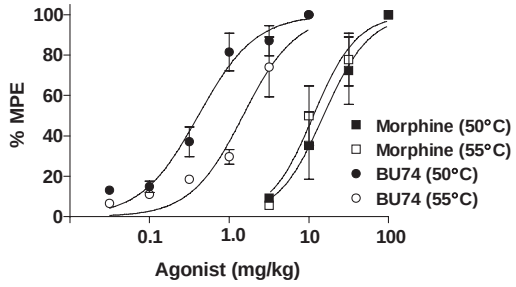

Fig. 4. Agonist dose–effect curves for morphine (i.p.) and BU74 (i.p.) in the mouse tail-withdrawal assay. Values are means±S.E.M. from six animals per group. Drugs were given in a cumulative dosing paradigm.

kappa opioid receptor agonist effect attributable to BU74 was evident in the mouse vas deferens. However, it is not unusual for high efficacy selective kappa opioid receptor agonists to show poor responses in the mouse vas deferens. The standard kappa opioid receptor agonist U69593 had high efficacy and potency in [^{35}S]GTPγS assays but had very low agonist potency in mouse vas deferens, 125-fold lower than its potency in guinea-pig ileum. This suggests that the kappa opioid receptor effects of BU74 would only be evident at much higher doses than those employed and are masked by the more potent delta opioid receptor agonist effects.

Compared with its high kappa opioid receptor efficacy, BU74 is devoid of mu opioid receptor efficacy. In stark contrast, its *N*-methyl derivative BU72 has very high mu opioid receptor efficacy (Husbands and Lewis, 2003; Neilan et al., 2004). Reduction in mu opioid receptor efficacy when the N-methyl group of benzomorphans, morphinans and epoxymorphinans is replaced by cyclopropylmethyl is a standard structure–activity relationship in opioid medicinal chemistry (Husbands and Lewis, 2000). Yet the complete absence of mu opioid receptor efficacy in BU74 is exceptional and makes BU74 one of the few examples of an almost full agonist at kappa opioid receptors but an antagonist at mu opioid receptors; the best known example is bremazocine (Romer et al., 1980).

In vivo, BU74 was a potent agonist in all antinociceptive tests in the mouse. In spite of the difficulty in antagonising

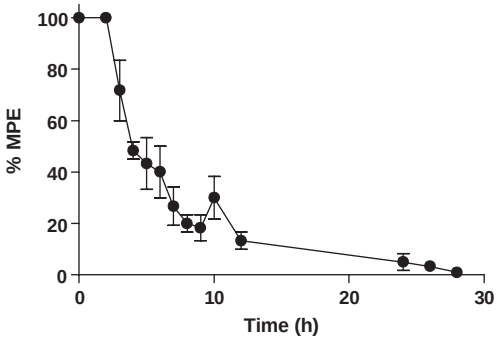

Fig. 5. BU74 (10 mg/kg, i.p.) time course in the tail-withdrawal assay at 50 °C. Values are means±S.E.M. from six animals. MPE=maximal possible effect.

Table 6
Antagonism of the antinciceptive activity of sufentanyl (mu) and enadoline (kappa) by BU74 in the mouse tail flick assay

BU74 dose (mg/kg, s.c.)	BU74 pretreatment time % antagonism of sufentanyl ED_{80}			BU74 pretreatment time % antagonism of enadoline ED_{80}		
	24 h	48 h	72 h	24 h	48 h	72 h
30	49	54	36	43	75	29
10	14	46	0	56	83	26
3	16	51	8	50	19	2
1	18	43	1	31	29	0

Experiments were performed as described in Materials and methods using ED_{80} doses of sufentanyl (mu, 0.01 mg/kg, s.c.) and enadoline (kappa, 1.0 mg/kg, s.c.) each given 20 min before the tail-flick test.

the in vivo antinociceptive activity with nor-BNI, probably due to the slow receptor kinetics shown by BU74 at the kappa opioid receptor in the isolated tissue assays, the available evidence strongly points to these antinociceptive effects being kappa opioid receptor-mediated. In the tail-withdrawal assay, the potency of BU74 in 50 °C water was higher than in 55 °C water. This loss of effect at the higher temperature is consistent with the relatively poor efficacy of kappa opioid receptor agonists in thermal assays; they are usually ineffective in 55 °C water (Tyers, 1980), but does confirm the high kappa opioid receptor efficacy of BU74 seen in vitro. The agonist activity of BU74 was long-lasting compared to morphine and replaced by antagonist activity of BU74 when its agonist effects had worn off. In the tail flick and writhing assays, the peak of antagonist activity was observed 48h after administration of BU74. At this time, BU74 antagonized mu, kappa and delta opioid receptors in line with its very high, and similar, affinity for all three opioid receptor types. The long-lasting antagonism of mu opioid receptor agonist effect is in line with persistent binding to the receptor. This may also be the mechanism underlying the kappa and delta opioid receptor antagonism. However, since BU74 is an agonist at kappa

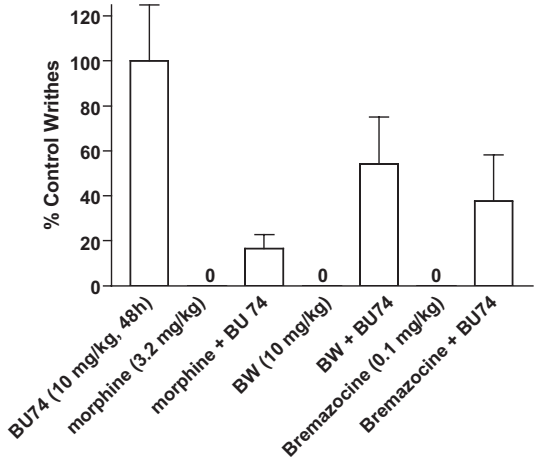

Fig. 6. BU74 antagonist selectivity in the acetic acid induced writhing assay. Agonists were added 15 min before the acetic acid and 48 h after 10 mg/kg BU74 or vehicle. All drugs were given s.c.

and delta opioid receptors, the delayed antagonism at these receptors could be due to receptor desensitization and/or down-regulation.

It is interesting to compare BU74 with DIPPA, since DIPPA has also been reported to have kappa opioid receptor agonist and long-term kappa opioid receptor antagonist actions as well as showing wash resistant binding in vitro (Chang et al., 1994a). The kappa opioid receptor agonist efficacy and duration of action of DIPPA in vivo are much lower than that of BU74, but the duration of kappa opioid receptor antagonism is similar (Chang et al., 1994b). DIPPA has an isothiocyanate group which can form a covalent bond to the kappa opioid receptor, although it has been suggested that some of the irreversible binding affinity of a DIPPA isomer for kappa opioid receptors is due to lipophilic interaction in addition to the covalent binding (Weerawarna et al., 1994). In contrast, BU74 has no electrophilic group to create an equivalent interaction, so the long duration of action is more likely due to the presence of highly lipophilic groups in the same way as the 14-cinnamoylaminomorphinone family of compounds (Woods et al., 1995; Broadbear et al., 2000) and buprenorphine (Hambrook and Rance, 1976).

In conclusion, BU74 has a unique opioid receptor efficacy profile as a kappa opioid receptor agonist, partial delta opioid receptor agonist and mu opioid receptor antagonist. If kappa opioid receptor agonists do have a role to play in the treatment of cocaine addiction, then the long-lasting activity of BU74 may be of interest, in a manner similar to the development of long-lasting opioids, such as buprenorphine, for the treatment of opioid abuse.

Acknowledgements

This work was supported by National Institute on Drug Abuse Grants DA07315 and DA00254. The National Institute of Drug Abuse-Opioid Treatment Discovery Program provided the isolated tissue data.

References

Aceto, M.D., Harris, L.S., Bowman, E.R., 1997. Etorphines: μ-opioid receptor-selective antinociception and low physical dependence capacity. Eur. J. Pharmacol. 338, 215–223.

Aceto, M.D., Bowman, E.R., Harris, L.S., May, E.L., 2001. Dependence studies of new compounds in the rhesus monkey, rat and mouse. NIDA Res. Monogr. 181, 156–202.

Broadbear, J.H., Sumpter, T.L., Burke, T.F., Husbands, S.M., Lewis, J.W., Woods, J.H., Traynor, J.R., 2000. Methocinnamox is a potent, long-lasting and selective antagonist of morphine-mediated antinociception in the mouse: comparison with clocinnamox, β-funaltrexamine and β-chlornaltrexamine. J. Pharmacol. Exp. Ther. 294, 933–940.

Chang, A.-C., Takemori, A.E., Ojala, W.H., Gleason, W.B., Portoghese, P.S., 1994a. κ opioid receptor selective affinity labels: electrophilic benzeneacetamides as κ-selective opioid antagonists. J. Med. Chem. 37, 4490–4498.

Chang, A.-C., Takemori, A.E., Portoghese, P.S., 1994b. 2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide: an opioid receptor affinity label that produces selective and long-lasting κ antagonism in mice. J. Med. Chem. 37, 1547–1549.

Cowan, A., Lewis, J.W., 1995. Buprenorphine. Wiley-Liss, New York. 326 pp.

Crawford, C.A., McDougall, S.A., Balanos, C.A., Hall, S., Berger, S.P., 1995. The effects of the κ-agonist U-50, 488 on cocaine-induced conditioned and unconditioned behaviours and Fos immunoreactivity. Psychopharmacology 120, 392–399.

Eddy, N.B., Leimbach, D., 1953. Synthetic analgesics: II. Dithienylbutenyl- and dithienylbutylamines. J. Pharmacol. Exp. Ther. 107, 385.

Hambrook, J.M., Rance, M.J., 1976. The interaction of buprenorphine with the opiate receptor: lipophilicity as a determining factor in drug-receptor kinetics. In: Kosterlitz, H.W. (Ed.), Opiates and Endogenous Opioid Peptides. Elsevier, Amsterdam, pp. 295–301.

Hughes, J., Kosterlitz, H.W., Leslie, F.M., 1975. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. Br. J. Pharmacol. 53, 371–381.

Husbands, S.M., Lewis, J.W., 1995. Morphinan cyclic imines and pyrrolidines containing a constrained phenyl group: high affinity opioid agonists. Bioorg. Med. Chem. Lett. 5, 2969–2974.

Husbands, S.M., Lewis, J.W., 2000. Structural determinants of efficacy for κ-opioid receptors in the orvinol series: 7,7-spiro analogues of buprenorphine. J. Med. Chem. 43, 139–141.

Husbands, S.M., Lewis, J.W., 2003. Opioid ligands having delayed long-term antagonist activity: potential pharmacotherapies for opioid abuse. Mini-Rev. Med. Chem. 3, 137–144.

Janssen, P.A.J., Niemgeers, J.E., Dony, J.G.H., 1963. The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawal reflex in rats. Arzneim.-Forsch. Drug Res. 13, 502–507.

Koster, R., Anderson, M., Debeer, E.J., 1959. Acetic acid for analgesic screening. Fed. Proc. 18, 412.

Lee, K.O., Akil, H., Woods, J.H., Traynor, J.R., 1999. Differential binding properties of oripavines at cloned mu- and delta-opioid receptors. Eur. J. Pharmacol. 378, 323–330.

Lowry, O.H., Rosenberg, N.G., Farr, A.L., Randell, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 139, 265–275.

McLaughlin, J.P., Hill, K.P., Jiang, Q., Sebastian, A., Archer, S., Bidlack, J.M., 1999. Nitrocinnamoyl and chlorocinnamoyl derivatives of dihydrocodeinone: in vivo and in vitro characterization of μ-selective agonist and antagonist activity. J. Pharmacol. Exp. Ther. 289, 304–311.

Mello, N.K., Negus, S.S., 2000. Interactions between kappa opioid agonists and cocaine-preclinical studies. New medications for drug abuse. Ann. N.Y. Acad. 909, 104–132.

Neilan, C.L., Husbands, S.M., Breeden, S., Ko, M.C.(Holden), Aceto, M.D., Lewis, J.W., Woods, J.H., Traynor, J.R., 2004. Characterization of the complex morphinan derivative BU72 as a high efficacy, long-lasting mu-opioid receptor agonist. Eur. J. Pharmacol. 499, 107–116.

Paton, W.D.M., Vizi, E.S., 1969. The inhibitory action of noradrenaline and adrenaline on acetylcholine output on guinea-pig longitudinal muscle strip. Br. J. Pharmacol. 35, 10–29.

Pearl, J., Harris, L.S., 1996. Inhibition of writhing by narcotic antagonists. J. Pharmacol. Exp. Ther. 154, 319.

Portoghese, P.S., Sayre, L.M., Larson, D.L., Fries, D.S., Takemori, A.E., 1980. A novel opioid receptor site-directed alkylating agent with irreversible narcotic antagonist and reversible agonist activities. J. Med. Chem. 23, 233–234.

Romer, D., Buscher, H., Hill, R.C., Maurer, R., Oetcher, T.J., Welle, H.B.A., Bakel, H.C.C.K., Kerman, A.M.A., 1980. Bremazocine, a potent, long-acting opiate kappa agonist. Life Sci. 27, 971–978.

Shippenberg, T.S., LeFevour, A., Heidbreder, C.H., 1996. κ-Opioid receptor agonists prevent sensitization to the conditioned rewarding effects of cocaine. J. Pharmacol. Exp. Ther. 276, 545–554.

Tallarida, R.J., Murray, R.B., 1987. Manual of Pharmacological Calculations with Computer Programs. Springer-Verlag, New York.

Toll, L., Berzetei-Gurske, I.P., Polgar, W.E., Brandt, S.R., Adapa, I.D., Rodriguez, L., Schwartz, R.W., Haggart, D., O'Brian, A., White, A., 1998. Standard binding and functional assays related to medications development division testing for potential cocaine and opiate narcotic treatment medications. NIDA Res. Monogr. 178, 440–466.

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

Tyers, M.B., 1980. A classification of opiate receptors that mediate antinociception in animals. Br. J. Pharmacol. 69, 503–512.

Ward, S.J., Portoghese, P.S., Takemori, A.E., 1982. Pharmacological characterisation in-vivo of the novel opiate β-funaltrexamine. J. Pharmacol. Exp. Ther. 220, 494–498.

Weerawarna, A.A., Davis, R.D., Nelson, W.L., 1994. Isothiocyanate-substituted κ-selective opioid receptor ligands derived from *N*-methyl-*N*-[(1*S*)-1-phenyl-2-(1-pyrrolidinyl)ethyl]phenylacetamide. J. Med. Chem. 37, 2856–2864.

Woods, J.H., Traynor, J.R., 2001. Evaluation of new compounds for opioid activity (2000). NIDA Res. Monogr. 181, 140–155.

Woods, J.H., Lewis, J.W., Winger, G., Butelman, E., Broadbear, J., Zernig, G., 1995. Methoclocinnamox: a μ partial agonist with pharmacotherapeutic potential for heroin abuse. NIDA Res. Monogr. 147, 195–219.

Zhu, J., Luo, L.Y., Li, J.G., Chen, C., Liu-Chen, L.Y., 1997. Activation of the cloned human kappa opioid receptor by agonist enhances [^{35}S]GTPgammaS binding to membranes: determination of potencies and efficacies of ligands. J. Pharmacol. Exp. Ther. 282, 676–684.