3-Methoxynaltrexone, a selective heroin/morphine-6β-glucuronide antagonist

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Abstract Recent work has suggested that heroin and morphine-6β-glucuronide (M6G) both act through a novel mu opioid receptor subtype distinct from those mediating morphine's actions. This very high affinity ^{3}H-M6G site is selectively competed by 3-methoxynaltrexone. In vivo, 3-methoxynaltrexone (2.5 ng, i.c.v.) selectively antagonizes the analgesic actions of heroin and M6G without interfering with mu (morphine and [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin), delta ([D-Pen^2,D-Pen^5]enkephalin), kappa$_1$ (U50,488H) or kappa$_3$ (naloxone benzoylhydrazone) analgesia. In dose–response studies, 3-methoxynaltrexone (2.5 ng, i.c.v.) significantly shifted the ED$_{50}$ values for heroin and its active metabolite, 6-acetylmorphine, without affecting the morphine curve. These results indicate that 3-methoxynaltrexone selectively blocks a novel ^{3}H-M6G binding site which is responsible for the analgesic actions of heroin and M6G. This ability to selectively antagonize heroin actions opens new possibilities in the development of therapeutics for the treatment of opioid abuse.

Key words: Opioid; Morphine; Opioid receptor; Analgesia; Mu receptor

1. Introduction

Pharmacokinetics have long been thought to play a crucial role in the addictive potential of many drugs of abuse, including heroin. Compared to morphine, heroin crosses the blood–brain barrier far more rapidly and to a greater extent [1,2], leading many investigators to conclude that pharmacokinetic differences between the two agents were responsible for their different pharmacology [1,3–6]. However, evidence now suggests that heroin and M6G both act through a novel receptor mechanism which is distinct from that of morphine [7–11].

Despite early evidence demonstrating its activity [12,13], the importance of morphine-6β-glucuronide (M6G) has only been recognized recently [14–16]. When given centrally to avoid the blood–brain barrier, M6G is 100-fold more potent than morphine [14]. Despite this dramatic difference in analgesic potency, morphine competes traditional mu receptor binding in either brain tissue or cells transfected with the MOR-1 clone with higher affinity than M6G. In addition, the efficacy of M6G in cyclase studies using MOR-1 transfected cells is not significantly different from morphine [17]. Thus, the extraordinary potency of M6G cannot be explained by either enhanced affinity or efficacy at traditional mu receptors.

Antisense mapping studies of the MOR-1 clone, which encodes a mu opioid receptor, reveal very different sensitivity profiles for morphine and M6G [7–9,18]. A series of three antisense oligodeoxynucleotides targeting exon 1 of MOR-1 effectively block morphine analgesia without affecting the actions of M6G [7–9,11]. Conversely, an additional three antisense probes based upon exon 2 which are inactive against morphine analgesia dramatically lower M6G analgesia. An exon 2 antisense probe which was inactive against morphine also blocked the analgesic actions of both heroin and its active metabolite, 6-acetylmorphine [11]. Morphine analgesia also can be differentiated from M6G and heroin by the G-proteins involved in their analgesic responses [7,11,19,20]. Morphine analgesia is sensitive to a $G_i\alpha 2$ antisense probe, but not to another targeting $G_i\alpha 1$. In contrast, the $G_i\alpha 1$ antisense blocks both M6G and heroin analgesia.

Finally, ^{3}H-M6G binding studies have identified a novel binding site with very high affinity for M6G (K_D 68 pM) [17]. During the characterization of this site, we came across a compound which appeared to selectively compete binding to this M6G site. We now report that this compound, 3-methoxynaltrexone, selectively competes this M6G site and antagonizes M6G and heroin analgesia, opening the possibility of novel therapeutic approaches towards the treatment of drug addiction.

2. Materials and methods

2.1. ^{3}H-M6G binding assays

^{3}H-M6G (85 Ci/mmol) was synthesized in our laboratory as previously described [21]. Binding was performed in mouse brain homogenates at 25°C for 150 min in potassium phosphate buffer (50 mM, pH 7.4) with $MgCl_2$ (5 mM) and filtered over glass fiber filters [22,23]. Nonspecific binding was determined with levallorphan (1 μM) [17]. Binding was linear with tissue and reached steady-state levels within 60 min. Binding parameters were determined using nonlinear regression analysis.

2.2. Analgesia

Male CD-1 mice were purchased from Charles River Breeding Laboratories (Raleigh, NC). Opiates were obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). Antinociception, referred to as 'analgesia' in the current study, was assessed in the tailflick assay, as previously described [8,11,15,24]. Baseline latencies, ranging from 2 to 3 s, were determined for each mouse prior to any testing. Analgesia, defined quantally as a doubling or greater of the baseline latency for an individual mouse, was determined 15 min after i.c.v. or 30 min after s.c. injection. Significance among single doses was assessed using the Fisher Exact Test. ED$_{50}$ values and 95% confidence limits were determined using either the Bliss [25] or a computerized Litchfield-Wilcoxin-derived program [26,27].

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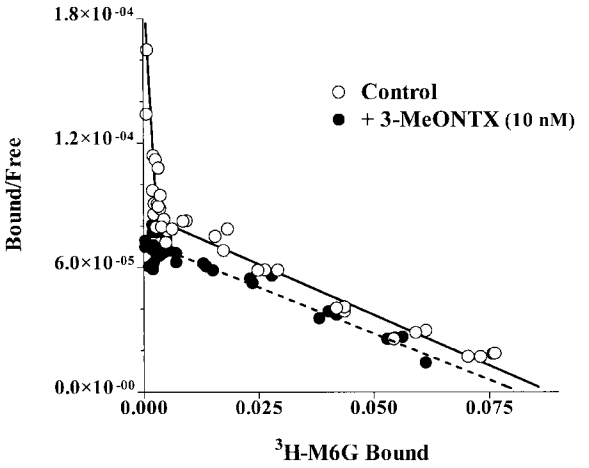

Fig. 1. Effect of 3-methoxynaltrexone on ^{3}H-M6G binding in brain homogenates. ^{3}H-M6G binding was determined in calf brain homogenates at various concentrations of radioligand (0.03–4 nM) alone or in the presence of a fixed concentration of 3-methoxynaltrexone (10 nM) and expressed as a Scatchard plot. Results are given from 3 replicate experiments. Nonlinear regression analysis of the binding curve of ^{3}H-M6G alone was best fit by two sites: Site (1) K_D 0.066 ± 0.01 nM, B_{max} 7 ± 1.6 fmol/mg protein; Site (2) K_D 1.8 ± 0.2 nM, B_{max} 85 ± 7 fmol/mg protein. Inclusion of the 3-methoxynaltrexone eliminates the higher-affinity binding component, leaving the lower component virtually unchanged (K_D 1.3 ± 0.1 nM; B_{max} 91 ± 5 fmol/mg protein).

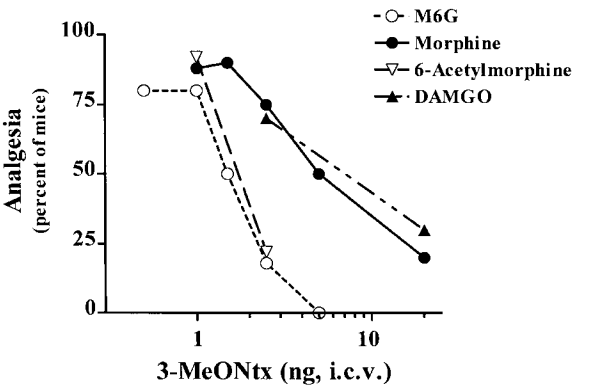

Fig. 2. Inhibition of opioid analgesia by 3-methoxynaltrexone. Groups of mice ($n \geq 10$) received a fixed dose of morphine (0.7 μg, i.c.v.), M6G (12.5 ng, i.c.v.), 6-acetylmorphine (1.2 μg, i.c.v.) or DAMGO (8 ng, i.c.v.) and the indicated dose of 3-methoxynaltrexone (i.c.v.). Analgesia was determined and expressed as the percent of mice responding. At 2.5 ng, 3-methoxynaltrexone significantly lowers the analgesic actions of both M6G ($P < 0.01$) and 6-acetylmorphine ($P < 0.01$), but not either morphine or DAMGO.

3. Results

In earlier binding studies, ^{3}H-M6G labeled a novel high-affinity site (K_D 68 pM) in addition to the traditional mu receptors. Competition experiments revealed several compounds with shallow slopes which could be resolved into two components using nonlinear regression analysis [17]. One compound, 3-methoxynaltrexone, was particularly interesting. With IC_{50} values of approximately 12 and 400 nM, 3-methoxynaltrexone discriminated quite well between the two ^{3}H-M6G binding components. Its relatively poor affinity against the transfected mu receptor encoded by MOR-1 (IC_{50} 250 nM) suggested that 3-methoxynaltrexone might have higher affinity against the high-affinity M6G site than traditional mu receptors. To define more fully the selectivity of 3-methoxynaltrexone for the ^{3}H-M6G binding sites, we performed ^{3}H-M6G saturation studies with or without a fixed concentration of 3-methoxynaltrexone. The control saturation study yielded a curvilinear Scatchard plot similar to ones previously observed [17]. Although the lower-affinity component (K_D 1.8 nM) corresponded quite well to the affinity of ^{3}H-M6G in CHO cells transfected with MOR-1 (K_D 3.3 nM) and to K_i values determined from competition studies against mu binding in brain [14], the high-affinity binding component (K_D 66 pM) was unique. Including a fixed concentration of 3-methoxynaltrexone (10 nM), a concentration far below its IC_{50} value against traditional mu binding, eliminated the high-affinity component of ^{3}H-M6G binding (Fig. 1), indicating that both 3-methoxynaltrexone and M6G bound with highest affinity to the same site.

CXBK are insensitive to morphine given systemically or intracerebroventricularly [28–30], which readily distinguishes them from CD-1 mice which are responsive to a wide variety of opioid analgesics (Table 1). Given intracerebroventricularly, morphine at a dose 15-fold higher than the ED_{50} value in CD-1 mice is analgesic in less then 25% of mice, a difference which is particularly dramatic at 0.7 μg, i.c.v., where the response in CD-1 mice (70%) is significantly different from that in the CXBK mice (8%, $P < 0.0001$). In contrast to morphine, CXBK mice display the same sensitivity as CD-1 mice to M6G, heroin and 6-acetylmorphine, the active metabolite of heroin (Table 1). Thus, M6G and heroin analgesia in CXBK mice involves mechanisms distinct from morphine analgesia, consistent with a novel heroin/M6G receptor.

Naltrexone is a potent opioid antagonist, raising the possibility that 3-methoxynaltrexone also might be an antagonist. We therefore examined its reversal of opioid analgesia (Fig. 2). 3-Methoxynaltrexone antagonized the analgesic actions of M6G and 6-acetylmorphine 3-fold more potently than either morphine or the mu peptide [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO). At 2.5 ng (i.c.v.), 3-methoxynaltrexone significantly lowered the response of M6G, heroin and 6-ace-

Table 1
Analgesic activity of opioids in CD-1 and CXBK mice

Drug	Route	ED_{50} value		Ratio
		CD-1	CXBK	
Morphine	i.c.v.	0.65 μg (0.25, 2.67)	> 10 μg	> 15
M6G	i.c.v.	13.0 ng (6.1, 31.8)	10.3 ng (5.1, 22.3)	0.8
Heroin	s.c.	0.33 mg/kg (0.23, 0.46)	0.41 mg/kg (0.2, 0.85)	1.2
6-Acetylmorphine	i.c.v.	1.76 μg (0.84, 3.7)	1.18 μg (0.89, 1.5)	0.7

ED_{50} values with 95% confidence limits were determined from at least three doses of each drug given to groups of mice ($n \geq 10$) through the indicated route. In the CXBK mice, the highest morphine dose tested was 10 μg, i.c.v., which was analgesic is less than 25% of mice tested.

Table 2
Effect of 3-methoxynaltrexone on the analgesic actions of opioid analgesics

Drug	Analgesic ED_{50} (95% confidence limits)		Ratio
	Control	With 3-MeONtx	
Supraspinal			
6-Acetylmorphine	566 ng (342, 937)	1730 ng (1443, 2074)	3.1 *
M6G	6.45 ng (4.2, 9.7)	18.0 ng (15, 22)	2.8 *
Morphine	410 ng (269, 621)	424 ng (272, 660)	1.0
Systemic			
Heroin	0.50 mg/kg (0.38, 0.67)	0.94 mg/kg (0.73, 1.2)	1.9 *
Morphine	3.11 mg/kg (2.4, 4.0)	2.85 mg/kg (2.1, 3.9)	0.9

ED_{50} values were determined using groups of mice ($n \geq 10$) and three agonist doses (i.c.v.) in the presence and absence of a fixed 3-methoxynaltrexone dose (2.5 ng, i.c.v.). All agonists were given through the indicated route of administration and results are the ED_{50} with 95% confidence limits. 3-Methoxynaltrexone significantly shifts the dose–response curves for 6-acetylmorphine, M6G and heroin ($P < 0.05$).

tylmorphine ($P < 0.03$) (Fig. 3). In contrast, the same 3-methoxynaltrexone dose was inactive against the analgesic actions of morphine, the mu peptide DAMGO, the delta analgesic [D-Pen^2,D-Pen^5]enkephalin (DPDPE), the kappa$_1$ drug U50,488H or the kappa$_3$ analgesic, naloxone benzoylhydrazone (NalBzoH; Fig. 3). Dose–response curves for supraspinal morphine, M6G and 6-acetylmorphine analgesia in the absence and presence of 3-methoxynaltrexone (2.5 ng, i.c.v.) revealed significant shifts of approximately 3-fold for M6G and for 6-acetylmorphine ($P < 0.05$) while the morphine dose–response curve remained unchanged (Table 2). The same 3-methoxynaltrexone dose also shifted the dose–response curve for systemically administered heroin but not for morphine.

4. Discussion

In addition to traditional mu receptors, ^{3}H-M6G labels with very high affinity a unique site with a low abundance in brain which appears to be selective for M6G [17]. In these studies, 3-methoxynaltrexone lowers ^{3}H-M6G binding with a shallow competition curve and nonlinear regression analysis indicated widely differing affinities for two ^{3}H-M6G binding components. The current studies confirm that 3-methoxynaltrexone and ^{3}H-M6G both bind with highest affinity to the same site. Methylating the 3-hydroxyl group lowers the affinity of opioids for traditional mu receptors, as illustrated by codeine and oxycodone. Thus, the poor affinity of 3-methoxynaltrexone for traditional mu receptors was expected. However, this structural change does not affect binding to the M6G site to the same degree.

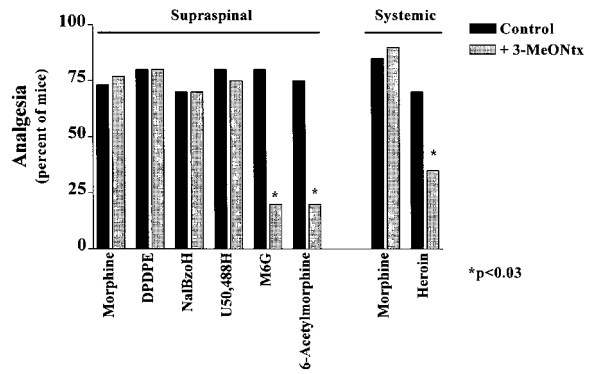

Fig. 3. Effect of 3-methoxynaltrexone on the dose–response curves of opioid analgesics. Left: Groups of mice ($n \geq 10$) received morphine (0.7 μg, i.c.v.), DPDPE (8 μg, i.c.v.), NalBzoH (20 μg, i.c.v.), U50,488H (75 μg, i.c.v.), M6G (12.5 ng, i.c.v.) or 6-acetylmorphine (1.2 μg, i.c.v.) alone or with 3-methoxynaltrexone (2.5 ng, i.c.v.). Right: Groups of mice (n ≥ 10) received morphine (5 mg/kg, s.c.) or heroin (0.8 mg/kg, s.c.) alone or with 3-methoxynaltrexone (2.5 ng, i.c.v.). Analgesia was determined and expressed as the percent of mice responding.

Like naltrexone, 3-methoxynaltrexone is an antagonist. It potently blocks the analgesic actions of a wide variety of mu analgesics. However, it shows a significant selectivity for M6G and heroin, antagonizing the actions of both analgesics at a dose which was inactive against morphine and the mu peptide, DAMGO. The selectivity of 3-methoxynaltrexone for M6G and heroin also extends to analgesics acting through delta and kappa receptors since these analgesics were not influenced by the antagonist. The possibility that M6G and heroin acted through traditional mu receptors also can be eliminated based upon their continued analgesic activity in mu opioid receptor knockout mice in which the first exon of MOR-1 had been targeted (A.G.P. Schuller, M. King, A. Chang, G. W. Pasternak, J.E. Pintar, in preparation). These observations strongly support the presence of a novel receptor which is responsible for M6G and heroin analgesia and which is uniquely sensitive to 3-methoxynaltrexone.

Although our results strongly suggest a novel receptor important in heroin actions, the pharmacology of heroin is complex. Heroin is rapidly converted to 6-acetylmorphine, which also acts through the M6G receptor and is believed to mediate the actions of heroin. However, 6-acetylmorphine can be further deacetylated to generate morphine [1], making it likely that heroin also has some morphine-like activity as well. It is likely that the rewarding properties of heroin, which are thought to be important for its addictive potential, are mediated through the M6G receptor, but this remains to be demonstrated. If the M6G receptor is responsible, the ability of 3-methoxynaltrexone to selectively antagonize M6G actions indicates that it may be possible to develop drugs which could selectively antagonize the addictive component of heroin actions, providing new therapeutic targets in the treatment of opioid abuse.

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