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Potential Irreversible Ligands for Opioid Receptors. Cinnamoyl Derivatives of β-Naltrexamine.

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Abstract

Cinnamoyl derivatives of β-naltrexamine (β-NTA) have been prepared and evaluated as potential irreversible opioid antagonists.

In receptor binding assays, isolated tissue preparations and mouse antinociception assays the *p*-methylcinnamoyl derivative BU42 was similar to the standard opioid ligand β-funaltrexamine (β-FNA). The main features were reversible κ agonism and irreversible μ antagonism. Surprisingly the *p*-chlorocinnamoyl derivative BU59 showed only modest competitive antagonist activity in-vivo despite appearing to bind irreversibly to μ receptors in the guinea-pig ileum (GPI) preparation. BU60, the dihydrocinnamoyl analogue of BU59, like BU59 displayed reversible κ agonism in GPI but in mouse antinociception assays its agonism was mediated by μ and δ receptors rather than κ.

The surprising changes of profile attributable to substitution in the aromatic ring of the cinnamoylamido group in this small series suggests that a larger range of substituted cinnamoylamido derivatives should be studied to further elucidate the effects of Michael acceptor activity and other factors.

Irreversible ligands have contributed greatly to the study of opioid receptors and mechanisms of opioid actions both in-vitro and in-vivo (Caruso et al 1979; Portoghese et al 1980; Rice et al 1983; Takemori & Portoghese 1985). Of particular utility for the study of μ opioid receptors has been the irreversible fumaramido derivative β-funaltrexamine (β-FNA; Fig. 1) (Portoghese et al 1980; Ward et al 1982; Ward & Takemori 1983; Takemori & Portoghese 1987; Zimmerman et al 1987; Rothman et al 1988; Mjanger & Yaksh 1991). The common feature of the irreversible ligands is that they all possess a reactive electrophilic group appropriately located so as to be able to form a covalent bond to a thiol group near the active site of the receptor. The fumaramido group at C-6 in β-FNA is a Michael acceptor to which a thiol group can add to create a covalently-bound ligand-receptor complex.

The primary drawback to the use of β-FNA is that in some in-vivo assays it has short-lasting agonist activity which has been shown to be mediated by κ-opioid receptors (Ward et al 1982). This property makes it difficult to use the selective μ-antagonist activity of β-FNA until a substantial time interval has elapsed after its administration (usually 24 h). An advantage of β-FNA in the investigation of opioid receptor mechanisms is that it can be administered systemically (Ward et al 1982; Mjanger & Yaksh 1991). This property is shared by clocinnamox (C-CAM; Fig 1) (Lewis et al 1988) in which a *p*-chlorocinnamoylamido group at C-14 acts as a putative Michael acceptor function. C-CAM shows no agonist actions after systemic administration (Aceto et al 1989) and as an irreversible antagonist is selective for μ receptors (Zernig et al 1994 (?)). However, in binding and isolated tissue assays, it appears to be somewhat less selective for μ receptors than β-FNA (Woods, unpublished data).

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Since the *p*-chlorocinnamoylamido group at C-14 in C-CAM was not associated with any opioid agonist activity, it was of interest to determine whether the κ-agonist activity of β-FNA could be avoided by replacing the fumaramido group at C-6 with a cinnamoylamido group. If it was and the greater μ selectivity of β-FNA was retained, the profile would be superior to that of either β-FNA or C-CAM. We here describe the synthesis and characterization of the *p*-chloro- and *p*-methylcinnamoyl derivatives (BU59 and BU42) of β-naltrexamine (β-NTA) and the *p*-chlorodihydrocinnamoyl analogue (BU60).

Materials and Methods

Synthesis

β-FNA and the cinnamoylamido analogues (BU42, 59 and 60) were prepared from β-naltrexamine (β-NTA; Fig. 1) in 60–70% yields by acylation with the appropriate acid chloride in dichloromethane in the presence of triethylamine.

Receptor-binding assays

Hartley guinea-pigs were decapitated and their brains were quickly removed and weighed. The brains were then homogenized in 50 mM Tris HCl, buffer pH 7·7, using a Polytron (~25 mL/brain). The homogenate was centrifuged at 40 000 *g* for 15 min, rehomogenized and centrifuged. The final pellet was resuspended in Tris HCl, pH 7·7, at a final concentration of 6·67 mg of original wet weight of tissue mL^{-1}.

The following radio-ligands (~1 nM) were used to label the receptor binding sites indicated in parentheses: [^{3}H]DAMGO (μ), [^{3}H]Cl-DPDPE (δ), [^{3}H]U69593 (κ).

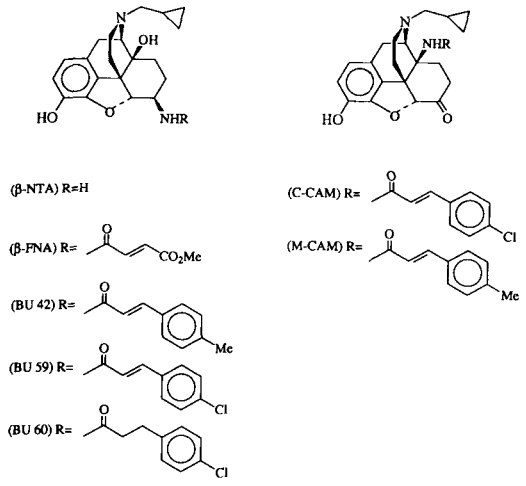

FIG. 1. Structures of β-NTA and its acyl derivatives, C-CAM and M-CAM.

The guinea-pig brain suspension (1.8 mL) was incubated in 50 mM Tris HCl, pH 7·7, for 1 h at 25°C with 100 mL of radioligand and 100 mL of test compound (10^{-5}–10^{-11} M). Non-specific binding was determined by incubating in the presence of 1 mM of the unlabeled counterpart of each labelled ligand. The samples were then filtered through glass fibre filters on a 48-well Brandel cell harvester. The filters were washed three times with 3 mL buffer. Filters were incubated overnight with 5 mL scintillation fluid before counting.

Guinea-pig ileum (GPI) preparation

Male Hartley guinea-pigs, 350–400 g, were decapitated and their small intestines were removed; about 20 cm of the terminal ileum was discarded. The longitudinal muscle with the myenteric plexus attached was gently separated from the underlying circular muscle by the method of Paton & Vizi (1969). The muscle strip was mounted in an 8-mL water-jacketed organ-bath containing Krebs-bicarbonate solution of composition (mM): NaCl 118, $CaCl_2$ 2·5, KCl 4·7, $NaHCO_3$ 25, mM 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 0·6–1·0 g was applied to the strips. The muscle strip was stimulated for 60 min before the start of each experiment.

Field electrical stimulation was delivered through platinum wire electrodes positioned at the top and bottom of the organ bath and kept at a fixed distance apart (3·5 cm). The upper electrode was a ring of 4-mm diameter. The parameters of rectangular stimulation were: supramaximal voltage, 1 ms impulse duration at a rate of 0·1 Hz. A Grass S-88 electrostimulator was used for stimulation. The electrically induced twitches were recorded using an isometric transducer (Metrigram) coupled to a Grass 7D Multichannel polygraph.

Mouse vas deferens (MVD) preparation

The vas deferens from Swiss-Webster mice, 30–35 g, were prepared as described by Hughes et al (1974). The tissues were mounted in an 8-mL, 31°C organ-bath containing a magnesium-free Krebs solution, which was bubbled with 5% CO_2 in oxygen. An initial tension of 150–200 mg was used.

The field stimulation parameters were as described by Ronai et al (1974); paired shocks of 100 ms delay between supramaximal rectangular pulses of 1 ms duration were delivered at a rate of 0·1 Hz. A Grass S-88 electrostimulator was used for stimulation. The contractions were recorded using an isometric transducer (Metrigram) coupled to a Grass 7D multichannel polygraph.

Warm-water tail-withdrawal procedure

Mice were restrained in plexiglass tubes with their tails protruding. The lower third of the tail was placed in warm water (50 or 55°C) and the latency until the mouse withdrew its tail was measured. Each test time was compared with the baseline latency (obtained following an injection of sterile water) and % maximum effect was calculated as:

$$100 \times (\text{test time} - \text{baseline})/(\text{maximum latency} - \text{baseline})$$

The maximum latency (designating 100% analgesia) was set at 15 s. Antagonists were administered 30 min or 24 h before beginning cumulative dosing with the agonist. Analgesia testing and administration of the agonist took place every 30 min. All injections were given intraperitoneally. Each data point represents the averaged results of five mice unless otherwise noted.

Acetic acid-induced writhing assay

The writhing assay is a very sensitive indicator of μ, δ and κ opioid activity. Use of this assay enabled the effects of test compounds at each opioid receptor subtype to be compared. The writhing response is an abdominal stretch accompanied by extension of the hind legs following an intraperitoneal injection of 0·6% acetic acid. Agonists and antagonists were administered at various times prior to the acetic acid, and the writhes of six mice over a 5-min period were compared with a control value (obtained when only sterile water was injected prior to the acid), yielding % control writhes for each group as follows:

$$100 \times \text{test writhes/control writhes}$$

Results

In the opioid receptor replacement binding assays the cinnamoyl derivatives showed high affinity for all three opioid receptor types (Table 1). They all showed highest affinity for μ receptors which was 2-5 fold than for δ. In each case κ affinity was substantially lower. These profiles are very similar to that of β-FNA but the cinnamoyl derivatives had higher affinity all-round which was most pronounced in the case of the dihydrocinnamoylamido compound BU60.

The novel derivatives were potent agonists in the GPI preparation (Table 2); *p*-chloro substitution in the aromatic ring of the cinnamoyl group (BU59 and BU60) conferred higher potency than methyl substitution (BU42). These effects were selectively antagonized by norbinaltorphimine (nor-BNI) showing that they were mediated by κ opioid receptors. The cinnamoyl derivatives (B59 and BU42) displayed irreversible binding in the GPI since they could not

Table 1. Opioid receptor binding assays.

	K_i (nM) [^{3}H]DAMGO (μ)	[^{3}H]DPDPE-Cl (δ)	[^{3}H]U 69593 (κ)
BU42	0·23	9·6	0·6
BU59	0·1	5	0·2
BU60	0·04	2·8	0·2
DAMGO	1·1	127·1	184·13
DPDPE-Cl	–	0.3	–
U 69593	710·2	10 333·9	0·7
β-FNA	0·4	22·4	0·9

be removed from the tissue by repeated washing. This characteristic was not shared by the dihydrocinnamoyl derivative BU60. None of the new derivatives were agonists in the MVD preparation but they showed evidence of potent antagonist activity.

The new derivatives were tested for in-vivo activity in mouse antinociception tests (Table 3). In the tail-withdrawal test using water at 50°C, they were low efficacy agonists similar to β-FNA having only 20-30% of the maximum possible effect at the highest dose tested. In the antiwrithing test in which the antinociceptive stimulus is of lower intensity than that provided by the tail withdrawal test, the cinnamoylamido derivatives were more efficacious. At the highest dose tested (32 mg kg^{-1}) the chlorocinnamoyl derivative (BU59) and its dihydrocinnamoyl congener (BU60) effectively showed total inhibition of the writhing response. The methyl-substituted analogue (BU42) was somewhat less efficacious. By pretreatment with the specific antagonists (β-FNA (μ), nor-BNI (κ) and naltrindole (δ)) the receptor types responsible for this agonist activity could be determined. The agonist activity of the two cinnamoyl derivatives was selectively inhibited by nor-BNI pretreatment showing that it was an effect primarily mediated by κ opioid receptors. β-FNA pretreatment had a lesser effect indicating some involvement of μ receptors but naltrindole had no effect. Surprisingly the chlorodihydrocinnamoyl derivative (BU60) was not κ selective; apparently the contribution of κ receptors to the antiwrithing response was less than that of μ and δ.

The modest agonist effects of the new derivatives in the tail-withdrawal test allowed their antagonist effects against morphine to be determined (Table 4). The chlorocinnamoyl (BU59) and dihydrochlorocinnamoyl (BU60) derivatives displaced the morphine dose-response curve by modest amounts and in parallel fashion. The methyl substituted derivative (BU42) was an altogether more impressive morphine antagonist, completely suppressing the agonist effect up to 320 mg kg^{-1}, the highest dose of morphine tested. The morphine dose-response curve determined 24 h after treatment with the chloro derivatives showed no rightward shift compared with the control dose-response curve. In contrast BU42 pretreatment at 24 h resulted in a substantial shift of the morphine dose-response curve in a non-parallel fashion. The selectivity of the opioid receptor antagonist activity of BU42 was determined in the antiwrithing assay by administration 1 h before the selective opioid agonists morphine (μ), BW 373U86 (δ) and bremazocine (κ) (Fig. 2). At this time the agonist activity of BU42 had substantially waned. The shift of the morphine dose-response curve (ca. 30-fold) was substantially greater than that of BW 373U86 (ca. 3-fold). BU42 had virtually no effect on the agonist activity of bremazocine. In a similar set of experiments, when BU42 (32 mg kg^{-1}) was administered 24 h before the specific agonists in the antiwrithing assay, the agonist effect of morphine (3·2 mg kg^{-1}) was totally inhibited; the effect of BW 373U86 (10 mg kg^{-1}) was also suppressed by about 50%. In a comparative study, β-FNA (32 mg kg^{-1}) displaced the morphine dose-response curve to a similar extent but had virtually no effect on the activity of BW 373U86 and bremazocine (data not shown).

Discussion

A principal finding of this work is that the pharmacological profile of the *p*-methylcinnamoyl derivative (BU42) of β-NTA is very similar to that of the fumaramyl derivative, β-FNA. Both are (reversible) κ agonists in the GPI and in mouse antinociception assays; in the latter they are of moderate efficacy and potency. Like β-FNA, BU42 also shows irreversible μ antagonist activity in the tail withdrawal test since the morphine dose-response curve was flattened. Although in the antiwrithing test, BU42 was also a selective μ antagonist, it was not irreversible. The effect of an irreversible antagonist is to reduce the number of available receptors, not the affinity of the agonist (Burke et al 1994). Flattening of the agonist dose-response curve to give

Table 2. Agonist effects of cinnamoyl derivatives in GPI and MVD in in-vitro assays.

	Guinea-pig ileum IC50 (nM)	K_e (nM) CTAP	K_e (nM) Nor-BNI	Mouse vas deferens IC50 (nM)	K_e (nM) Naltrindole
BU42	12·57 ± 4·04	a	0·16 ± 0·05	17 114 ± 3005	N.D.
BU59	2·21 ± 0·61	a,b	0·29 ± 0·04	b	
BU60	3·24 ± 0·32	322	0·30 ± 0·02	c	
U 69593	1·66 ± 0·63	N.D.	0·06 ± 0·017	208·3 ± 139·7	N.D.
DAMGO	8·25 ± 2	25·31 ± 2·54	27·67 ± 4·52	177·60 ± 134	N.D.
DPDPE	4130 ± 870	25·83 ± 15·3	50·68 ± 35·1	4·11 ± 1·32	0·021 ± 0·007

[a]The inhibition produced by this compound could neither be washed out nor reversed by CTAP. [b]No IC50 could be determined up to 10^{-5} M in the MVD; the compound was a μ and δ antagonist and its pA_2 at μ was 8·72 and at δ, 8·28. [c]No IC_{50} could be determined up to 10^{-5} M in the MVD; the compound was a δ antagonist and its pA_2 was 8·53. N. D. = assay not performed.

Table 3. Agonist effects of cinnamoyl derivatives in mouse antinociception assays.

	Tail withdrawal[a] % max. possible effect at 32 mg kg^{-1}	Antiwrithing % max. possible effect at 32 mg kg^{-1}	Selectivity[b]
BU 42	20	60	$\kappa > \mu$: no δ
BU 59	30	100	$\kappa \gg \mu$: no δ
BU 60	< 30	90	$\delta = \mu > \kappa$
β-FNA	23	100	ND

[a]Warm water at 50°C. [b]Selectivity of agonist response measured using specific antagonists—μ (β-FNA 32 mg kg^{-1}; 24-h pretreatment); κ (nor-BNI 32 mg kg^{-1}; 24-h pretreatment); δ (naltrindole 10 mg kg^{-1}; 15-min pretreatment).

a maximum response below that achievable in the absence of the irreversible antagonist is a result of there being insufficient receptors available to express a full agonist response (Adams et al 1990; Mjanger & Yaksh 1991; Comer et al 1992). In an assay, e.g. antiwrithing, requiring a lower level of agonist efficacy for full response, a greater proportion of receptors can be inactivated before the agonist dose-response curve is flattened (Stevens & Yaksh 1989). Thus the effect of BU42 in effecting only a parallel rightward shift of the morphine dose-response curve does not invalidate the conclusion that it is an irreversible μ antagonist. Other evidence for irreversible opioid receptor binding of BU42 was obtained in GPI from which BU42 could not be removed by continuous washing.

The only significant difference between BU42 and β-FNA is that the latter appears to have somewhat superior μ selectivity in its in-vivo antagonist actions. In the antiwrithing assay β-FNA pretreatment suppressed only μ agonist activity whereas BU42 also had some effect on δ though not κ agonist activity. The similarity in pharmacological profile between the *p*-methylcinnamoyl and fumaramyl derivatives of β-NTA cannot simply be attributed to the presence of the Michael acceptor function since the electrophilic character of the two groups is significantly different. Fumaramyl is a much better electrophile than *p*-methylcinnamoyl. Sebastian et al (1993) were unable to demonstrate in-vitro that a *p*-nitrocinnamoylamido group in the irreversible μ antagonist N-CPM-MET-CAMO could react as a Michael acceptor with *N*-acetylcysteine in buffered solution. Moreover, the *p*-methylcinnamoylamido group will be a weaker electrophile than *p*-nitrocinnamoylamido. In view of the profound morphine antagonist activity of the

Table 4. Morphine antagonism in the mouse tail withdrawal test (50°C water).

BU No.	Shift (-fold) of morphine dose-response curve: 30-min pretreatment	24-h pretreatment
42	Total suppression* (32)	25 (32)
59	2·5 (10)	No shift (10)
60	3·3 (32)	No shift (10)

*Test performed with water at 55°C; morphine dose-response curve totally suppressed up to 320 mg kg^{-1}.

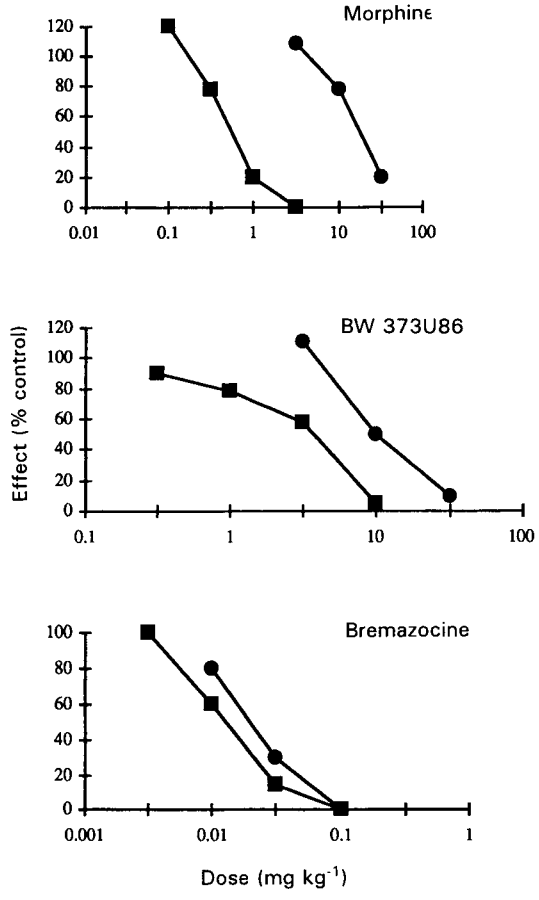

FIG. 2. Dose-response curves for specific agonists at different types of opioid receptor, alone and in the presence of BU42 (10 mg kg^{-1}), in the acetic acid-induced writhing test. BU42 was administered 1 h before the agonist which itself was administered 30 min before the acetic acid. The number of writhes was measured over a 5 min period beginning 5 min after the acetic acid administration.

p-methylcinnamoyl derivative it was surprising that the *p*-chloro analogue (BU59) had very modest in-vivo antagonist activity despite being a very potent antagonist in GPI and MVD and, like BU42, binding irreversibly in the latter. This profound change brought about by substituting chlorine for methyl had not been found in the C-14 substituted analogues C-CAM and M-CAM. The latter are both μ-selective irreversible antagonists lacking in-vivo agonist effects (Lewis et al 1988; Aceto et al 1989) differentiated only by the greater μ selectivity of M-CAM (Woods et al; unpublished data). The difference in antagonist activity in-vivo between BU42 and BU59 may be attributed to difficulty of access to the central nervous system (CNS) of the *p*-chloro derivative.

There was very little difference in morphine antagonist activity in-vivo between BU59 and the dihydrocinnamoyl derivative BU60 in which unsaturation in the cinnamoyl group was missing. There was however a surprising difference between them in the selectivity of their agonist response in the antiwrithing test. The antinociceptive activity of the cinnamoyl derivative, BU59, was mediated primarily through κ receptors, consistent with its agonist effects in GPI. The dihydrocinnamoyl derivative BU60, by contrast

appeared to owe its antiwrithing response to μ and δ receptors rather than κ despite being a κ agonist in GPI. This apparent discrepancy may be due to the particular sensitivity of GPI to κ agonists (C. F. C. Smith, personal communication). In GPI the finding that BU59, but not BU60, could not be removed by repeated washing of the tissue lends support to the role of the cinnamoylamido group as a Michael acceptor capable of forming a covalent bond to the receptor.

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