Antinociception, tolerance and withdrawal symptoms induced by 7-hydroxymitragynine, an alkaloid from the Thai medicinal herb *Mitragyna speciosa*

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Abstract

7-Hydroxymitragynine is a potent opioid analgesic alkaloid isolated from the Thai medicinal herb *Mitragyna speciosa*. In the present study, we investigated the opioid receptor subtype responsible for the analgesic effect of this compound. In addition, we tested whether development of tolerance, cross-tolerance to morphine and naloxone-induced withdrawal signs were observed in chronically 7-hydroxymitragynine-treated mice. Subcutaneous (s.c.) administration of 7-hydroxymitragynine produced a potent antinociceptive effect mainly through activation of μ-opioid receptors. Tolerance to the antinociceptive effect of 7-hydroxymitragynine developed as occurs to morphine. Cross-tolerance to morphine was evident in mice rendered tolerant to 7-hydroxymitragynine and vice versa. Naloxone-induced withdrawal signs were elicited equally in mice chronically treated with 7-hydroxymitragynine or morphine. 7-Hydroxymitragynine exhibited a potent antinociceptive effect based on activation of μ-opioid receptors and its morphine-like pharmacological character, but 7-hydroxymitragynine is structurally different from morphine. These interesting characters of 7-hydroxymitragynine promote further investigation of it as a novel lead compound for opioid studies.

Keywords: 7-Hydroxymitragynine; Morphine; μ-Opioid receptor; Antinociception; Tolerance; Withdrawal

Introduction

7-Hydroxy-7*H*-mitragynine (7-hydroxymitragynine, Fig. 1) is a minor constituent of *Mitragyna speciosa* (Ponglux et al., 1994), which has long been used in Thailand for its opium-like effect (Burkill, 1935) and its coca-like stimulant ability to combat fatigue and enhance tolerance to under a scoring sun (Grewal, 1932; Suwanlert, 1975). We previously compared the antinociceptive effect of *Mitragyna speciosa* and mitragynine, its major constituent (about 66% of crude base extract), in in vivo experiments, but the antinociceptive effect of mitragynine was less potent than that of the crude extract of *Mitragyna speciosa* (Watanabe et al., 1992, 1999). This finding means that minor constituents of *Mitragyna speciosa* may have a very potent antinociceptive effect. Therefore, we investigated the opioid effects of 7-hydroxymitragynine (Matsumoto et al., 2004).

In guinea-pig ileum, 7-hydroxymitragynine inhibited electrically induced contraction through the opioid receptors, and its effect was about 13-fold more potent than morphine. Receptor-binding assays revealed that 7-hydroxymitragynine has a higher affinity for μ-opioid receptors than for the other opioid receptors (Takayama et al., 2002; Matsumoto et al., 2004). 7-Hydroxymitragynine induces potent antinociceptive effects in mouse tail-flick and hot-plate tests when subcutaneously or orally administered. It is noteworthy that the antinociceptive effect of 7-hydroxymitragynine is more potent

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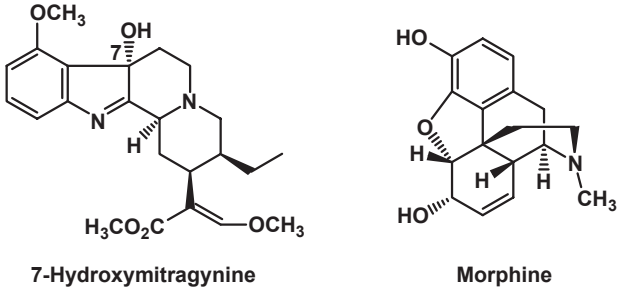

Fig. 1. Chemical structures of 7-hydroxymitragynine and morphine.

than that of morphine in both tests (Matsumoto et al., 2004). However, the opioid receptor selectivity profile of 7-hydroxymitragynine in in vivo experiments has not yet been determined. In the present study, we investigated the selectivity of opioid receptors involved in the 7-hydroxymitragynine-induced antinociception by using selective opioid receptor antagonists.

It is well known that chronic administration of opiates such as morphine leads to the development of tolerance and dependence (Pasternak, 1993). Suwanlert (1975) reported that the chronic exposure to *Mitragyna speciosa* elicits withdrawal symptoms in humans. However, pharmacological studies on the possible side effects of mitragynine-related compounds have been lacking (Jansen and Prast, 1988). In the present study, we tried to clarify the opiate properties of 7-hydroxymitragynine, including the side effects. We studied development of tolerance, cross-tolerance to morphine, and naloxone-precipitated withdrawal signs in mice chronically treated with 7-hydroxymitragynine.

Methods

Animals

Male *ddY* mice (25–39 g) purchased from Japan SLC Inc. (Shizuoka, Japan) were used. Animals were housed with controlled temperature (24±2 °C) and a 12-h light/dark cycle (lighting on at 7:00 AM) for at least 1 week before the start experiment. Food and water were available ad libitum. The experiments were performed in compliance with "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society and the guidelines approved by the Ethical Committee on Animal Care and Animal Experimentation of the Graduate School of Pharmaceutical Sciences, Chiba University. This study was carried out in accordance with the guidelines of the Ethics Committee of the International Association for the Study of Pain (Zimmermann, 1983).

Tail-flick test

The method was adapted from that of D'Amour and Smith (1941). Mice respond to a focused heat stimulus by flicking or moving their tail from the path of the stimulus, thereby exposing a photocell located in the tail-flick analgesia meter (Ugo Basile Tail-flick Unit 7360, Ugo Basile, Comerio, Italy) immediately below the tail. The reaction time is automatically recorded. Prior to injection, the nociceptive threshold was measured three times, and the mean of the reaction times was used as the pre-drug latency for each mouse. A cut-off time of 10 s was used to prevent tissue damage. Antinociception was quantified as the maximum possible effect (MPE) using the following formula:

$$\mathrm{MPE}(\%)=\frac{\text{Post-drug latency}-\text{Pre-drug latency}}{\text{Cut-off time}-\text{Pre-drug latency}}\times 100. \quad (1)$$

Development of tolerance

Morphine or 7-hydroxymitragynine tolerance was produced by twice daily injection of morphine (10 mg/kg, s.c.) or 7-hydroxymitragynine (10 mg/kg, s.c.) for 5 consecutive days. The effect of an agonist was measured daily 30 min after the first administration. The development of tolerance was defined as a significant reduction of the analgesic effect of the agonist compared with the effect produced by the first treatment.

Determination of cross-tolerance

Animals were pretreated with morphine (10 mg/kg, s.c.), 7-hydroxymitragynine (10 mg/kg, s.c.) or vehicle (10 ml/kg, s.c.) by administration twice per day for the first 5 days. On day 6, animals tolerant to morphine, 7-hydroxymitragynine or non-tolerant (i.e., treated with vehicle for 5 days) received morphine (10 mg/kg, s.c.) or 7-hydroxymitragynine (10 mg/kg, s.c.), and the antinociceptive effects were evaluated 30 min later by the tail-flick test.

Naloxone-induced withdrawal

Morphine or 7-hydroxymitragynine was injected s.c. daily at 9:00 A.M. and 7:00 P.M. according to the schedule reported previously (Suzuki et al., 1995; Kamei and Ohsawa, 1997; Tsuji et al., 2000). The dose of morphine or 7-hydroxymitagynine was progressively increased from 8 to 45 mg/kg over a period of 5 days. The doses of morphine or 7-hydroxymitragnine (mg/kg) injected at 9:00 A.M. and 7:00 P.M. were: 1st day (8, 15), 2nd day (20, 25), 3rd day (30, 35), 4th day (40, 45), 5th day (45 at 9:00 A.M. only), respectively. Withdrawal signs were precipitated by injecting naloxone (3 mg/kg, s.c.) 2 hr after the final morphine or 7-hydroxymitragnine administration. After the naloxone challenge, mice were immediately placed on a circular platform (30 cm in diameter × 70 cm height). Naloxone-precipitated signs were recorded for 60 min.

Molecular modeling

Morphine and 7-hydroxymitragynine were subjected to energy minimization using the semiempirical quantum

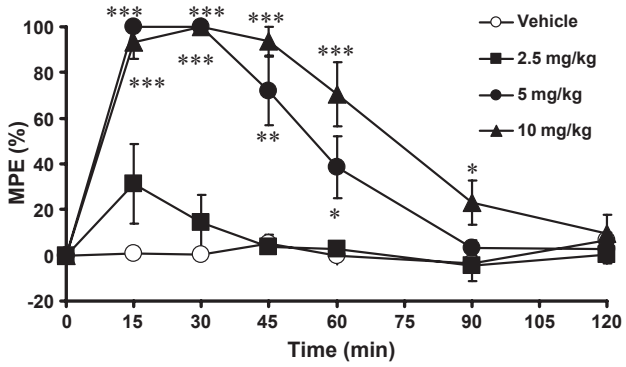

Fig. 2. Antinociceptive effects of 7-hydroxymitragynine in mouse tail-flick test. 7-Hydroxymitragynine was administered subcutaneously. Each point represents the mean ± S.E.M. of six mice. * $P<0.05$; ** $P<0.01$; *** $P<0.001$, versus the vehicle group.

mechanisms method AM1 as implemented in the MOPAC 5.0 programs. The superimposed ensemble of morphine/7-hydroxymitragynine was subjected to the overlay program implemented in Chem 3D 6.0.

Drugs

The drugs used in this study were morphine hydrochloride (Takeda Chemical Industries, Osaka, Japan), naloxone hydrochloride, cyprodime hydrobromide, naltrindole hydrochloride, nor-binaltorphimine dihydrochloride (Sigma Chemical Co., St. Louis, USA). 7-Hydroxymitragynine was synthesized as described previously (Ponglux et al., 1994; Takayama et al., 2002). The purity (>99%) of these compounds was checked by HPLC and ^{1}H NMR (500 MHz) analysis (Takayama et al., 2002). Morphine, cyprodime and 7-hydroxymitragynine were first dissolved in 100% dimethylsulfoxide and then subsequently diluted with 0.5% carboxyl methylcellulose. The final concentration of dimethylsulfoxide was 4.8%. The vehicle did not affect the pain response in the tail-flick test (data not shown). Other drugs were dissolved in saline. All drug solutions were prepared just before the experiments. The solution was injected in a volume of 10 ml/kg body weight.

Naloxone (2 mg/kg, s.c.), cyprodime (3 mg/kg, s.c.) or naltrindole (3 mg/kg, s.c) was administered 15 min before morphine or 7-hydroxymitragynine administration. Nor-binaltorphimine (20 mg/kg, s.c.) was administered 3 h before morphine or 7-hydroxymitragynine administration. The dose and schedule for each opioid antagonist in this study were determined as described previously (Kamei et al., 1994; Santos et al., 1999).

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with a two-tailed Student's *t*-test for comparison of two groups, and by a one-way analysis of variance followed by a Bonferroni multiple comparison test for comparison of more than two groups. A P value <0.05 was considered statistically significant.

Results

Time course and dose–response curve of the tail-flick inhibition induced by s.c. administration of 7-hydroxymitragynine

Subcutaneous injection of 7-hydroxymitragynine at 2.5, 5 and 10 mg/kg induced a dose-dependent antinociception in the tail-flick response. The tail-flick inhibition developed in 15 min, reached its maximum 15–30 min after injection, declined slowly and had returned to the pre-injection level at 120 min after injection (Fig. 2).

Effects of naloxone, cyprodime, naltrindole and nor-binaltorphimine on inhibition of the tail-flick response induced by 7-hydroxymitragynine or morphine

In order to characterize the antinociceptive activities of 7-hydroxymitragynine, mice were pretreated with selective antagonists (Fig. 3). Pretreatment with the non-selective antagonist naloxone (2 mg/kg, s.c.) fully inhibited antinociception induced by 7-hydroxymitragynine or morphine. Similar results were observed with the μ-selective antagonist cyprodime (3 mg/kg, s.c.). The δ-selective antagonist naltrindole (3 mg/kg, s.c) did not affect 7-hydroxymitagynine-induced antinociception, but partial inhibition was observed in the morphine-induced one. The κ-selective antagonist nor-binaltorphimine

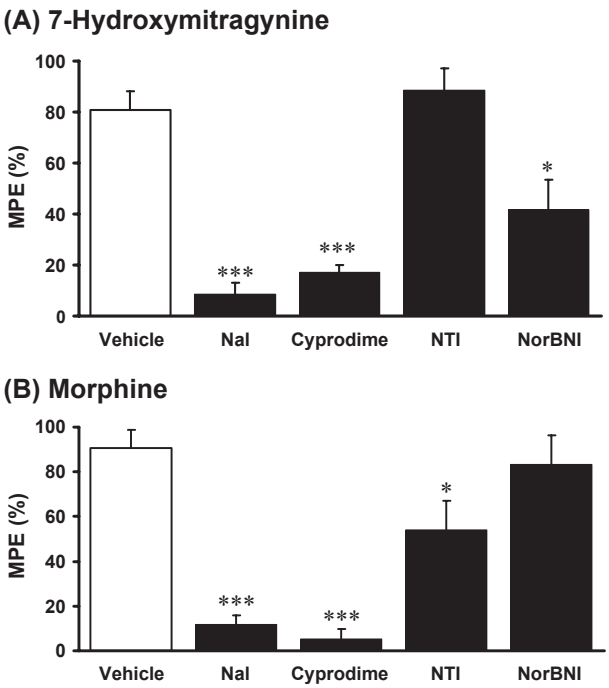

Fig. 3. Effects of naloxone (Nal), cyprodime, naltrindole (NTI) and nor-binaltorphimine (NorBNI) on (A) 7-hydroxymitragynine (5 mg/kg, s.c.)- and (B) morphine (10 mg/kg, s.c.)-induced antinociception in the mouse tail-flick test. Naloxone (2 mg/kg), cyprodime (3 mg/kg) or naltrindole (3 mg/kg) was subcutaneously administered 15 min before the tail-flick test. Nor-binaltorphimine (20 mg/kg, s.c.) was administered 3 h before the test. Each column represents the mean ± S.E.M. of six mice. * $P<0.05$; *** $P<.001$, versus the corresponding control group.

(20 mg/kg, s.c.) inhibited 7-hydroxymitagynine-induced antinociception, but did not inhibit the morphine-induced one.

Development of tolerance following repeated s.c. administration of 7-hydroxymitragynine or morphine

Antinociceptive effects in mice treated for 5 days with repeated administration of 7-hydroxymitragynine (10 mg/kg, s.c., twice daily) or morphine (10 mg/kg, s.c., twice daily), are shown in Fig. 4. No difference was found between the morphine-treated group and the control group on day 5 (Fig. 4), showing the development of tolerance to morphine. The repeated administration of 7-hydroxymitagynine produced a development of tolerance similar to that of morphine.

Development of cross-tolerance

The animals pretreated with 7-hydroxymitragynine (10 mg/kg, s.c., twice daily for 5 days) exhibited significant and complete tolerance to the antinociceptive effects induced by 7-hydroxymitragynine or morphine (open columns) when compared with the animals pretreated with vehicle (solid columns) (Fig. 5A). As was seen in the 7-hydroxymitragynine-pretreated group, the animals pretreated with morphine (10 mg/kg, s.c., twice daily for 5 days) showed tolerance to morphine and cross-tolerance to 7-hydroxymitragynine (Fig. 5B).

Naloxone-induced withdrawal signs following chronic treatment with 7-hydroxymitragynine or morphine

Morphine-dependent mice, which were chronically treated with morphine, showed withdrawal signs such as jumping, rearing, urination, ptosis, forepaw tremor and diarrhea after naloxone (3 mg/kg, s.c.) was administered. 7-Hydroxymitragynine-dependent mice, which were chronically treated with 7-hydroxymitragynine, also showed fewer but significant withdrawal signs after naloxone injection (3 mg/kg, s.c.), compared with the group of morphine-dependent mice (Table 1).

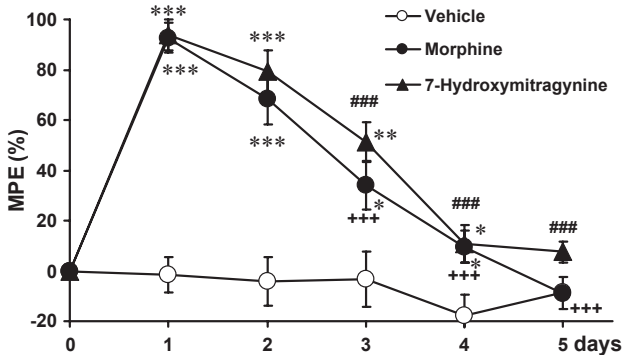

Fig. 4. Development of tolerance to the antinociceptive activities of morphine (10 mg/kg, s.c.) and 7-hydroxymitragynine (10 mg/kg, s.c.) in mouse tail-flick test. Each point represents the mean±S.E.M. of eight mice. * $P<0.05$; ** $P<0.01$; *** $P<0.001$, versus the vehicle group. ### $P<.001$, versus the antinociceptive activities on the first day of 7-hydroxymitragynine administration. +++ $P<0.001$, versus the antinociceptive activities on the first day of morphine.

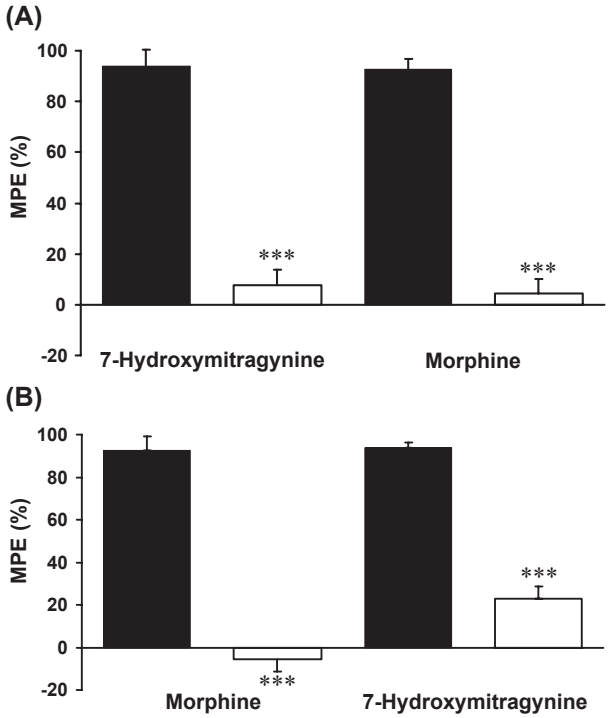

Fig. 5. Cross-tolerance between morphine and 7-hydroxymitragynine. Groups of eight mice received (A) 7-hydroxymitragynine (10 mg/kg, s.c.) or vehicle and (B) morphine (10 mg/kg, s.c.) or vehicle twice daily for 5 days. On day six, morphine (10 mg/kg, s.c.) or 7-hydroxymitragynine (10 mg/kg, s.c.) was administered to mice. Open columns, vehicle; solid columns, agonists. Each column represents the mean±S.E.M. of eight mice. *** $P<0.001$, versus the corresponding control group.

Computational superposition of morphine and 7-hydroxymitragynine

We explored the structural similarity between morphine and 7-hydroxymitragynine using molecular modeling techniques (Fig. 6). At the outset, we examined the respective superimpositions of the nitrogen atom, benzene ring and oxygen function on the benzene ring in morphine and 7-hydroxymitragynine. Not all functional groups of the two molecules were superimposed (Fig. 6).

Discussion

We previously surveyed constituents of *Mitragyna speciosa* and found a new compound, 7-hydroxymitragynine, as a minor

Table 1
Naloxone (3 mg/kg, s.c.)-precipitated withdrawal responses in morphine- and 7-hydroxymitragynine-dependent mice

Withdrawal signs	Positive mice/total mice		
	Vehicle	Morphine	7-Hydroxymitragynine
Jumping	0/7	6/8	5/7
Rearing	0/7	8/8	4/7
Urination	0/7	8/8	6/7
Ptosis	0/7	5/8	2/7
Forepaw tremor	3/7	6/8	5/7
Diarrhea	0/7	3/8	1/7

Each value represents the number of positive animals/the total number of animals.

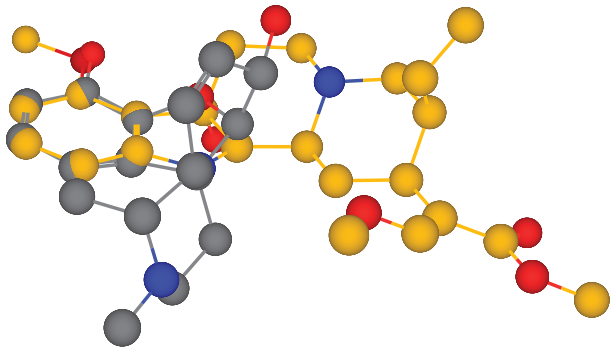

Fig. 6. Overlay of the low-energy conformation of 7-hydromitragynine (yellow) and morphine (gray). Hydrogen atoms are omitted. Red and blue balls represent oxygen and nitrogen atoms, respectively.

constituent of this plant (Ponglux et al., 1994; Takayama et al., 2002). 7-Hydroxymitragynine exhibited potent opioid activities in in vitro and in vivo assays (Takayama et al., 2002; Matsumoto et al., 2004). In the present study, we investigated the properties of 7-hydroxymitragynine on opioid receptor selectivity, development of tolerance, cross-tolerance to morphine and naloxone-induced withdrawal symptoms in mice.

7-Hydroxymitragynine-induced antinociception

Antinociceptive activities of subcutaneously injected 7-hydroxymitragynine were characterized by selective antagonists of opioid receptors. The μ-opioid selective antagonist cyprodime significantly antagonized and the κ-opioid selective antagonist nor-binaltorphimine inhibited the 7-hydroxymitragynine-induced antinociception, but the δ-selective antagonist naltrindole did not. Based on functional in vitro binding and in vitro bioassays, the compound appeared to act predominantly at μ-opioid receptors (Takayama et al., 2002; Matsumoto et al., 2004). The present result that cyprodime significantly antagonized the antinociceptive effect of 7-hydroxymitragynine confirmed its stimulatory effect on μ-opioid receptors in vivo. These results suggest that the antinociceptive effect of 7-hydroxymitragynine is produced mainly by the activation of μ-opioid receptors and partially by κ-opioid receptors.

Evaluation of tolerance and cross-tolerance

Repeated exposure to opioid drugs such as morphine leads to the development of tolerance. The study of cross-tolerance is a valuable method to define common mechanisms of opioid activities. In this study, the development of tolerance and cross-tolerance to 7-hydroxymitragynine and morphine following repeated administration of 7-hydroxymitragynine was compared with the morphine-pretreated group. Repeated administration of 7-hydroxymitragynine resulted in the development of tolerance to its antinociceptive effect. Animals rendered tolerant to 7-hydroxymitragynine clearly displayed cross-tolerance to morphine antinociception and vice versa. It is well known that morphine tolerance is based mainly on μ-opioid receptors (Pasternak, 2001). Furthermore, the antinociceptive effects of both 7-hydroxymoitragynine and morphine occur mainly through the activation of μ-opioid receptors in mouse tail-flick tests (Fig. 3). Taken together, the development of tolerance and antinociceptive effects of morphine and 7-hydroxymitragynine are supposed to be mediated through the stimulation of μ-opioid receptors.

Evaluation of physical dependence

As is generally accepted, the potent and repeated stimulation of μ-opioid receptor agonists leads to the development of physical dependence (Cowan et al., 1988; Matthes et al., 1996; Narita et al., 2001). Physical dependence following chronic treatment with 7-hydroxymitragynine was studied. Withdrawal signs were observed after naloxone injection, demonstrating that repeated administration of 7-hydroxymitragynine induces physical dependence. As described above, the antinociceptive effects of 7-hydroxymitragynine were mainly mediated by μ-opioid receptors in the mouse tail-flick test. Furthermore, the mice rendered tolerant to 7-hydroxymitragynine clearly displayed cross-tolerance to morphine antinociception in the tail-flick test (Fig. 5A). These results possibly show similarities between naloxone-precipitated withdrawal in morphine- and 7-hydroxymitragynine-dependent mice.

Structural similarity between 7-hydroxymitragynine and morphine

Next, we investigated structural similarities between morphine and 7-hydroxymitragynine using molecular modeling techniques. As shown in Fig. 6, we cannot superimpose all three functional groups, i.e., a nitrogen atom, a benzene residue and a oxygen function on the benzene ring in the structures of morphine and 7-hydroxymitragynine. These functional groups play a significant role in producing analgesic activity (Dhawan et al., 1996). Therefore, it is speculated that 7-hydroxymitragynine binds opioid receptor sites other than those morphine does.

Conclusion

7-Hydroxymitragynine, the Mitragyna alkaloid, induced potent antinociceptive effects mainly through μ-opioid receptors in the tail-flick test in mice. The development of tolerance in the antinociceptive effect of 7-hydroxymitragynine, the formation of bidirectional cross-tolerance and withdrawal signs suggested similar opioid mechanisms for morphine and 7-hydroxymitragynine. However, 7-hydroxymitragynine has a different chemical structure than the morphine skeleton. 7-Hydoxymitragynine may be a seed for novel analgesics because of its unique structure and strong potency.

Acknowledgments

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