Pharmacology, Biochemistry and Behavior 90 (2008) 651–657

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Inhibition of the development of morphine tolerance by a potent dual μ-/δ-opioid antagonist, H-Dmt-Tic-Lys-NH-CH_2-Ph

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ARTICLE INFO

Article history:
Received 23 January 2008
Received in revised form 23 April 2008
Accepted 16 May 2008
Available online 23 May 2008

Keywords:
H-Dmt-Tic-Lys-NH-CH_2-Ph
Antinociception
Tolerance
Spinal
Dual μ-/δ-opioid antagonist

ABSTRACT

Three analogues of the dual μ-/δ-antagonist, H-Dmt-Tic-R-NH-CH_2-Ph (R=1, Lys-Z; 2, Lys-Ac; 3, Lys) were examined in vivo: 1 and 2 exhibited weak bioactivity, while 3 injected intracerebroventricularly was a potent dual antagonist for morphine- and deltorphin C-induced antinociception comparable to naltrindole (δ-antagonist), but 93% as effective as naloxone (nonspecific opioid receptor antagonist) and 4% as active as CTOP, a μ antagonist. Subcutaneous or oral administration of 3 antagonized morphine-induced antinociception indicating passage across epithelial and blood-brain barriers. Mice pretreated with 3 before morphine did not develop morphine tolerance indicative of a potential clinical role to inhibit development of drug tolerance.

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1. Introduction

The incorporation of a Lys linker in H-Dmt-Tic-R-NH-CH_2-Ph (R=side-chain Lys unprotected or protected with benzyloxy-carbonyl (Z) or acetyl (Ac) groups) provides new lead compounds with opioid activities that differ from those of the parent compound, H-Dmt-Tic-Gly-NH-CH_2-Ph, an opioid receptor ligand with mixed μ-agonism/δ-antagonism profile (Balboni et al., 2006). Lys derivatives exhibited high affinity for both μ- and δ-opioid receptors without selectivity for either opioid receptor subtype. Functional bioactivity studies in vitro using guinea-pig ileum (GPI) and mouse vas deferens (MVD) revealed that they were pure dual antagonists at both μ- and δ-opioid receptor sites (Balboni et al., 2006). Increasing evidence reveals the existence of an interaction between opioid receptors in the cell membrane, in which the stimulation of one opioid receptor can enhance the activity and sensitivity of another receptor type (George et al., 2000; He and Lee, 1998; Heyman et al., 1989; Larson et al., 1980) due to the formation of receptor heterodimers (Gomes et al., 2004; Rozenfeld and Devi, 2007). Thus, the stimulation of δ-opioid receptors can modulate μ-receptor-mediated antinociception, such that supraspinally and spinally injected opioids can synergistically potentate each other (He and Lee, 1998; Heyman et al., 1989; Larson et al., 1980). Interactions between different types of opioid receptors may contribute to the pharmacological properties of various opioid drugs (George et al., 2000).

Dimeric opioid agonists which interact with multiple homologous receptors, for example enkephalin derivatives (Lipkowski et al., 1982; Shimohigashi et al., 1982), dermorphin analogues (Lazarus et al., 1989), and *bis*-[H-Dmt-NH(CH_2)$_n$]-2(1*H*)-pyrazinone opioidmimetics (Jinsmaa et al., 2004; Okada et al., 2003) enhance bioactivity, stability and permeability of ligands through gastrointestinal membranes and eventually the blood-brain barrier to produce antinociception (Jinsmaa et al., 2004; Okada et al., 2003). Furthermore, unique opioid compounds with both agonist and antagonist properties or dual μ-/δ-bioactivities (agonism or antagonism), which have an ability to interact simultaneously with opioid receptor subtypes (Fujita et al., 2004; Jinsmaa et al., 2005; Salvadori et al., 1999; Schiller et al., 1999), may have important clinical potential to combat reward-driven mechanisms associated with opiate drug dependency (Heidbreder and Hagan, 2005), alcoholism (Bryant, 2005; Herz, 1997; Li et al., 2007) and obesity (Statnick et al., 2003). Such compounds may be also effective in preventing the development of opiate tolerance, which involves interactions between μ- and δ-opioid receptors (Riba et al., 2002; Zhang et al., 2006).

The synthesis of a new class of opioid compounds created by the alteration of a single amino acid in the Dmt-Tic pharmacophoric derivatives provides an interesting approach to generate dual acting compounds. This was evident in the replacement of Gly in H-Dmt-Tic-Gly-NH-Ph by Lys, Lys(Ac) or Lys(Z) that altered the in vitro functional

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0091-3057/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.pbb.2008.05.008

bioactivity from mixed μ-agonists/δ-antagonists to dual μ-/δ-receptor-mediated antagonists (Balboni et al., 2006). In this study, we measured the in vivo activity of these Lys derivatives on both μ- and δ-opioid receptor-induced antinociception, and investigated the ability of the most potent ligand (3) to nullify the development of morphine tolerance in mice.

2. Materials and methods

2.1. Animals

Male Swiss–Webster mice (20–25 g, Taconic, Germantown, NY) were used and housed in plastic cages and maintained on a 12-h light/dark cycle with free access to food and water. All experiments with animals were carried out according to protocols approved by and on file with the NIEHS Animal Care and Use Committee.

2.2. Opiate drugs and opioid peptides

Morphine, naloxone HCl and U50488 were obtained from Sigma (St. Louis, MO, USA); naltrindole hydrochloride and CTOP from Tocris (Ellisville, MO, USA); deltorphin C from Bachem (Torrance, CA, USA). The dual μ-/δ-opioid antagonist compounds 1, 2 and 3 were synthesized as described previously and used the same numbering system in this article for direct ease of comparison (Balboni et al., 2006).

2.3. Intracerebroventricular injection

Icv injection was performed in immobilized animals with Hamilton microsyringe fitted with disposable 26-gauge needle inserted 2.3–3 mm deep as described by Laursen and Belknap (1986). Briefly, the bregma was found by lightly rubbing the point of the needle over the skull until the suture was felt through the skin (about 1–3 mm rostral to a line drawn through the anterior base of the ears). The needle was inserted about 2 mm lateral to the midline; the total volume injected was 4 μl. Shortly after testing, the animals were euthanized with carbon dioxide according to approved protocols by the Animal Care and Use Committee of NIEHS, a slit was made along the midline of the scalp and mice having needle tract 2 mm lateral from the bregma were counted as having been injected correctly.

2.4. Hot-plate test

Mice with pre-response time less than 15 s were selected for the experiment. Animals were placed on an electrically heated plate (55°±0.1 °C, IITC MODEL 39D Hot Plate analgesia meter, IITC Inc, Woodland Hills, CA) 10 min after icv administration of the compounds. Hot-plate latency (HPL) was the interval between the placement of mice onto the hot plate and observing movement consisting of either jumping, licking or shaking their hind paws with cut off time of 30 s. Measurements were repeated every 10 min and testing was terminated when the HPL was close to the pre-response time. For studying the icv effect of antagonists, mice were injected with solution containing saline with morphine (control) or different doses of antagonists with morphine. For sc and oral administrations, the antagonists were injected 5 and 20 min before morphine (icv), respectively, and the effect was measured after 10 min post-morphine injection. Test doses of compounds used were selected from preliminary dose finding study starting from low (1 nmol/mouse and 1 mg/kg) to high doses (10 nmol/mouse and 30 mg/kg for icv and oral administrations, respectively).

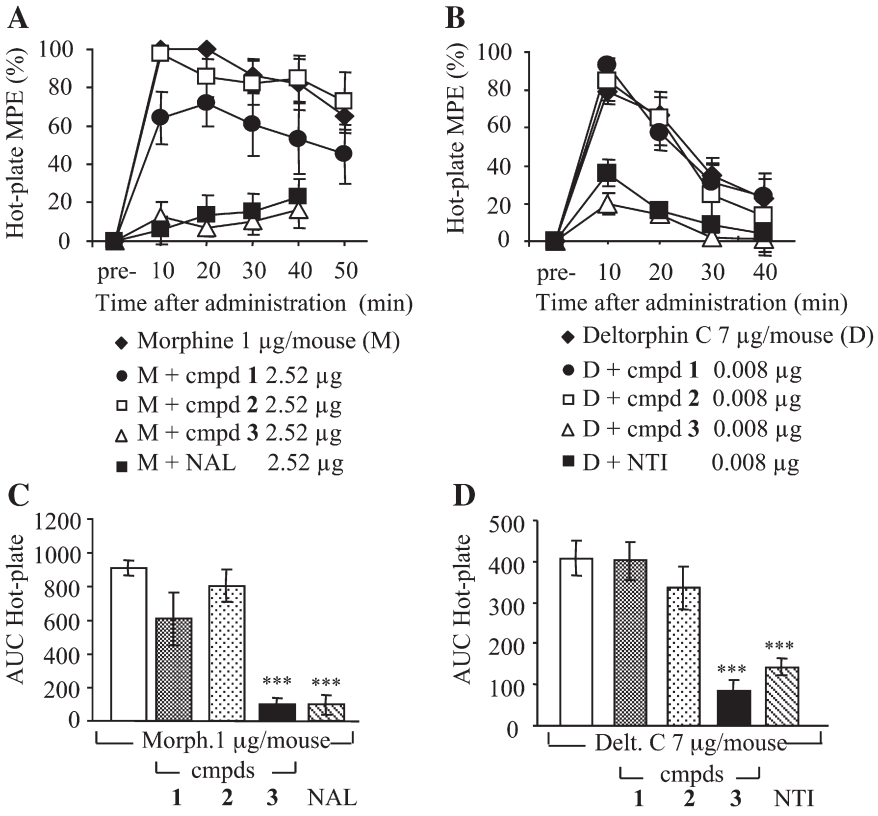

Fig. 1. Effect of 1, 2 and 3 on morphine- (A, C) and deltorphin C- (B, D) induced antinociception in the hot-plate test. Compounds were injected icv at 2.52 μg/mouse and 0.008 μg/mouse for morphine (1 μg/mouse) and deltorphin C (7 μg/mouse)-induced antinociception, respectively. (A, B) Time course, (C, D) Area Under the Curve (AUC). Each value is the mean±SEM (n=5–6 mice). (*) Denotes AUC values that are significantly different from saline-treated mice by Dunnett's test (*** $p<0.001$) following ANOVA (panel C: $p<0.0001$: $F=18.95$, d.f. 4; panel D: $p<0.0001$, $F=11.73$, d.f. 4).

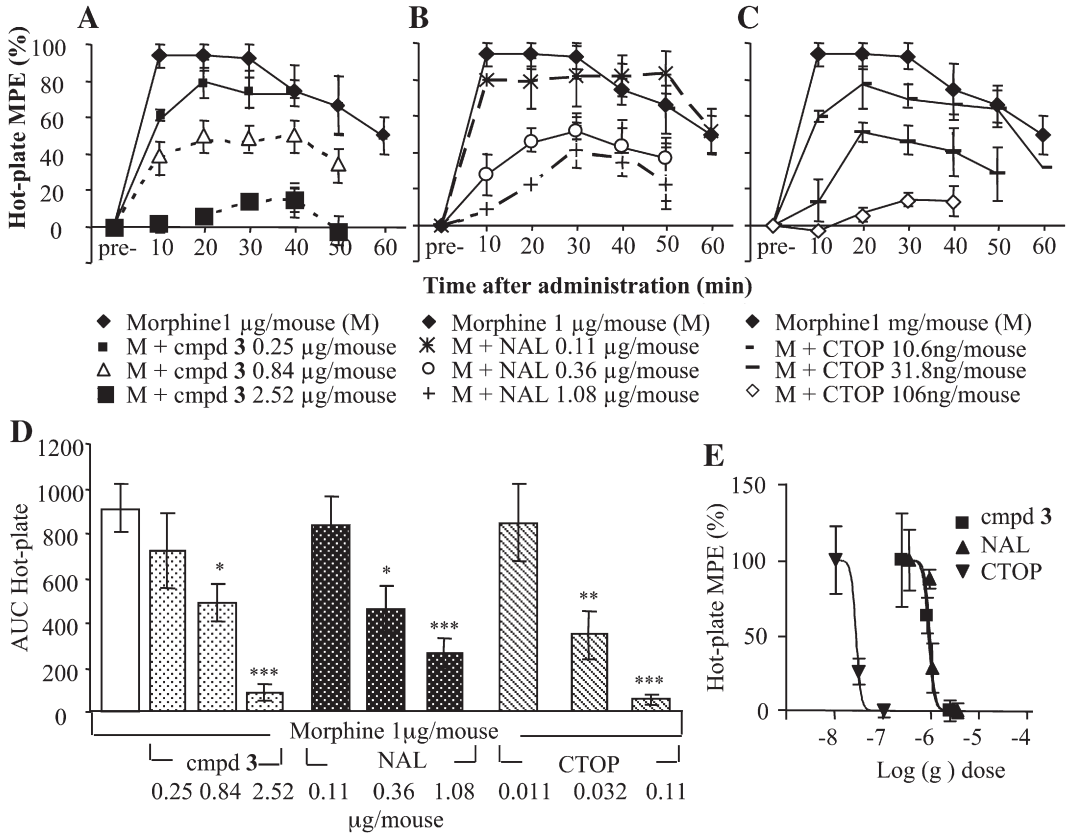

Fig. 2. Effect of intracebroventricularly injected 3 (A), naloxone (B) and CTOP (C) on morphine-induced antinociception in the hot-plate test. (A,B,C) Time course, (D) Area Under the Curve (AUC), (E) % MPE was calculated after 10 min following icv injection of the compounds. Morphine (1 μg/mouse) was injected icv. Each value is the mean ± SEM (n = 5 to 6 mice). (*) Denotes AUC values that are significantly different from saline-treated mice by Dunnett's test (*** $p<0.001$, ** $p<0.01$, * $p<0.05$) following ANOVA ($p<0.0001$: $F=20.78$, d.f. 9).

2.5. Tail-flick test

Spinal effects were measured by use of a tail-flick instrument (Columbus Instruments, Columbus, OH). Radiant heat was applied on the dorsal surface of the tail and the latency for removal of the tail from the onset of the radiant heat is defined as the tail-flick latency (TFL). The baseline of TFL was adjusted between 2 and 3 s (pre-response time) and a cut off time was set at 8 s to avoid external heat-related damage. The measurement of time was the same as described for the HP test.

2.6. Effect of compound 3 on morphine tolerance development

Tolerance was developed by injecting mice with morphine (10 mg/kg, sc) for 6 consecutive days, saline or 3 (0.01 mg/kg, sc) were injected 30 min before morphine. On days 1 and 6, the TFL and HPL were measured 30, 60 and 90 min after injection of morphine. Separate groups of mice were used for each test. In control groups injected with saline or 3 daily, the antinociceptive effect of morphine was tested on day 6. On day 7 the potency of morphine for all treatment groups was determined by cumulative dosing according to procedure of Duttaroy et al. (1997) with minor modifications. Mice were injected subcutaneously with a starting dose of morphine (1 mg/kg) and the antinociceptive effect was tested 25 min later. Within 5 min of testing, the next dose of morphine was administered and mice were retested for their antinociceptive effect. The final cumulative doses of morphine were as follow: 1, 2, 3, 5, 7, 9, 19, 29 and 59 mg/kg. This cumulative dose–response protocol was continued until all mice demonstrated a full antinociceptive effect.

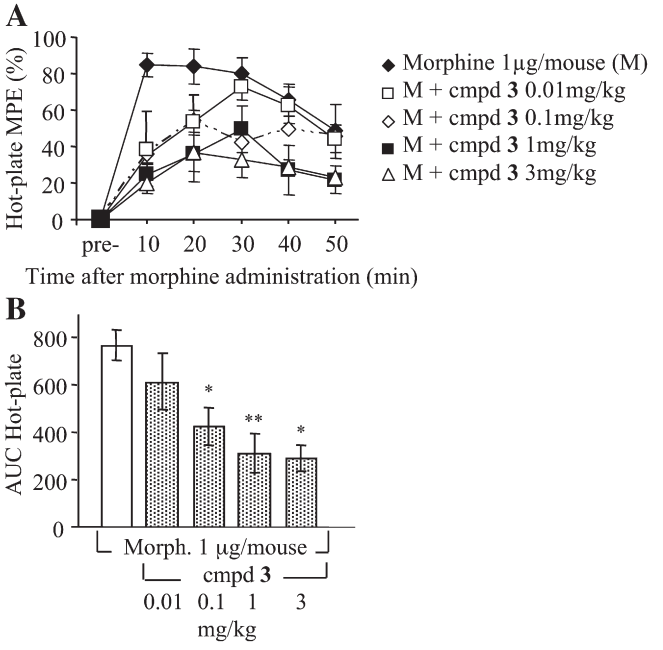

Fig. 3. Effect of subcutaneously injected 3 on morphine-induced antinociception determined by the hot-plate test. (A) Time course, (B) AUC. Morphine (1 μg/mouse) was injected icv 5 min after administration of 3. Each value is the mean ± SEM (n = 5–6 mice). (*) Denotes AUC values that are significantly different from saline-treated mice by Dunnett's test (*** $p<0.001$, ** $p<0.01$, * $p<0.05$) following ANOVA ($p<0.001$: $F=7.114$, d.f. 4).

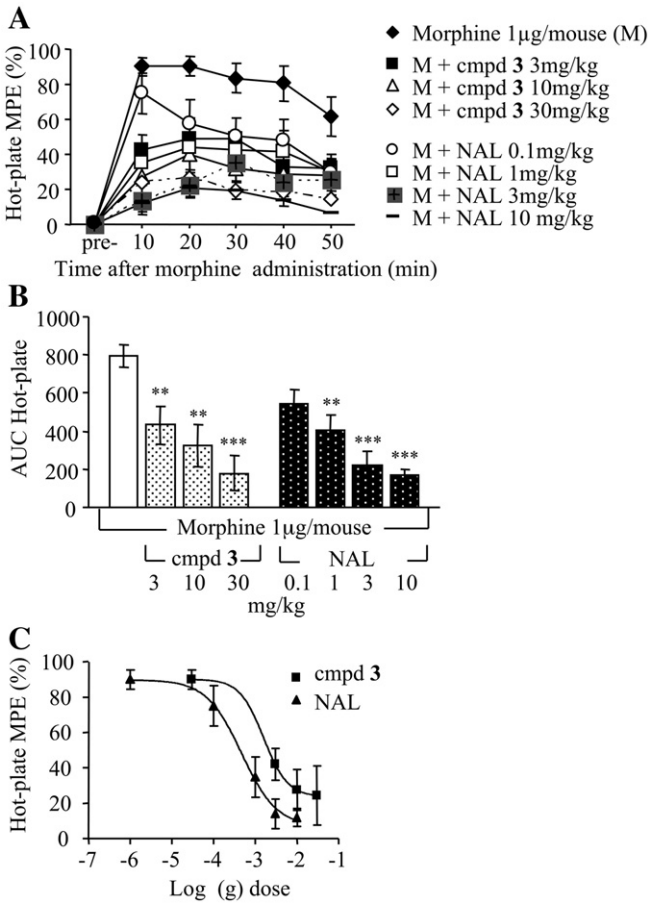

Fig. 4. Effect of orally administered 3 and naloxone on morphine-induced antinociception determined by the hot-plate test. (A) Time course, (B) AUC, (C) % MPE was calculated 30 min following oral injection of the 3 or naloxone. Morphine (1 μg/mouse) was injected icv 20 min after administration of 3 or naloxone. Each value is the mean ± SEM (n=5–6 mice). (*) Denotes AUC values that are significantly different from saline-treated mice by Dunnett's test (*** $p<0.001$, ** $p<0.01$) following ANOVA ($p<0.0001$, F=21.31, d.f. 7).

2.7. Statistical analysis

Statistical significance of the data was estimated by one-way analysis of variance (ANOVA) followed by Dunnett's test using the computer software program JMP (SAS Institute Inc, Cary, NC). The data were considered significant at $p<0.05$. Minimum Effective Dose (MED) is the minimum dose of compound showing statistically significant antinociceptive effect expressed as area under the curve (AUC) value in comparison with saline-treated group. The area under the time-response curve (AUC) was obtained by plotting the response time (s) on the ordinate and time (min) on the abscissa after administration of the compounds. AD_{50} and Hill slope values with their 95% confidence intervals were calculated with a curve-fitting program (Prism 4™, GraphPad Software, Inc., San Diego, CA). The percent maximum possible effect (% MPE) was calculated as ([post-drug response latency − pre-drug response latency] / [cut-off time (30 s for HP and 8 s for TF) − pre-drug response latency]) × 100.

3. Results

3.1. Antagonism of antinociception: demonstration of mode of action

While compounds 1 (H-Dmt-Tic-Lys(Z)-NH-CH_2-Ph), 2 (H-Dmt-Tic-Lys(Ac)-NH-CH_2-Ph) and 3 (H-Dmt-Tic-Lys-NH-CH_2-Ph) exhibited in vitro antagonism at both μ- and δ-opioid receptor sites (Balboni et al., 2006), only 3 had demonstrated potent antagonism in vivo: it blocked both morphine- (1 μg/mouse, icv) and deltorphin C (7 μg/mouse, icv)-induced antinociception (Fig. 1A, B, C and D). Compounds 1 and 2 (at the doses range of 1–10 nmol/mouse) had very weak μ-opioid antagonism and both were almost 10-fold less potent than 3. In terms of δ antagonism, 1 was inactive, independent of the dose (data not shown), and 2 exerted about of the 30% activity of 3.

In the case of HP, inhibition of morphine-induced antinociception by 3 occurred in a dose-dependent manner and was statistically significant at 0.84 μg/mouse (AD_{50} at 10 min was 0.899 μg; $CL_{95\%}$: 0.512–1.298); the effect began within 10 min post-injection and lasted for nearly an hour (Fig. 2A, D and E). Although 3 interacts as a dual opioid antagonist toward μ- and δ-opioid receptors, it was 93% as active as the non-specific opiate antagonist naloxone (AD_{50} 0.837 μg, $CL_{95\%}$: 0.499–1.401) (Fig. 2B, D and E), and had 4% the activity of the selective μ-opioid antagonist CTOP [D-Phe-c(Cys-Tyr-D-Trp-Orn-Thr-Pen)-Thr-NH_2] (AD_{50} 0.0279 μg; $CL_{95\%}$: 0.0257–0.0302) (Fig. 2C, D and E). On the other hand, similar potencies were observed between 3 and naltrindole (Fig. 1B and D).

Subcutaneous (sc) injection of 3 antagonized morphine antinociception in dose-dependent manner between 0.1–3 mg/kg (Fig. 3A, B), reached peak potency at 15 min and diminished thereafter depending on the dose. Furthermore, 3 injected sc at the dose of 10 mg/kg did not have any effect on antinociception induced by κ-opioid agonist, U50488 (100 nmol/mouse icv) in both HP and TF tests (data not shown). In another systemic validation of activity, orally administered 3 exhibited a dose-dependent antagonism toward morphine antinociception between 30 and 60 min post-injection (Fig. 4A and B); however, 3 (AD_{50} 1.64 mg/kg, $CL_{95\%}$: 0.155–3.126) was a quarter as active as naloxone (AD_{50} 0.38 mg/kg, $CL_{95\%}$: 0.175–0.845) (Fig. 4C).

The TF assay for the measurement of spinal involvement of opioid compounds revealed that 3 exhibited potent δ-antagonism (0.1 mg/kg), while it was much less effective at μ-receptor sites, even at doses as high as 10 mg/kg (Fig. 5A and B).

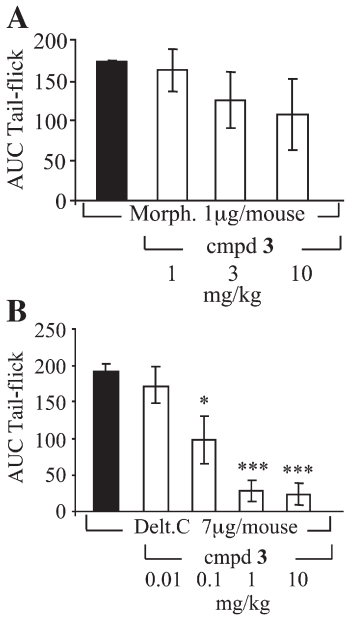

Fig. 5. Effect of subcutaneously injected 3 on (A) morphine and (B) deltorphin C-induced antinociception using the tail-flick test measurement. Morphine (1 μg/mouse) and deltorphin C (7 μg/mouse) were injected icv 5 min after administration of 3. Each value is the mean ± SEM (n=5–6 mice). (*) Denotes AUC values that are significantly different from saline-treated mice by Dunnett's test (*** $p<0.001$, * $p<0.05$) following ANOVA (panel A: p=0.1159: F=2.303, d.f. 3; panel B: $p<0.0001$: F=38.76, d.f. 4).

3.2. Elimination of morphine tolerance

To assess the effect of 3 on the development of morphine tolerance, mice were injected daily with morphine (10 mg/kg, sc) for 6 days in the absence or presence of 3 (0.01 mg/kg, sc, a dose which did not inhibit morphine- or deltorphin C-induced antinociception) and TF and HP latencies were measured. On day one, both groups (morphine only, and morphine plus 3) exhibited antinociception to a similar degree in both tests (AUC = 403 ± 12 and 391 ± 15 in the TF test, respectively; and 376 ± 9 and 369 ± 12 in HP test, respectively, in which neither difference is significant). While the morphine only group significantly reduced AUC 62% in TF (AUC 151 ± 43) and 21% in HP (AUC 299 ± 11) on day 6, the morphine plus 3 group remained unchanged. Treatment of mice with saline or 3 for 6 days had no effect on acute morphine antinociception (Fig. 6A,B,D,F). These results correlate with changes of morphine potency (Fig. 6C, E) which depends on the treatment. The dose–response curves of morphine-induced antinociception in mice administered with morphine (10 mg/kg) for 6 days is shifted to the right in comparison to saline treated groups in both TF and HP tests (Fig. 6C,E). Consequently, treatment with morphine caused an increase in TF ED_{50} from 7.3 mg/kg (6.6–8.2) in saline to 15.6 mg/kg (12.0–20.3) in the morphine-treated group; and HP ED_{50} from 14 mg/kg (11.2–17.1) in saline to 22.9 mg/kg (21.0–27.6) in morphine-treated mice. Co-administration of 3 with morphine prevented the decrease of morphine potency [ED_{50} 8.1 mg/kg (7.5–8.7) in TF and 14.2 mg/kg (11.3–18.8) in HP], and administration of 3 alone did not have any effect on morphine potency in comparison with saline treated group [ED_{50} 7.2 mg/kg (6.2–8.5) in TF and 14.0 mg/kg (11.2–17.1) in HP].

4. Discussion

Increasing evidence reveals that opioid receptors interact in vivo to form homo- or heterodimeric complexes, such that the stimulation of one type of opioid receptors can affect the activity and sensitivity of other receptor type (Gomes et al., 2004; Heyman et al., 1989). In fact,

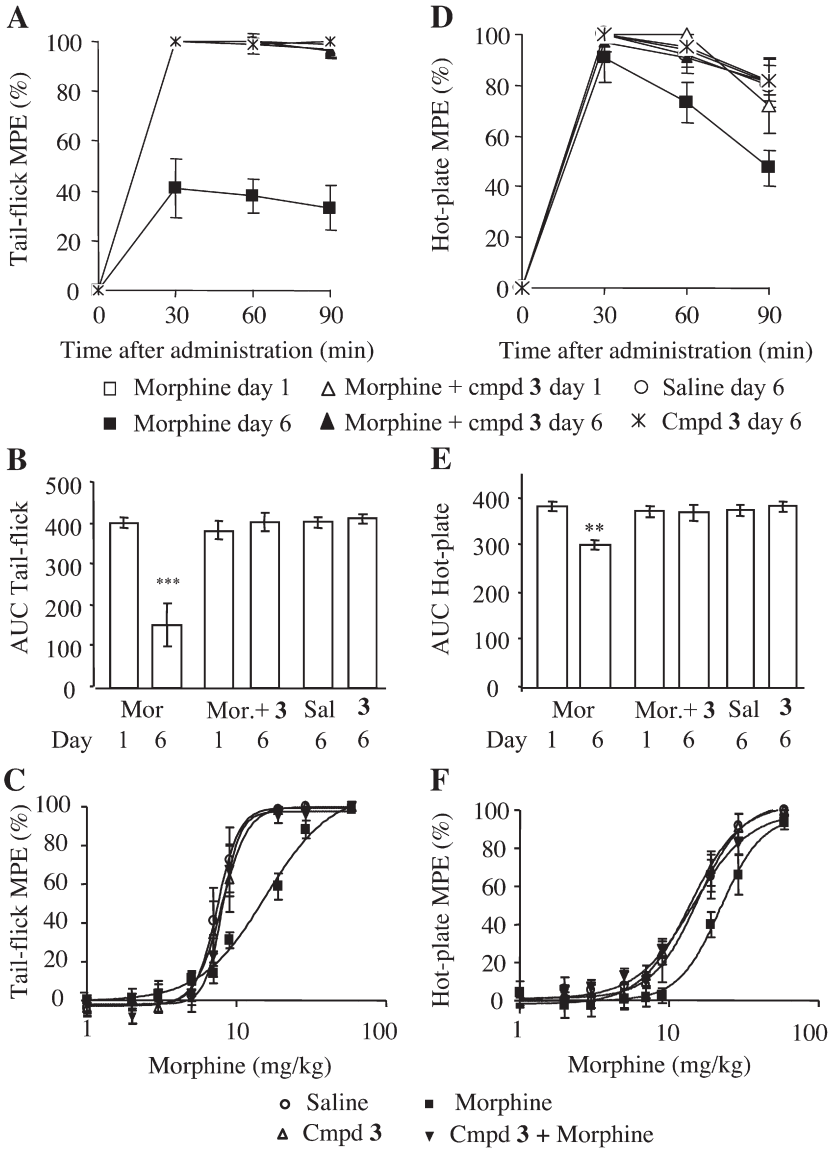

Fig. 6. Effect of 3 (0.01 mg/kg, sc) on development of morphine tolerance in the tail-flick (A, B, C) and hot-plate (D, E, F) tests. (A, D) Time course, and (B, E) Area Under the Curve (AUC) of morphine (10 mg/kg) injected subcutaneously at day 1 and 6 of the treatment regime, (C, F) Cumulative dose–response for morphine-induced analgesia measured on day 7. Each value is the mean ± SEM (n = 5 mice). (*) Denotes AUC values that are significantly different from saline-treated mice by Dunnett's test (*** $p < 0.001$, ** $p < 0.01$) following ANOVA (panel B: $p < 0.0001$: $F = 36.77$, d.f. 5; panel E: $p < 0.0006$: $F = 6.529$, d.f. 5).

animal studies revealed that stimulation of δ-opioid receptors can modulate μ-opioid receptor mediated antinociception and supraspinally and spinally injected opioids can synergistically potentate each other (He and Lee, 1998; Heyman et al., 1989; Larson et al., 1980). Therefore, interactions between different opioid receptor types may contribute to the pharmacology of opioid drugs and designing opioids that interact with multiple opioid receptor types, which may have important pharmacological and clinical roles (Fujita et al., 2004; Jinsmaa et al., 2005; Li et al., 2005; Lipkowski et al., 1982; Salvadori et al., 1999; Schiller et al., 1999).

The bioactivity of 3, which exhibits antagonism toward both μ- and δ-opioid receptors in vitro, revealed the critical importance of a free amino group in Lys for the appearance of antagonism (Balboni et al., 2006, 2007) and is supported by the in vivo studies herein. Since 3 was active following both subcutaneous and oral administrations, the data indicate transit through the gastrointestinal epithelial and blood-brain barriers. The mode of action, however, depends on the specific assay: in the supraspinal HP test, 3 clearly behaves as a dual μ-/δ-antagonist, while in the spinal TF assay, 3 acts prevalently as a potent δ-antagonist with much weaker μ-antagonistic properties. Differences in the potency of 3 in the HP and TF assays may be related to different receptor subtypes involved in the antinociceptive effects of agonists in these tests.

It is generally accepted that even in the case of δ-agonists, μ opioid receptors are preferentially involved in analgesia when heat is used as the nociceptive stimulus (Scherrer et al., 2004; Sora et al., 1997). In μ receptor knock-out mice, the antinociceptive effect of morphine was completely eliminated and the effect of δ agonist was significantly reduced in both HP and TF tests indicating involvement of μ-opioid receptors in both tests (Scherrer et al., 2004; Sora et al., 1997). In δ-opioid receptor knockout mice, icv injected δ agonists, DPDPE and deltorphin II, produced a full antinociceptive effect, which was reversed in both tests by the μ-selective antagonist CTOP (Scherrer et al., 2004). These data suggest that μ rather than δ receptors are recruited by the two agonists for the TF and HP tests. On the other hand, studies involving different routes of administration of compounds demonstrated that in δ opioid receptor knockout mice icv administered δ agonists show antinociceptive effect similar to wild type mice, while intrathecal administration caused significant decrease of their potency (Zhu et al., 1999). In μ-opioid receptor-deficient mice, in which morphine had a significant decrease in antinociceptive potency when administered icv, but exhibited an effect comparable to progenitor mice after intrathecal administration, δ agonists were fully antinociceptive (Vaught et al., 1988) suggesting differential involvement of μ- and δ-opioid receptors in spinal and supraspinal antinociception. These observations are consistent with the existence of antinociceptive cross-tolerance between morphine and the specific δ agonist DPLPE at the spinal level (Porreca et al., 1987). The degree of δ-opioid receptors involvement in antinociceptive effect measured by TF and HP tests differs, which could be observed as the involvement of different receptor subtypes (Pasternak, 1993) and is probably related to different degrees of receptor heterodimerization (Gomes et al., 2000).

Long term systemically administrated morphine develops tolerance, which is related to the interactions between μ- and δ-opioid receptors (Riba et al., 2002; Zhang et al., 2006). These interactions may change receptor properties (Traynor and Elliott, 1993). One of the explanations for the development of morphine tolerance involves heterodimerization of μ- and δ-opioid receptors, which leads to a switch in signaling from G-protein-dependent in the case of μ- and δ-opioid receptors, to β-arrestin-dependent in the case of μ-δ-opioid receptor heterodimers (Rozenfeld and Devi, 2007). Further, up-regulation of δ-opioid receptors occurs during chronic morphine treatment (Ma et al., 2006). These newly available δ-receptors on a cell surface may recruit μ-receptors into a heterodimer complex, thereby changing μ-opioid receptor signaling. There is evidence that blocking δ-opioid receptor action or depletion of δ-opioid receptors in mice attenuated the development of morphine tolerance and withdrawal symptoms (Abdelhamid et al., 1991; He and Lee, 1998; Hepburn et al., 1997; Riba et al., 2002; Zhang et al., 2006) probably by switching signaling from the μ-δ-opioid receptor heterodimer to a μ-opioid receptor-driven action. It was reported (Hepburn et al., 1997) that although the antinociceptive response of rats treated daily with naltrindole alone was no different from the controls, animals co-treated with morphine and naltrindole had significantly greater antinociceptive response (reduced tolerance) than animals treated with morphine alone. Our data revealed that 3 inhibited development of morphine tolerance in mice similar to that observed with the δ-antagonist naltrindole, but with greater potency. Mice co-treated with morphine plus 3 for 6 days failed to exhibit an attenuation in morphine antinociception suggesting the complete absence of morphine tolerance in these animals. Compound 3 may assist the recovery of μ-opioid receptor functions by binding to receptors existing in a heterodimeric complex. The presence of only 3 for 6 days failed to exhibit an effect on a single, acute dose of subcutaneously injected morphine, suggesting that 3 acts only during long term use of morphine where alteration of opioid receptors interaction may readily occur.

Thus, we demonstrated that the in vivo activity of a novel dual μ-/δ-opioid antagonist, H-Dmt-Tic-Lys-NH-CH_2-Ph, may have an important role in opioid research to understand opioid receptor interactions and in the design of new compounds having unique activities for potential clinical application in prevention of the development of drug tolerance.

Acknowledgements

This work was supported in part by the Intramural Research Program of the NIH and NIEHS, and in part by the University of Cagliari (PRIN 2004) and University of Ferrara (PRIN 2004). The authors appreciate the expertise and assistance of the library staff and the Comparative Medicine Branch at NIEHS.

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