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MD-354 potentiates the antinociceptive effect of clonidine in the mouse tail-flick but not hot-plate assay

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Abstract

Albeit conflicting, evidence suggests that 5-HT_3 receptor partial agonists as well as $\alpha_{2NON\text{-}A}$-adrenoceptor agonists might be involved in antinociception. MD-354 (*m*-chlorophenylguanidine) can be viewed as the first example of a rather selective 5-HT_3/α_{2B}-adrenergic ligand. In a tail-flick test in mice, subcutaneous administration of MD-354 doses up to 30 mg/kg did not produce antinociception and failed to antagonize the effect of clonidine (ED_{50} = 0.5 mg/kg), but a combination of an inactive dose of clonidine (0.25 mg/kg) that produced only 13% maximal possible effect (MPE) with an inactive dose of MD-354 (10 mg/kg, MPE = 8%) produced an antinociceptive effect (MPE = 83%). In the hot-plate assay, neither subcutaneous administration of MD-354 (3 to 30 mg/kg) alone nor in combination with clonidine (ED_{50} = 0.8 mg/kg) produced an antinociceptive effect. MD-354 was demonstrated to potentiate the antinociceptive effect of clonidine in the tail-flick assay, but its underlying mechanism remains to be determined.

Keywords: α_2-Adrenoceptor; 5-HT_3 receptor; MD-354 (*m*-chlorophenylguanidine); Antinociception; In vitro and in vivo model

1. Introduction

We have previously identified a novel class of 5-HT_3 receptor ligands—arylguanidines—of which MD-354 (*m*-chlorophenylguanidine) is a representative member (Dukat et al., 1996). MD-354 binds with high affinity at 5-HT_3 receptors (K_i = 35 nM; Dukat et al., 1996) and behaves as an agonist in several assays typical of 5-HT_3 receptor agonists (Dukat et al., 1996, 2000), but has also been shown capable, at higher doses, of antagonizing certain 5-HT_3-mediated actions (e.g., antagonism of cisplatin-induced emesis in shrews; Dukat et al., 2000). All indications are that arylguanidines, at least MD-354, are 5-HT_3 receptor partial agonists. In an effort to further characterize MD-354, a binding profile was obtained (data presented herein), and MD-354 was found to bind at α_2-adrenoceptors. Furthermore, MD-354 was reasonably selective for α_{2B}- versus α_{2A}- or α_{2C}-adrenoceptors. Its affinities (K_i values) for each of the α_2-adrenoceptor subtypes were determined to be α_{2A}-K_i = 825 nM, α_{2B}-K_i = 25 nM, and α_{2C}-adrenoceptor K_i = 140 nM. As such, MD-354 is a reasonably selective α_{2B}-adrenoceptor ligand.

MD-354 is unique in that it is the only agent identified to date that is a 5-HT_3 receptor partial agonist with selectivity for α_{2B}-adrenoceptors. Furthermore, its affinity for 5-HT_3 receptors is comparable to its affinity for α_{2B}-adrenoceptors. Because both 5-HT_3 receptor ligands (i.e., antagonists and partial agonists) and α_{2B}-adrenoceptor agonists have been implicated as playing a role in pain (reviewed by Dukat, 2004; Maze and Fujinaga, 2000), it was of interest to examine MD-354 as an antinociceptive agent.

Analgesic effects evoked by α_2-adrenoceptor agonists have been well documented in animals and humans including patients with opioid tolerance (reviewed by Ongjoco et al., 2000). Relatively free of side-effects (e.g., respiratory depression, addiction), α_2-adrenoceptor agonists seem to be attractive and promising targets and alternatives to currently available analgesic agents; however, their clinical use remains limited by sedation and hypotension as undesirable side-effects. This triggered new studies to elucidate what functions are associated with each of the three subtypes (α_{2A}, α_{2B}, and α_{2C}) of α_2-adrenoceptors and to identify their distribution. Clonidine, a nonselective α_2-adrenoceptor agonist, produces

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antinociception both in animals and humans mainly through α_{2A}-adrenoceptors (Furst, 1999). Unfortunately, sedation and hypotension restrict the clinical use of clonidine, and both side-effects are also thought to reflect action at α_{2A}-adrenoceptors. Epidural clonidine has been exclusively approved for cancer pain (Kamibayashi and Maze, 2000). It has been demonstrated that a prazosin-sensitive α_2-adrenoceptor subtype inhibits neurotransmitter release from spinal cord preparations, indicating a role for α_{2B}- or α_{2C}-adrenoceptor subtypes (Maze and Fujinaga, 2000). Takano and Yaksh (1992) confirmed this finding by demonstrating that ST-91 (2-(2,6-diethylphenylamino)-2-imidazoline), a α_{2NON-A}-adrenoceptor subtype agonist induces antinociception in the hot-plate assay in rats, and that the effect was blocked by prazosin. Activation of the α_{2B}-adrenoceptor subtype is thought to be the target of nitrous oxide (a potent inhalative analgesic) to produce its antinociceptive effect. In knockout mice lacking α_{2B}-adrenoceptors, the analgesic effect of nitrous oxide was undetectable (Philipp et al., 2002). Graham et al. (1997) demonstrated that the α_2-adrenoceptor subtype involved in rat pain is species- and strain-dependent, with the α_{2B}-adrenoceptor subtype predominantly involved in the hot-plate assay in Harlan rats, while both α_{2A}- and α_{2B}-adrenoceptor subtypes modulate nociceptive responses in the tail-flick test in Harlan and Sasco rats (Graham et al., 1997). Lack of subtype-selective α_2-adrenoceptor agonists and antagonists prevent a more sophisticated analysis by classical pharmacological techniques, and mechanistic involvement remains another unmet challenge. Using molecular genetics, an antinociceptive role for the α_{2A}-adrenoceptor subtype in thermal analgesia in transgenic mice along with sedation, hypotension, and bradycardic actions were confirmed (reviewed by Maze and Fujinaga, 2000). Similar studies showed involvement of the α_{2B}-adrenoceptor subtype in vasoconstriction and the α_{2C}-adrenoceptor subtype in hypothermia, modulation of dopaminergic activity, and possible involvement in antinociception (reviewed by Maze and Fujinaga, 2000). However, one has to be aware that experiments with genetically modified subjects might result in data that differ from that obtained with wild-type mice. At present, literature data suggest that the α_{2B}- and/or α_{2C}-adrenoceptors might be primary targets for α_2-mediated pain modulation. The main advantage of α_{2B}- or α_{2C}-adrenoceptor-selective agonists over nonselective adrenoceptor agonists for antinociception would be their lack of side-effects associated with the α_{2A}-adrenoceptor subtype (e.g., sedation).

Despite the inconsistency in clinical reports on pain modulation by 5-HT_3 receptor ligands, their role seems promising. Granisetron, a 5-HT_3 receptor antagonist, was shown to reduce pain in patients with systematic inflammatory disorders, while chronic low-back pain and cervical pain responded to the 5-HT_3 receptor antagonist tropisetron (Israili, 2001). 5-HT_3 receptor antagonists seem to play a role in pathophysiological pain associated with migraine and irritable bowel syndrome (Fozard, 1994). Less is known about the role of 5-HT_3 receptor agonists in pain management due to contradictory findings related to the inability of current ligands to penetrate the blood–brain barrier, different doses, routes of administration, types of nociceptive tests employed (e.g., thermal, mechanical, chemical), and most importantly, possible differences in the density of 5-HT_3 receptors in various neuronal systems (reviewed by Dukat, 2004). Nevertheless, there are indications that 5-HT_3 receptor agonists or partial agonists could be effective in the treatment of pain. Recently, a novel 5-HT_3 receptor agonist YM-31636 (2-(1*H*-imidazol-4-ylmethyl)-8*H*-indeno[1,2-*d*]thiazole) was developed for the treatment of constipation. Gastrointestinal hypermotility might be associated with visceral pain. Fortunately, YM-31636 facilitates defecation without reducing or increasing visceral pain at doses higher than those that increase defecations (reviewed by Dukat, 2004).

MD-354 might represent a novel, dual-mechanism type of analgesic agent. It was of interest to determine if MD-354 possesses antinociceptive activity. We used two thermal stimuli tests to study the possible antinociceptive properties of this compound in mice using clonidine as a reference (i.e., positive control) agent. The tail-flick test was used to study any possible involvement of spinal α_{2B}-adrenoceptors, whereas the hot-plate test was used to study the possible involvement of supraspinal receptors.

2. Materials and methods

2.1. Animals

Male ICR mice (24–28 g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Mice were housed in groups of five, with free access to food and water. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility, and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Mice were allowed to adapt to the testing environment for at least 2 h prior to any treatment. Animals were weighed on the day of the experiment(s) for the calculation of drug dosages.

2.2. Drugs

Clonidine hydrochloride and morphine hydrochloride were purchased from Sigma-Aldrich Chemical, (Milwaukee, WI). MD-354 nitrate was resynthesized as reported previously (Dukat et al., 1996). Imiloxan hydrochloride and zacopride hydrochloride were obtained from Tocris (Ballwin, MO). All drugs were dissolved in physiological saline (0.9% sodium chloride) and given in a total volume of 10

ml/1000 g body weight for subcutaneous (s.c.) and intraperitoneal (i.p.) injections to mice.

2.3. Radioligand binding

The receptor binding assays were conducted by the NIMH Psychoactive Drug Screening Program (PDSP) using their standard assay protocols. Details of the assay protocols can be found at: http://pdsp.cwru.edu. MD-354 was examined in triplicate at >30 receptor populations, and for binding at the serotonin, norepinephrine, and dopamine transporters, at a concentration of 10,000 nM. Where >50% inhibition of binding was observed, a K_i value was determined in triplicate; where < 50% inhibition occurred, the K_i was reported as >10,000 nM.

2.4. Behavioral assays

2.4.1. Antinociception

2.4.1.1. Tail-flick test. Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941) as modified by Dewey et al. (1970) using a Columbus Tail-Flick Analgesia Meter. A control response (1.7–4.0 s) was determined for each mouse before treatment, and a test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 s was imposed. The antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE=[(test – control)/(10 – control)] × 100. Groups of 5 to 12 animals were used for each dose and for each treatment.

The experimental protocol for testing the effects of drugs was as follows: 15 min prior to s.c. injection of drugs, baseline tail-flick was determined for each mouse. The animal then was injected with MD-354 using various (5 to 45 min) pretreatment intervals. In combination tests using (i) MD-354 with clonidine, MD-354 was injected 25 min prior to the clonidine dose and 45 min before the test; (ii) MD-354 with morphine, MD-354 was injected 15 min prior to the morphine dose and 45 min before the test. Imiloxan was administered i.p. 10 min prior to MD-354 and 35 min before the clonidine dose. Zacopride was administered i.p. 5 min prior to MD-354 and 30 min before the clonidine dose. The order and timing of administration of clonidine (20 min—the time of peak effect; Spaulding et al., 1979; Kameyama et al., 1986) and morphine (30 min—the time of peak effect; Spaulding et al., 1979) ensured that the times of their peak antinociceptive effects coincided.

2.4.1.2. Hot-plate test. The method is a modification of that described by Eddy and Leimbach (1953) and Atwell and Jacobson (1978). Mice were placed into a 10-cm-wide glass cylinder on a hot plate (Columbus Hot-Plate Analgesia Meter) maintained at 55.0 °C. Two control latencies at least 10 min apart were determined for each mouse. The control latency (reaction time) was 6 to 10 s. The antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE=[(test – control)/(40 – control) × 100]. The reaction time was scored when the animal jumped or licked its paws. A cutoff of 40 s was used to avoid any paw damage. Groups of 5 to 14 animals were used for each dose and for each treatment. The dosing schedule for MD-354 and clonidine was the same as used in the tail-flick assay.

2.4.2. Spontaneous activity

Mice were placed into individual Tru Scan Infrared Locomotor Activity System (Coulbourn Instruments, Allentown, PA) photocell activity cages (40 cm cube) after s.c. administration of either 0.9% saline or MD-354 (6.0 mg/kg). Ambulatory movement was measured by the number of times the animal interrupted the infrared beams traversing the cage for a period of 15 min. Measurements were taken 15, 30, and 45 min following drug treatment. The analysis was focused only on main measures (three main measures: total moves, move time, move distance) of activity to determine whether MD-354 (n=6/dose) depressed this action relative to saline (n=6) control.

2.5. Statistical analysis

Data were analyzed statistically by an analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple post hoc comparison test. The null hypothesis was rejected at the 0.05 level. For the time-course studies, each animal was used once. Data were analyzed by one-way or a two-factor ANOVA as applicable. ED_{50} values with 95% CL for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987).

3. Results

3.1. Radioligand binding

The data from the NIMH Psychoactive Drug Screening Program (PDSP) indicate that MD-354 lacked affinity (K_i>10,000 nM) for human 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_6 receptors, dopamine D1–D5 receptors, mu and kappa opioid peptides, m_1–m_5 muscarinic receptors, β_1- and β_2-adrenoceptors, rat H_1 histamine receptors, phencyclidine (PCP) receptors, NMDA (*N*-methyl-D-aspartate) receptors, benzodiazepine receptors, and the serotonin, norepinephrine, and dopamine transporters. MD-354 displayed low to modest affinity for 5-HT_{1A} (K_i=4100 ±1440 nM), 5-HT_{5A} (K_i=4160 ± 770 nM), and 5-HT_7 (K_i=680 ± 190 nM) receptors, and α_{1A}- and α_{1B}-adrenoceptors (K_i=300 ± 20 and 1900 ± 113 nM, respectively). In

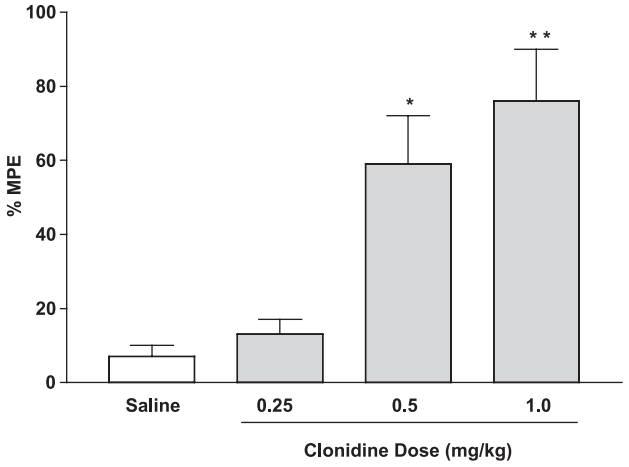

Fig. 1. Antinociceptive actions (± S.E.M.) of clonidine in the tail-flick assay ($n = 6–12$ mice/treatment). Asterisks denote significant differences compared to control group; $^*P < 0.01$ and $^{**}P < 0.001$; one way ANOVA ($F_{3,33} = 11.85$) followed by Newman–Keuls post hoc test.

addition, MD-354 was shown to bind at the α_2-adrenoceptor family with moderate selectivity for α_{2B}- ($K_i = 25 \pm 5$ nM) over α_{2C}- ($K_i = 140 \pm 40$ nM) and α_{2A}-adrenoceptors ($K_i = 825 \pm 160$ nM).

3.2. Antinociceptive activity

3.2.1. Tail-flick and hot-plate assays

The antinociceptive properties of MD-354 were compared with those of clonidine. In the tail-flick assay, 1.0 mg/kg of clonidine produced 76% of the maximal possible effect (MPE; Fig. 1) when administered via the s.c. route 20 min prior to evaluation. Lower doses produced less antinociception (Fig. 1). The potency of clonidine in the tail-flick assay ($ED_{50} = 0.5$ mg/kg; 95% CL = 0.4–0.7 mg/kg) was consistent with that previously reported in the literature (Kameyama et al., 1986). MD-354 failed to produce a statistically significant antinociceptive effect (Fig. 2). MD-354 produced 1–24% MPE at doses of 1.0, 3.0, 6.0, 10, and 30 mg/kg when administered 45 min prior to testing (Fig. 2). To determine an optimal pretreatment time, selected MD-354 doses were initially examined using various pretreatment times (in addition to the 45-min pretreatment time) but failed to produce >13% MPE (data not shown). Consequently, the 45-min pretreatment time was subsequently selected for all studies reported herein (unless otherwise specified).

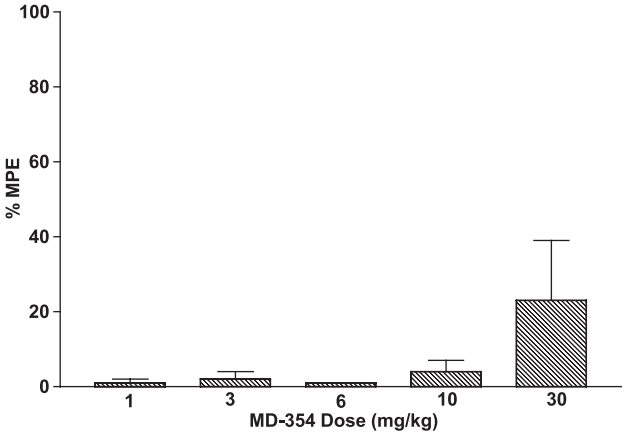

Fig. 2. Effect (± S.E.M.) of MD-354 doses administered 45 min prior to examination in the tail-flick assay ($n = 6–9$ mice/treatment) as compared to saline control (1 ± 1% MPE).

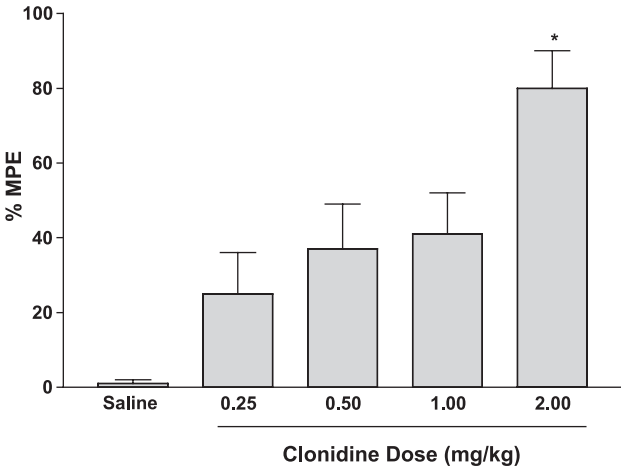

Fig. 3. Antinociceptive actions (± S.E.M.) of clonidine in the hot-plate assay ($n = 5–8$ mice/treatment). Asterisks denote significant differences compared to control group; $^*P < 0.001$; one way ANOVA ($F_{4,30} = 6.98$) followed by Newman–Keuls post hoc test.

In the hot-plate test, 2.0 mg/kg of clonidine produced 80% MPE ($ED_{50} = 0.8$ mg/kg; 95% CL = 0.6–1.1 mg/kg; Fig. 3), whereas MD-354 produced 8–26% MPE at doses of 3.0, 10, and 30 mg/kg after 10-, 20-, 45-, or 90-min post drug administration (data not shown). The observed effect was time-dependent and diminished following the 45-min MD-354 pretreatment time; for example, 30 mg/kg of MD-

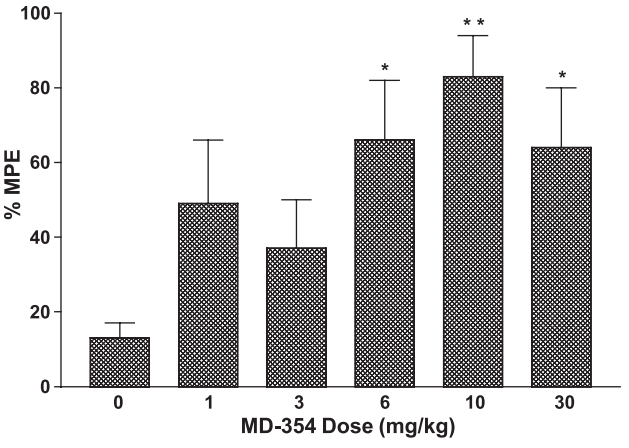

Fig. 4. Potentiation of the antinociceptive actions (± S.E.M.) of clonidine (0.25 mg/kg) by MD-354 in the tail-flick assay ($n = 6–9$ mice/treatment). Asterisks denote significant differences compared to control group; $^*P < 0.05$ and $^{**}P < 0.01$; one way ANOVA ($F_{5,40} = 4.44$) followed by Newman–Keuls post hoc test.

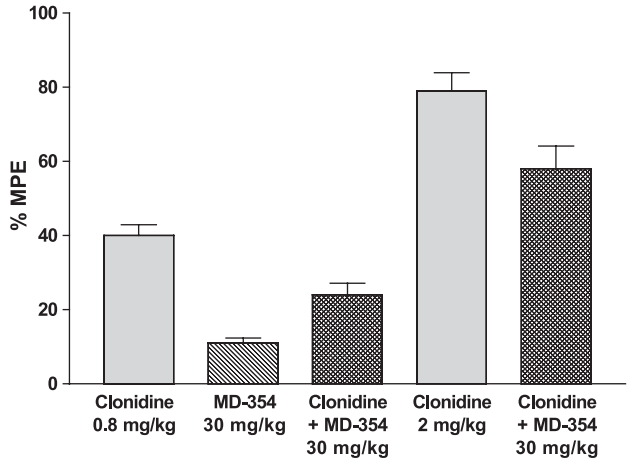

Fig. 5. Effect (± S.E.M.) of MD-354 doses administered in combination with clonidine (0.8 and 2 mg/kg) in the hot-plate assay ($n = 5–14$ mice/treatment).

354 produced 12 ± 4% MPE after 10 min of pretreatment, 17 ± 6% MPE after 20 min, 26 ± 3% MPE after 45 min, and 1 ± 1% MPE after 90 min.

3.2.2. Combination studies

MD-354 was examined in both thermal assays as a possible clonidine antagonist. In the tail-flick assay, doses of 1.0, 3.0, and 10 mg/kg of MD-354 failed to antagonize the antinociceptive actions of the ED_{50} dose (i.e., 0.5 mg/kg) of clonidine when administered 25 min prior to clonidine, and tested 20 min thereafter. However, a combination of the ED_{50} dose of clonidine (MPE = 59%) plus an "inactive" dose of MD-354 (10 mg/kg, MPE = 8%) augmented the antinociceptive effect in mice (MPE = 74%; data not shown). Similarly, a lower, "inactive" dose of clonidine (0.25 mg/kg, MPE = 13%) in combination with "inactive" doses (1.0, 3.0, 6.0, 10, and 30 mg/kg) of MD-354 produced significant antinociception (49–83% MPE) in a dose-dependent manner (Fig. 4). In contrast, in the hot-plate assay, a dose of 30 mg/kg of MD-354 (MPE = 11%) slightly antagonized the effect of the ED_{50} dose (0.8 mg/kg, 40% MPE) of clonidine (combination: 24% MPE), as well as a higher (2.0 mg/kg, 79% MPE) dose of clonidine (combination: 58% MPE; Fig. 5). However, the observed antagonist effect was not statistically significant. Interestingly, a lower dose of clonidine (0.25 mg/kg, 12% MPE) in combination with doses (10 and 30 mg/kg, 10% and 11% MPE, respectively) of MD-354 slightly elevated the antinociceptive effect (MPE = 35% and 32%, respectively; Fig. 6). Here too, the observed effect was not statistically significant.

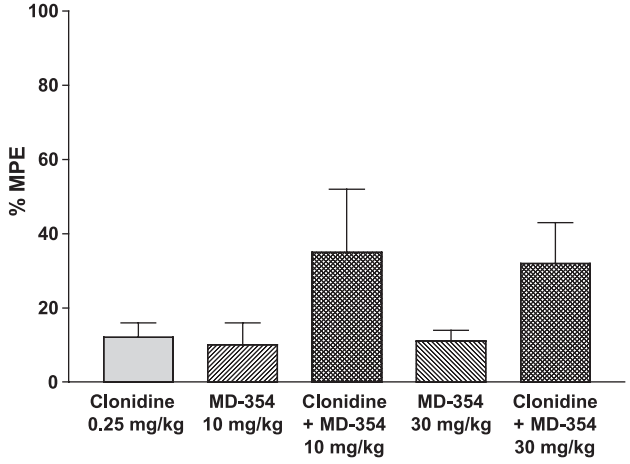

Fig. 6. Effect (± S.E.M.) of MD-354 doses administered in combination with clonidine (0.25 mg/kg) in the hot-plate assay ($n = 5–7$ mice/treatment).

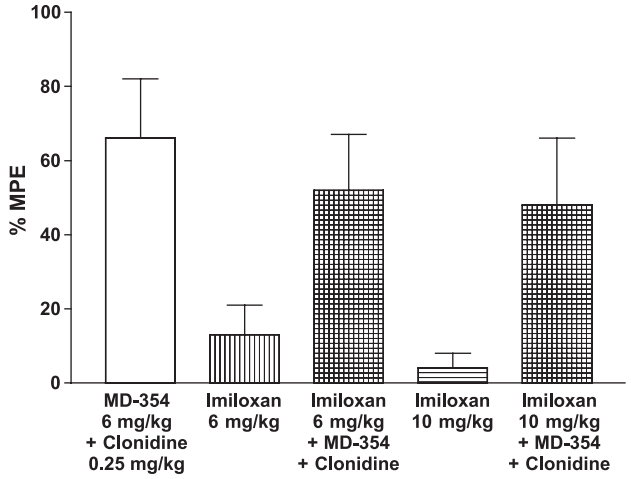

Fig. 7. Effect (± S.E.M.) of imiloxan on the antinociceptive actions of a combination of clonidine (0.25 mg/kg) and MD-354 (6 mg/kg) in the tail-flick assay ($n = 6–8$ mice/treatment).

3.2.3. Mechanistic studies

An attempt to determine the mechanism behind the observed effect (potentiation of clonidine antinociception by MD-354) in the tail-flick assay was undertaken. Neither

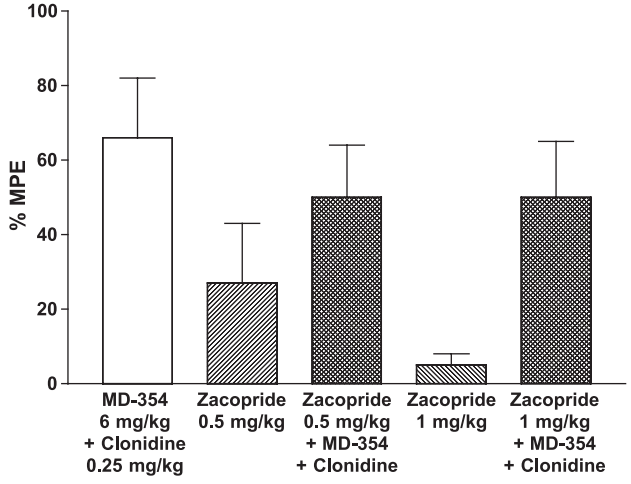

Fig. 8. Effect (± S.E.M.) of zacopride on the antinociceptive actions of a combination of clonidine (0.25 mg/kg) and MD-354 (6 mg/kg) in the tail-flick assay ($n = 6–7$ mice/treatment).

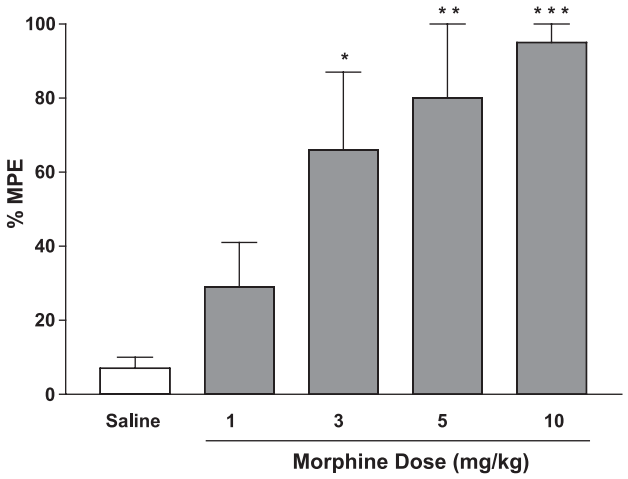

Fig. 9. Antinociceptive actions (± S.E.M.) of morphine in the tail-flick assay ($n = 5–11$ mice/treatment). Asterisks denote significant differences compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; one way ANOVA ($F_{4,28} = 7.13$) followed by Newman–Keuls post hoc test.

imiloxan, a purportedly selective α_{2B}-adrenoceptor antagonist (6.0 and 10 mg/kg i.p.; 13% and 4% MPE, respectively) nor the 5-HT_3 receptor antagonist zacopride (0.5 and 1.0 mg/kg i.p.; 27% and 5% MPE, respectively) produced a statistically significant antinociceptive effect in the tail-flick assay when administered alone. Pretreatment with imiloxan (6.0 and 10 mg/kg i.p.) or zacopride (0.5 and 1.0 mg/kg i.p.) failed to significantly attenuate the increase (MPE = 66%) in the tail-flick latency produced by s.c. administration of MD-354 (6.0 mg/kg) in combination with clonidine (0.25 mg/kg; Figs. 7 and 8).

In the tail-flick assay, 10 mg/kg of morphine produced 95% MPE (Fig. 9) when administered via the s.c. route. Lower doses produced less antinociception (Fig. 9). The potency of morphine in the tail-flick assay ($ED_{50} = 2.0$ mg/kg; 95% CL = 1.2–3.2 mg/kg) was consistent with that previously reported in the literature (Narita et al., 2002). In a combination study, doses of 6.0, 10, and 30 mg/kg of

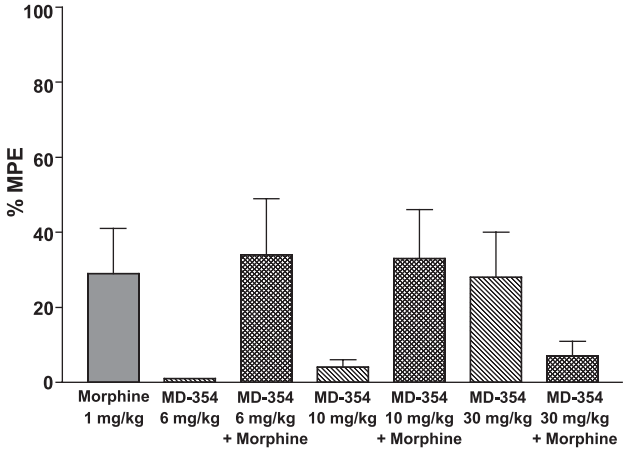

Fig. 10. Antinociceptive actions (± S.E.M.) of morphine/MD-354 combination in the tail-flick assay ($n = 5–11$ mice/treatment).

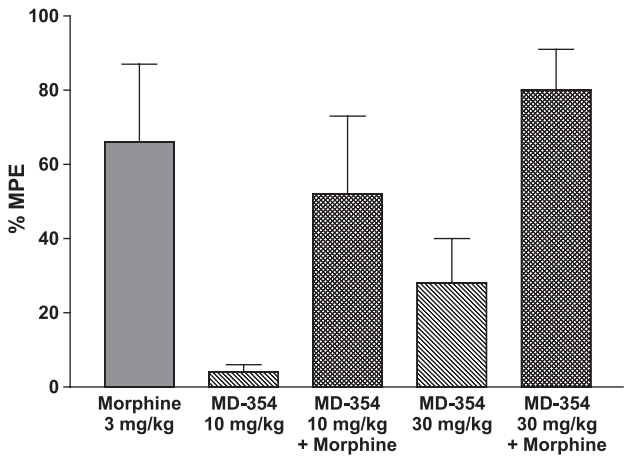

Fig. 11. Antinociceptive actions (± S.E.M.) of morphine/MD-354 combination in the tail-flick assay ($n = 5–11$ mice/treatment).

MD-354 failed to potentiate the antinociceptive action of 1.0 mg/kg (29% MPE) of morphine (Fig. 10). Similarly, no significant increase in antinociceptive effect was observed when a higher dose of morphine (3.0 mg/kg, 66% MPE) was administered in combination with 10 and 30 mg/kg of MD-354 (Fig. 11).

3.3. Spontaneous activity

Subcutaneous administration of 6.0 mg/kg of MD-354 after 15, 30, and 45 min produced saline-like effects in all three measurements. For example, after 45 min the measure of (i) *total movements* was recorded as 384.6 ± 18.8 and 365.9 ± 8.2 for saline and MD-354, respectively; (ii) *total movement time* in seconds was 1914.0 ± 99.3 for saline and 2016.6 ± 30.4 for MD-354; (iii) *total movement distance* in centimeters was 8304 ± 1309 and 9033 ± 667 for saline and MD-354, respectively.

4. Discussion

Because MD-354 binds in a relatively selective fashion to 5-HT_3 receptors and α_{2B}-adrenoceptors, and because both receptor populations have been implicated as playing a role in pain modulation, the antinociceptive character of MD-354 was examined. Clonidine, a nonselective α_2-adrenoceptor agonist, produces antinociception in thermal tests in animals and served as a positive control. We examined clonidine ($ED_{50} = 0.5$ mg/kg) in the tail-flick assay in mice and obtained results (Fig. 1) consistent with that reported in the literature (Kameyama et al., 1986). Interestingly, MD-354 was without antinociceptive actions at the doses evaluated (e.g., Fig. 2). One possible explanation for the lack of activity is that MD-354 is an α_{2A}-adrenoceptor antagonist. However, MD-354 failed to antagonize the antinociceptive effect produced by the ED_{50} dose of clonidine. A combination of the ED_{50} dose of

clonidine (0.5 mg/kg, MPE = 59%) with an "inactive" dose of MD-354 (10 mg/kg, MPE = 8%) seemed to enhance the antinociceptive effect in mice (MPE = 74%), and the observed effect was comparable to the antinociceptive effect produced by 1.0 mg/kg of clonidine alone (MPE= 76%). Because at its ED_{50} dose clonidine still produces a significant antinociceptive effect, it was of interest to examine the combination using a lower dose of clonidine. A relatively inactive dose of clonidine (0.25 mg/kg, MPE = 13%) in combination with MD-354 (10 mg/kg, MPE = 8%) produced a significant antinociceptive effect in mice (MPE = 83%; Fig. 4). Hence, pretreatment of animals with MD-354 apparently enhanced the antinociceptive character of clonidine. The action is not simply additive because the effect of certain dose combinations (e.g., 0.25 mg/kg of clonidine plus 6.0 or 10 mg/kg of MD-354) is greater than the sum of the two treatments when the agents were examined alone. Furthermore, it is unlikely that the action of MD-354 is attributable to a general central depressant effect, because MD-354 failed to influence locomotor activity as determined in the spontaneous motor assay.

In the hot-plate assay, clonidine was used as a positive control as well. Effectiveness of clonidine was examined in the hot-plate test (ED_{50} = 0.8 mg/kg; Fig. 3), and the results replicate that in the literature (Capasso and Loizzo, 2001). Analogous to the tail-flick studies, MD-354 (doses up to 30 mg/kg) was ineffective (8–26% MPE) in the hot-plate paradigm. Moreover, MD-354 failed to antagonize the effect of the ED_{50} dose of clonidine. A combination of MD-354 (30 mg/kg) with the ED_{50} and higher doses of clonidine slightly attenuated the effect of clonidine (Fig. 5). In contrast, combination of different doses (10 and 30 mg/kg) of MD-354 with a lower dose of clonidine (0.25 mg/kg) slightly enhanced the antinociceptive effect (Fig. 6). Neither effect was statistically significant. The action of clonidine in combination with MD-354 in the two assays was different; there are several possible explanations. The tail-flick assay might involve different receptor subpopulations or a different receptor mechanism (or combinations thereof) than the hot-plate assay (e.g., spinal versus supraspinal receptors, reflexive responses versus behavioral responses). Differences might also be related to the low density of α_{2B}-adrenoceptors present in the brain (MacDonald et al., 1997), and access to pertinent receptors might vary; for example, MD-354 is not a very lipophilic agent and distributional factors could be involved. MD-354 has an experimentally determined Log *P* value of −0.64 (Rahman et al., 2003), which suggests that it might have difficulty penetrating the blood–brain barrier. In addition, strain differences have been previously shown to account for nonparallel actions of drugs in the two assay systems (Graham et al., 1997).

How can the observed potentiation of clonidine's effects by MD-354 in the tail-flick assay be explained? And, does the effect involve a 5-HT_3 and/or α_{2B}-adrenergic component? The potentiation of clonidine antinociception by MD-354 was not antagonized either by the 5-HT_3 antagonist zacopride (doses of up to 1.0 mg/kg, i.p.) nor by the supposedly α_{2B}-selective antagonist imiloxan (at 3.0 mg/kg, s.c. at doses of up to 10 mg/kg, i.p.). It would seem unlikely, then, that the effect involves either a 5-HT_3 or α_{2B}-adrenergic mechanism. Because MD-354 binds at α_{2C}-adrenoceptors, and with lower affinity at α_{2A}-adrenoceptors, involvement of these receptor subpopulations cannot be ruled out at this time. However, it might be noted that the selectivity of imiloxan as an α_{2B}-antagonist is not certain and has been questioned. For example, a report by Takano et al., (1992) indicated that the i.t. administered α_2-adrenoceptor agonist ST-91 acts predominantly at α_{2B}-adrenoceptors, and its antinociceptive effect (hot-plate in rats) was antagonized by imiloxan. Recently Graham et al. (1997) conducted isobolographic analysis (tail-flick and hot-plate in rats) and concluded that ST-91 produces its antinociceptive effect by acting at α_{2C}-adrenoceptors. Moreover, ST-91 showed very low affinity for α_{2A} and α_{2B}-adrenoceptors (Renouard et al., 1994). On the basis of these studies, it might be argued that imiloxan's selectivity as an α_{2B}-adrenoceptor antagonist is uncertain. Thus, further investigation of the mechanism underlying the antinociceptive actions of the clonidine/MD-354 combination are required.

Clonidine, a nonselective α_2-adrenoceptor agonist, has been shown to potentiate the antinociceptive effect of morphine (e.g., Spaulding et al., 1979; Fairbanks and Wilcox, 1999; Özdoğan et al., 2003). The exact adrenergic receptor subpopulation(s) responsible for this potentiation is (are) unknown. Hence, we wished to determine if MD-354 would similarly potentiate the effect of morphine (in the tail-flick assay) in a manner consistent with its potentiation of clonidine's antinociceptive effects. In addition, potentiation of the morphine effect by MD-354 could provide further evidence for which adrenoceptor subpopulation is involved if potentiation were to occur. As shown in Figs. 10 and 11, MD-354 failed to potentiate the antinociceptive actions of morphine. Thus, it is tempting to conclude that the potentiation of morphine by clonidine might not involve an α_{2B}-adrenoceptor mechanism.

In summary, MD-354 was found to lack antinociceptive action of its own in the mouse tail-flick and hot-plate assays. However, MD-354 was demonstrated to potentiate the antinociceptive effect of clonidine. Its underlying clonidine-potentiating mechanism remains to be determined and further studies will be focused in this area.

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