Pharmacological Studies on 7-Hydroxymitragynine, Isolated from the Thai Herbal Medicine *Mitragyna speciosa*: Discovery of an Orally Active Opioid Analgesic

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2006

Contents

General Remarks

Substances derived from natural products have been utilized since the beginning of time for various medical purposes including the treatment of pain. Opium, for example, has been mentioned in the earliest historical records, some 7000 years ago. In fact, research in the area of pain management and drug addiction originally focused on natural products exclusively. Prototypical examples of such natural products are the opium poppy (*Papaver somniferum*). Morphine, an alkaloid component of the opium poppy, is the most widely used compound among narcotic analgesics and remains the gold standard. Recently, analogs have been produced from natural substances, and completely synthetic compounds based on natural pharmacophores have been introduced to the market. However, the research and medical fields still struggle with the undesirable side-effects of these analgesic substances (McCurdy and Scully 2005).

Our research group has studied uniquely structured, nitrogen-containing compounds isolated from the traditional Thai herb *Mitragyna speciosa*. This herb has long been used in tropical areas for its opium- and coca-like effects (Burkill, 1935). It has been used also as a substitute for opium and to wean addicts off morphine (Grewal, 1932; Suwanlert, 1975). We have been investigating the pharmacological properties of this herb, individual components of its extracts, and structurally related compounds since the 1980s. We compared the antinociceptive effects of *Mitragyna speciosa* and mitragynine, the major alkaloid of this herb, in *in vivo* experiments, and found that 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 one or more minor constituents of *Mitragyna speciosa* may have a very potent antinociceptive effect. We have investigated mitragynine-related compounds that express interesting opioid activities: an oxidative derivative of mitragynine, mitragynine pseudoindoxyl, was found to exhibit potent opioid agonistic activity *in vitro* and antinociceptive activity *in vivo* (Yamamoto et al., 1999; Takayama et al., 2002). These findings prompted us to embark on the development of novel compounds based on the mitragynine skeleton, which is quite different from the skeleton of morphine.

In the present study attempting to find a new analgesic from the Thai herbal medicine, I surveyed the opioid effects of the other constituents of *Mitragyna speciosa* and synthetic derivatives of

mitragynine by the Magnus method in isolated smooth muscle preparations. Among them, I found a novel alkaloid, 7-hydroxymitragynine, a minor constituent of *Mitragyna speciosa*, and investigated the involvement of opioid receptor subtypes by *in vitro* assays. Furthermore, I investigated the antinociceptive and side effects of 7-hydroxymitragynine *in vivo* and compared them to the effects of morphine to evaluate the clinical utility of 7-hydroxymitragynine.

1. Historical overview of *Mitragyna speciosa*

Mitragyna speciosa Korth has been used for many years in Thailand, Malaysia, Borneo, the Philippines, and New Guinea; the Thai and Malay natives use it as a substitute for opium. It is called "kratom" by the natives of Thailand and "biak-biak" in Malaysia. Natives used the leaves of the plant in fresh or dried forms, and they also prepared syrup by evaporating a solution made from dried leaves. The leaves were chewed, or the syrup was drunk after dissolving it in hot water, or even smoked in a way similar to opium. Besides the use of leaves of *Mitragyna speciosa* as a substitute for opium, other uses are a cure for fever, treatment for diarrhea, and a cure for opioid withdrawal syndrome (Burkill, 1935). Furthermore, Suwanlert (1975) reported the use of *Mitragyna speciosa* as a stimulant in Thailand by market gardeners, peasants, and laborers to overcome the burden of hard work as well increasing work efficiency under a scoring sun. His study on *Mitragyna speciosa* users in Thailand describes the stimulant effect and strong desire to work induced by the plant as leading to its regular use, which progresses to addiction. In these addicts, symptoms such as anorexia, weight loss, stomach distention, insomnia, darkening of the skin, constipation, and withdrawal syndrome were reported (Grewal, 1932; Suwanlert, 1975).

In contrast, there are reports that *Mitragyna speciosa* use causes much less aggressiveness and hostility than opium smoking, that is, the absence of adverse physical conditions and character changes (Jansen and Prast, 1988). *Mitragyna speciosa* has a psychostimulant effect like coca and a depressive effect like opium and cannabis, which seem to be contradictory. It is also reported that it is weaker than morphine, has a milder withdrawal syndrome compared to opioids, and is less harmful

than cocaine. Although the medical use of *Mitragyna speciosa* to treat opium addicts in Thailand has been documented (Jansen and Prast, 1988; Burkill and Haniff, 1930), its use has been prohibited by Thai law since 1943 because of its narcotic effects. However, this law is not effective because the tree of *Mitragyna speciosa* is indigenous to the country. The fact is that the herb is not under any control in many other countries and is readily available on the Internet for purchase by anyone (McCurdy and Scully 2005).

Chewing the leaves of *Mitragyna speciosa*

Mitragyna speciosa (kratom) leaves

2. Pharmacology of mitragynine and its metabolite

Mitragynine (Figure 1) is the major alkaloid of *Mitragyna speciosa*, and for this reason mitragynine was assumed to be the major chemical responsible for the effects of this herb. Hooper (1907) was the first person to isolate mitragynine, and this was repeated by Field (1921). Its structure was first fully determined by Zacharias et al. (1964). In the 1960s, the Chelsea group in the U.K. reported the isolation of several indole alkaloids from the leaves of *Mitragyna speciosa* from Thailand (Beckett et al., 1965, 1966a, b). Almost ten years later, Shellard et al. (1974) isolated more than twenty kinds of Corynanthe-type alkaloids, including oxindole derivatives, in their investigation of the alkaloid constituents in various samples of *Mitragyna speciosa* from Thailand. They pointed out that the variation in the constituents among different batches of leaves may be an indication of the presence of geographical variants of the species within Thailand (Shellard, 1974).

The pharmacology of *Mitragyna speciosa* and mitragynine was first explored by K. S. Grewal at the University of Cambridge in 1932. He performed a series of experiments on animal tissues and a group of five male volunteers. He described mitragynine as having a central nervous system stimulant effect resembling that of cocaine. Macko et al. (1972) reported that mitragynine exhibited antinociceptive and antitussive actions in mice comparable those of codeine. Their findings were that, unlike opioid analgesics at equivalent doses, mitragynine did not possess the side effects common to opioids. Moreover, the absence of an antagonistic effect of nalorphine on mitragynine-induced antinociception led them postulate noninvolvement of the opioid system in the action of mitragynine.

We have studied the pharmacological effects of mitragynine on guinea-pig ileum, mouse vas deferens, radioligand binding, and the tail-flick test in mice, and found that mitragynine acts on opioid receptors and possesses antinociceptive effects (Watanabe et al., 1997; Yamamoto et al., 1999; Takayama et al., 2002). But the effect of mitragynine was less potent than that of morphine. Some pharmacological investigations of mitragynine have also revealed that it has an antinociceptive action through the supraspinal opioid receptors, and that its action is dominantly mediated by μ- and δ-opioid receptors in *in vivo* and *in vitro* studies (Matsumoto et al., 1996a, b; Tohda et al., 1997; Thongpradichote et al., 1998).

Another alkaloid of interest is mitragynine pseudoindoxyl (Figure 1), which was at first isolated as a metabolite of mitragynine by microbial biotransformation. Macko et al. (1972) reported that oral administration of mitragynine was more effective than subcutaneous administration. This finding suggested that the antinociceptive effect of mitragynine exists predominantly in its derivatives. Our previous study demonstrated a potent opioid agonistic property of compound mitragynine pseudoindoxyl in *in vitro* experiments (Yamamoto et al., 1999). In guinea-pig ileum, mitragynine and mitragynine pseudoindoxyl inhibit the twitch contraction through opioid receptors. The effect of mitragynine pseudoindoxyl was 20 fold more potent than that of morphine. In mouse vas deferens, the effect of mitragynine pseudoindoxyl was 35 fold more potent than that of morphine. In spite of its potent opioid effect, mitragynine pseudoindoxyl induced only a weak antinociceptive effect in the mouse tail-flick test in comparison with morphine (Takayama et al., 2002).

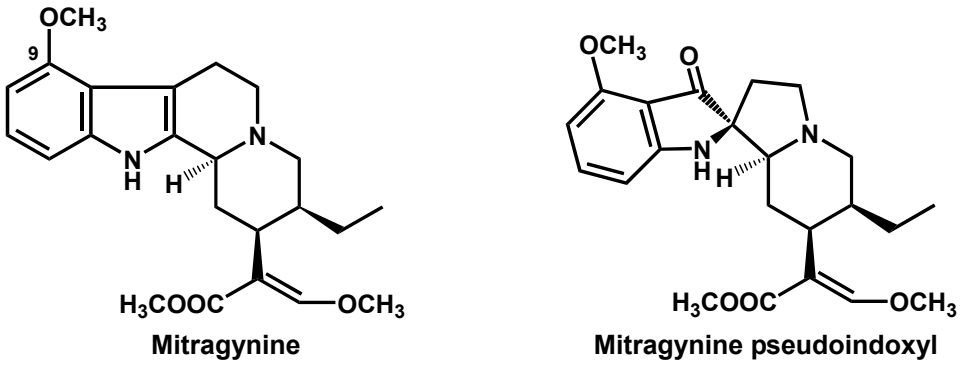


Figure 1 Chemical structures of mitragynine and mitragynine pseudoindoxyl

3. Opioid receptor and analgesics

Opioid is the common name for all compounds that have the same mechanism of action as the constituents of opium. All opioids interact with the endogenous opioid receptor system, which presently includes four known receptor subtypes (Dhawan et al., 1996) that are designated μ, δ, κ, and ORL-1 (opioid receptor-like receptor 1). These receptors are widely distributed in the mammalian system and have been found in all vertebrates. Their density is relatively high in the brain and spinal cord, but they are also found in the gastrointestinal system and the cells of the immune system.

Although all three major types of opioid receptors, μ, δ, and κ-opioid receptors, are able to mediate analgesia/antinociception, their individual binding profiles and other pharmacological activities clearly distinguish one from another. Highly selective ligands that allow for receptor-type labeling have become available, and are summarized in Table 1. Of the three major classes of opioid receptors the μ-opioid receptor has proven to be the major target of opioid analgesics. Morphine is the prototypical μ-opioid analgesic that serves as the standard drug against which all analgesics are compared in determining their relative analgesic potencies. It has been recommended as the drug of choice in the management of patients with chronic cancer pain by the World Health Organization Cancer Unit in its Cancer Pain Relief Program.

However, morphine also has undesirable effects, such as tolerance, withdrawal symptoms, constipation, respiratory depression, nausea, and vomiting. The majority of clinically available opioid

analgesics are μ-agonists derived from chemical templates that relate to the natural opium alkaloids, with progressive simplification through the morphinans to the benzomorphans and the piperidines, to the phenylpropylamines, e.g., fentanyl, pethidine, and methadone (Corbett et al., 2006).

The major goals of opioid research are to understand the underlying biology of the endogenous opioid systems, to discover new analgesic drugs devoid of the unwanted side effects associated with morphine, and to develop new therapies for the treatment of opioid addicts. To find the ideal opioid analgesic, thousands of analogues have been synthesized, but an ideal analgesic that has a powerful effect yet is free from undesirable side effects has not been found at this stage.

Table 1 Opioid receptor and ligand relationship

Currently accepted name	μ	δ	κ	ORL1
Currently IUPHAR name	OP3	OP1	OP2	OP4
Endogeneous ligands	β-Endorphin Endomorphin-1 Endomorphin-2	Enkephalins	Dynorphin	Nociceptin/Orphanin FQ
Exogeneous agonists	DAMGO Fentanyl Morphine	DPDPE (δ_1) DSLET (δ_2)	U69593 (κ_1) U50488 (κ_1)	Nociceptin/OrphaninFQ
Selective antagonists	β-FNA (μ_1, μ_2) CTOP (μ_1, μ_2) Cyprodime (μ_1, μ_2) Naloxonazine (μ_1)	Naltrindole (δ_1, δ_2) DALCE (δ_1) naltriben (δ_2)	norBNI (κ_1, κ_2)	
Non selective antagonists	Naloxone Naltrexone	Naloxone Naltrexone	Naloxone Naltrexone	
Radioligands of choice	[^{3}H]DAMGO [^{3}H]Naloxone	[^{3}H]Naltrindole [^{3}H]DPDPE	[^{3}H]U69593 [^{3}H]norBNI	[^{3}H] Nociceptin/Orphanin FQ

Abbreviations

norBNI, nor-Binaltorphimine; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Phe-Thr-NH_2; DALCE, [D-Ala^2,Leu^5,Cys^6]-Enkephalin; DAMGO, [D-Ala^2,N-Me-Phe4,Gly-ol5]-Enkephalin; DPDPE: [D-Pen2,5]-Enkephalin; DSLET, [D-Ser2,Leu5,Thr^6]-Enkephalin; Endomorphin 1, Tyr-Pro-Trp-Phe-NH_2; Endomorphin 2, Tyr-Pro-Phe-Phe-NH_2; β-FNA, β-Funaltrexamine; U69593, (+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl)benzeneacetamide; U50488, 3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide

Part I. Exploration of compounds acting on opioid receptors in components of *Mitragyna speciosa* and its related synthetic alkaloids

1. Introduction

Mitragyna speciosa (called kratom in Thailand) has been used in Thailand for its opium- and coca-like effects. Additionally, it has been used to treat diarrhea and to wean addicts off morphine (Jansen and Prast, 1988). This medicinal herb contains many indole alkaloids (Houghton et al., 1991; Takayama et al., 1999, 2000). Mitragynine (Figure 1) is a main constituent of the leaves of *Mitragyna speciosa* (Takayama et al., 2000). Recently, we found that mitragynine acts on μ-opioid receptors in guinea-pig ileum (Watanabe et al., 1997; Yamamoto et al, 1999). In addition, some pharmacological studies have also revealed that mitragynine has an antinociceptive action through the supraspinal μ- and δ-opioid receptors (Matsumoto et al., 1996a, b; Tohda et al., 1997; Thongpradichote et al., 1998). The antinociceptive effect of mitragynine, however, is less potent than that of the crude extract of this plant (Watanabe et al., 1999). That is, the opium-like effect of *Mitragyna speciosa* cannot be fully explained by that of mitragynine. This finding suggests that minor constituents of *Mitragyna speciosa* have a very potent antinociceptive effect. However, this plant has not so far been investigated systematically for isolation of opioid agonistic constituents. In the present chapter, we fractionated the crude extract of *Mitragyna speciosa*, and explored active constituents that have opioid agonistic activities using an *in vitro* guinea-pig ileal contraction test. Furthermore, we surveyed the opioid agonistic activities of semi-synthetic compounds derived from mitragynine in order to elucidate specific structure necessary for its pharmacophore binding on opioid receptors.

2. Materials and methods

Animals

All experiments were performed in compliance with the “Guiding Principles for the Care and

Use of Laboratory Animals" approved by the Japanese Pharmacological Society. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data, and animal discomfort was kept to the minimum. Male albino guinea pigs (320–540 g, Takasugi Lab. Animals, Japan) were killed by CO_2 inhalation.

Isolation of guinea-pig ileum

The guinea-pig ileum was dissected and placed in Krebs-Henseleit solution (mM): NaCl, 112.08; KCl, 5.90; $CaCl_2$, 1.97; $MgCl_2$, 1.18; NaH_2PO_4, 1.22; $NaHCO_3$, 25.00 and glucose, 11.49. The ileum was placed under 1 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37ºC and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a needle 5 mm in length) electrode. After 60 min equilibration in Krebs-Henseleit solution, the ileum was transmurally stimulated (Cox and Weinstock, 1966) with monophasic pulses (0.2 Hz and 0.1 ms duration) by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Contractions were isotonically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by transmural stimulation elicited through the ring electrodes were examined. At the start of each experiment, the maximum response to acetylcholine (3 μM) in each tissue was obtained to check its stability. The mean amplitude of the electrically-stimulated contraction was about 30% of the maximal response to acetylcholine (3 μM). The electrically-induced twitch contraction was almost abolished by tetrodotoxin (1 μM) and atropine (0.1 μM), as described previously (Watanabe et al., 1997). Thus, the electrical stimulation induced cholinergic contraction in guinea-pig ileum (Brookes et al., 1991). The height of the twitch response to transmural stimulation was measured before and after the drug challenge. Contraction (%) is expressed as a percentage of the twitch response to the transmural stimulation before the drug challenge.

Plant material

The leaves of *Mitragyna speciosa* were collected on the campus of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The plant was identified by Dr. Nijsiri Ruangrungsi, Faculty of Pharmaceutical Sciences, Chulalongkorn University. A voucher sample (#1991Dec-MS) was deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and isolation

Big, young leaves were powdered (165.5 g) and extracted five times with hot methanol. The solvent was concentrated under reduced pressure to give a crude extract (53.5 g), a part of which was dissolved in 10% aqueous acetic acid (AcOH). The insoluble material was removed by filtration through Celite to give the AcOH-insoluble fraction solution (AcOH-insoluble fraction, 50.3 g). The aqueous layer was basified with Na_2CO_3 at 0°C and extracted with chloroform ($CHCl_3$). The organic layer was washed with water, dried over $MgSO_4$, and then evaporated to give the crude base fraction (2.43 g). The aqueous layer was further extracted with n-Butanol (BuOH), which was concentrated under reduced pressure to yield the n-BuOH fraction (4.77 g). A part of the residual aqueous solution (10 ml) was lyophilized to give a hygroscopic solid (ca. 2 g), which was isolated with ethanol using a Soxhlet extractor in order to remove the inorganic materials. The ethanol extract was evaporated to give a residue containing the water-soluble organic materials (water-soluble fraction, 1.08 g).

The crude base fraction (2.0 g), which exhibited an opioid agonistic effect on the guinea-pig ileum, was purified by SiO_2 column chromatography (6 × 17 cm) using $CHCl_3$/ethyl acetate (AcOEt) (9:1, 370 ml; fraction A), $CHCl_3$/AcOEt (4:1, 240 ml; fraction B), $CHCl_3$/AcOEt (1:1, 320 ml; fraction C), AcOEt (80 ml; fraction D), MeOH/AcOEt (1:19, 120 ml; fraction E), MeOH/AcOEt (1:4, 160 ml; fraction F), MeOH/AcOEt (1:1, 80 ml; fraction G), and MeOH (150 ml; fraction H). The combined fractions C and D were further purified by SiO_2 column chromatography (3 × 17 cm) using an n-hexane/AcOEt (3:2, 1:1, 1:5, 30 ml each) gradient that afforded 24 fractions. Fractions 2–8 contained mitragynine (1343 mg, 66% based on the crude base, $[\alpha]_D^{24}$: –126° {c 1.2, $CHCl_3$}) and fractions 18–22 yielded 7-hydroxymitragynine (40 mg, 2% based on the crude base, $[\alpha]_D^{23}$: + 47.9° {c

0.55, $CHCl_3$}). From fraction E, paynantheine (178 mg, 8.9% based on the crude base, $[\alpha]_D^{25}$: + 29.4° (c 1.2, $CHCl_3$}) was obtained. Fraction F afforded speciogynine (132 mg, 6.6% based on the crude base, $[\alpha]_D^{24}$: + 26.8° (c 0.85, $CHCl_3$}). Fraction G was subjected to MPLC (SiO_2, 2.5 × 10 cm) with MeOH/CHCl3 (1:9, 3 mL/min) to provide speciociliatine (t_R: 18 min, 15 mg, 0.8% based on the crude base, $[\alpha]_D^{24}$: –10.5° (c 1.2, $CHCl_3$}). The isolated compounds were identified by direct comparison with the corresponding authentic samples. The purity (> 99%) of the above compounds was checked by HPLC and ^{1}H-NMR (500 MHz) analyses.

Chemistry

To investigate the structure-activity relationship, mitragynine was isolated from the extract of leaves of *Mitragyna speciosa*. Mitragynine-related indole alkaloids (Figure 2) were synthesized from mitragynine as described previously (Takayama et al., 2002, 2004). The purity (> 99%) of these compounds was checked by HPLC and ^{1}H-NMR (500 MHz) analysis (Takayama et al., 2002).

Drugs

The drugs used in this study were acetylcholine chloride (Dai-ichi Pharmaceutical Co., Tokyo, Japan), atropine sulfate (Nacalai Tesque Inc., Tokyo, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), morphine hydrochloride (Takeda Chemical Industries, Osaka, Japan), and naloxone hydrochloride (Sigma Chemical Co., St. Louis, USA). For bioassays, mitragynine-related alkaloids were first dissolved in 100% dimethylsulfoxide to yield a 10 mM solution and then subsequently diluted with distilled water. Other drugs were dissolved in distilled water.

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with two-tailed *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.

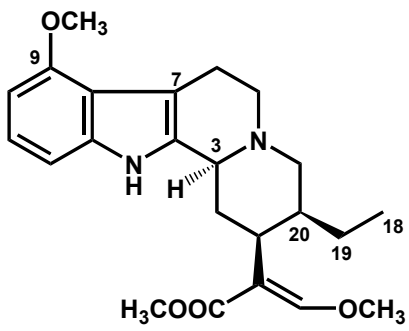
3. Results

Effect of crude extract on electrically-stimulated twitch contraction

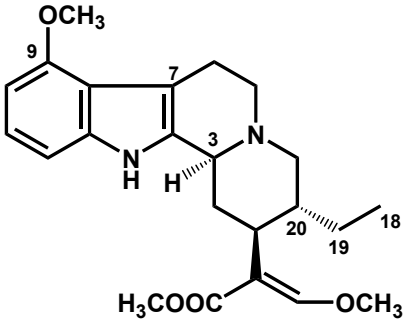
First, the opioid agonistic activity of the crude extract of *Mitragyna speciosa* was evaluated using the twitch contraction induced by electrical stimulation in guinea-pig ileum. The crude extract (1–300 μg/ml) inhibited the twitch contraction in a concentration-dependent manner (Table 1). The effect of the opioid receptor antagonist naloxone on the contraction inhibition was examined to verify that the extract acts on opioid receptors. The effect of the crude extract was reversed by naloxone (Table 2). Naloxone (30–300 nM) also inhibited the effect of morphine, but did not affect the effect of verapamil, an L-type Ca^{2+} channel blocker, on the twitch contraction (Table 2), suggesting that the antagonistic effect of naloxone is specific to the opioid receptors.

This crude extract was successively fractionated into crude base, n-BuOH, and water fractions. Among them, only the crude base extract was found to exhibit the inhibition of the twitch contraction (Table 1). The inhibitory effect was concentration-dependent (1–100 μg/ml). The AcOH-insoluble, n-BuOH and water-soluble fractions (10–300 μg/ml) showed hardly any effect on ileal twitch contraction.

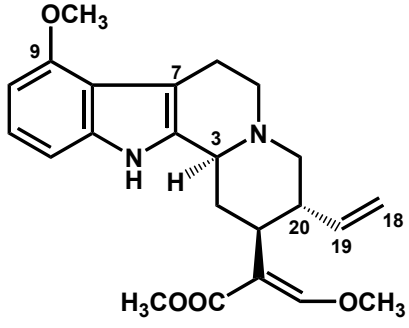
Silica gel column chromatography of the crude base fraction isolated five alkaloids: 7-hydroxymitragynine, mitragynine, speciogynine, speciociliatine, and paynantheine (Figure 1). Each alkaloid inhibited the electrically-induced twitch contraction in a concentration-dependent manner. Among them, 7-hydroxymitragynine showed the most potent effect on the ileal contraction (Table 1). The potency was 30 and 17 fold higher than that of mitragynine and morphine, respectively. Naloxone (30, 300 nM) restored the twitch contraction inhibited by 7-hydroxymitragynine (Table 2).



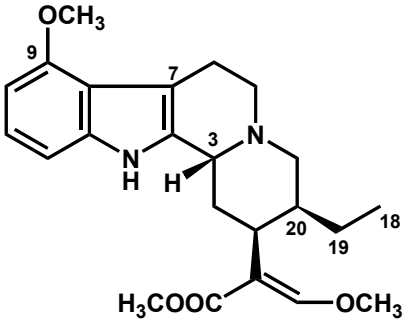
Mitragynine



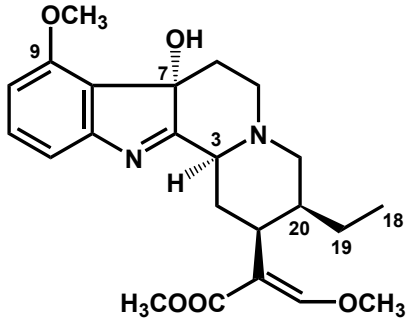
Speciogynine



Paynantheine



Speciocilliatine



7-Hydroxymitragynine

Figure 1 Chemical structures of constituents of *Mitragyna speciosa*.

Table 1 Opioid agonistic activities of extracts and constituents of *Mitragyna speciosa* in guinea-pig ileum preparation

Compound	pD_2 Value	Relative Potency (%)	Maximum Inhibition (%)	Relative Inhibitory Activity (%)
Morphine (positive control)	7.15 ± 0.05	100	87.2 ± 1.8 [c]	100
Crude extract	5.05 ± 0.24	0.8	42.3 ± 6.0 [b]	49
Crude base fraction	4.32 ± 0.15	0.1	72.5 ± 8.1 [c]	83
n-BuOH fraction	NE	NE	-11.3 ± 4.0 [a]	-13
AcOH-insoluble fraction	NE	NE	13.6 ± 8.9	16
Water-soluble fraction	NE	NE	-12.3 ± 7.3	-14
7-Hydroxymitragynine	8.38 ± 0.12	1698	86.3 ± 4.8 [c]	99
Mitragynine	6.91 ± 0.04	58	84.0 ± 2.0 [c]	96
Speciogynine	5.61 ± 0.06	3	75.1 ± 8.3 [c]	86
Paynantheine	4.99 ± 0.06	1	74.9 ± 5.0 [c]	86
Speciociliatine	5.55 ± 0.15	3	86.3 ± 2.1 [c]	99

Effects of samples on electrically-induced twitch contraction were examined in guinea-pig ileum. Potency is expressed as the pD_2 value, which is the negative logarithm (–log g/ml for extracts, –log M for compounds) of the concentration required to produce 50% of the maximum response to each compound (EC_{50}). Relative potency is expressed as a percentage of the pD_2 value of each compound against that of morphine. Maximum inhibition (%), which is elicited by the compound when the response reaches a plateau, was calculated by regarding the twitch contraction as 100%. Relative inhibitory activity, which means intrinsic activity on opioid receptors, is expressed as a percentage of the maximum inhibition by each compound against that by morphine. Each value represents mean ± S.E.M. of five animals. When significant inhibition was not obtained at 30 μM of the compound, the effect was recorded as "not effective (NE)". [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, significantly different from the values before the addition of each compound.

Table 2 Effects of naloxone on twitch contraction inhibited by crude extract and constituents of *Mitragyna speciosa* in guinea-pig ileum preparation

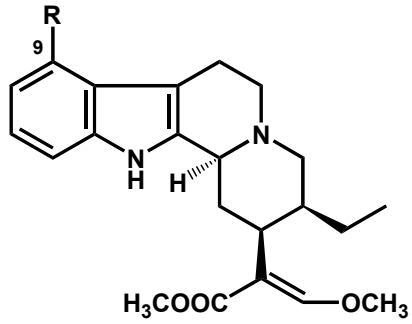
Compound	(Concentration)	Contraction (%) Inhibited by Samples	Contraction (%) Reversed by Naloxone	
			30 nM	300 nM
Crude extract	(300 μg/ml)	56.5 ± 11.2	65.7 ± 8.4	83.2 ± 5.2 [b]
7-Hydroxymitragynine	(100 nM)	24.4 ± 4.4	56.3 ± 8.2 [b]	129.5 ± 8.1 [c]
Mitragynine	(3 μM)	18.9 ± 2.3	29.9 ± 2.5	117.4 ± 5.7 [c]
Speciogynine	(30 μM)	22.6 ± 9.1	25.9 ± 9.0	42.3 ± 12.0
Paynantheine	(30 μM)	43.0 ± 5.8	42.4 ± 5.8	41.6 ± 6.5
Speciociliatine	(30 μM)	25.2 ± 6.4	19.1 ± 5.4	25.2 ± 5.2
Morphine	(1 μM)	15.3 ± 2.0	68.0 ± 5.3 [c]	121.6 ± 6.1 [c]
Verapamil	(3 μM)	7.9 ± 2.6	7.9 ± 2.6	6.1 ± 1.6

Each value represents mean ± S.E.M. of five animals. [b] $P < 0.01$, [c] $P < 0.001$, significantly different from the values before the addition of naloxone.

Effects of mitragynine-related indole alkaloids on electrically-stimulated twitch contraction

The opioid agonistic activities of the natural analogue of mitragynine and semi-synthetic compounds derived from mitragynine were evaluated by measuring the twitch contraction induced by electrical stimulation in the guinea-pig ileum preparation (Table 3). Mitragynine inhibited the twitch contraction induced by electrical stimulation at a potency of about 58% of that of morphine. In investigating the structure-activity relationship, we initially directed our attention to the presence of a methoxyl group at the C9 position on the indole ring in mitragynine. The 9-demethoxy analogue of mitragynine, corynantheidine, did not show any opioid agonistic activity at all, but reversed the morphine-inhibited twitch contraction in guinea-pig ileum. Its antagonistic effect was concentration-dependent (data not shown). The 9-demethyl analogue of mitragynine, 9-hydroxycorynantheidine, also inhibited the electrically-induced twitch contraction, but its maximum inhibition percentage was lower than that of mitragynine. 9-Acetoxymitragynine produced a marked reduction of both intrinsic activity and potency compared with those of mitragynine. 9-Methoxymethylcorynantheidine did not show any opioid agonistic activities.

Next, we investigated the transformation of the substituent at C7 position. 7-Hydroxymitragynine inhibited the electrically-stimulated twitch contraction in a concentration-dependent manner, as mitragynine and morphine did. The pD_2 values were 8.38 ± 0.12 for 7-hydroxymitragynine, 6.91 ± 0.04 for mitragynine and 7.15 ± 0.05 for morphine. The introduction of a methoxy, ethoxy, or acetoxy group at the C7 position (7-methoxymitragynine, 7-ethoxymitragynine, or 7-acetoxymitragynine) led to a marked reduction in both maximum inhibition and relative potency of the opioid receptors (Table 3). The pD_2 values were 6.45 ± 0.04 for 7-methoxymitragynine, 5.29 ± 0.12 for 7-ethoxymitragynine, and 6.50 ± 0.16 for 7-acetoxymitragynine.



R

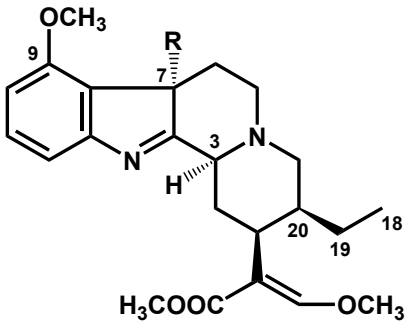
OCH_3: Mitragynine

H: Corynantheidine

OH: 9-Hydroxycorynantheidine

$OCOCH_3$: 9-Acethoxycorynantheidine

OCH_2OCH_3: 9-Methoxymethylcorynantheidine



R

OH: 7-Hydroxymitragynine

OCH_3: 7-Methoxymitragynine

OCH_2CH_3: 7-Ethoxymitragynine

$OCOCH_3$: 7-Acethoxymitragynine

Figure 2 Chemical structures of mitragynine-related indole alkaloids

Table 3 Opioid agonistic activities of mitragynine-related compounds and morphine in electrically-stimulated guinea-pig ileum preparation

Compound	pD_2 Value	Relative Potency (%)	Maximum Inhibition (%)	Relative Inhibitory Activity (%)
Morphine (positive control)	7.15 ± 0.05	100	87.2 ± 1.8 [c]	100
Mitragynine	6.91 ± 0.04	58	84.0 ± 2.0 [c]	96
Corynantheidine	NE	NE	-18.1 ± 8.6	NE
9-Hydroxycorynantheidine	6.78 ± 0.23	41	49.4 ± 3.1 [c]	57
9-Acetoxycorynantheidine	5.39 ± 0.12	2	33.2 ± 8.8 [c]	38
9-Methoxymethylcorynantheidine	NE	NE	NE	NE
7-Hydroxymitragynine	8.38 ± 0.12	1698	86.3 ± 4.8 [c]	99
7-Methoxymitragynine	6.45 ± 0.04	19	60.9 ± 0.2 [b]	70
7-Ethoxymitragynine	5.29 ± 0.12	1	22.9 ± 1.1 [c]	26
7-Acethoxymitragynine	6.50 ± 0.16	21	13.4 ± 12.7 [c]	15

Opioid agonistic activities of the compounds were evaluated by their ability to inhibit the electrically-induced twitch contraction, which was reversed by naloxone (300 nM). Relative potency is expressed as a percentage of the pD_2 value of the compound against that of morphine. Maximum inhibition (%), which is elicited by the compound when the response reaches a plateau, was calculated by regarding electrically-induced contraction as 100%. Relative inhibitory activity, which means intrinsic effect on opioid receptors, is expressed as a percentage of the maximum inhibition by compounds against that by morphine. Each value represents mean ± S.E.M. of five to six animals. [b] $P < 0.01$, [c] $P < 0.001$, significantly different from the morphine group. When significant inhibition was not obtained at 30 μM of the compound, the effect was regarded as "no effect" (NE).

4. Discussion

Opioid effect of extract

Mitragyna speciosa has been traditionally used as a substitute for opium in tropical areas (Jansen and Prast, 1988). We found that mitragynine, a major constituent of this plant, elicits an opioid agonistic effect in guinea-pig ileum (Watanabe et al., 1997; Yamamoto et al, 1999). In the present study, we attempted to find opioid agonistic principles other than mitragynine. The opioid agonistic activities of the crude and fraction extracts were evaluated using the twitch contraction induced by electrical stimulation in guinea-pig ileum. The crude extract inhibited the twitch contraction, which was reversed by naloxone. This result demonstrates that it has an opioid agonistic effect.

Opioid effect of alkaloids

Based on the results of activities of various fractions, the active components were extracted from the crude base fraction. This fraction extract was chromatographed to yield five compounds. They were identified as 7-hydroxymitragynine, mitragynine, speciogynine, paynantheine, and speciociliatine by direct comparison with corresponding authentic samples. Each alkaloid inhibited the electrically-induced twitch contraction in a concentration-dependent manner. The opioid agonistic effect of mitragynine was also obtained as reported previously (Watanabe et al., 1997; Yamamoto et al., 1999). 7-Hydroxymitragynine is an oxidized derivative of mitragynine and a minor constituent of the leaves of *Mitragyna speciosa* (Ponglux et al., 1994). The inhibitory effect of 7-hydroxymitragynine was abolished by naloxone, suggesting the involvement of opioid receptors.

Among the components isolated in this study, 7-hydroxymitragynine exhibited the most potent activity. The potency, calculated using pD_2 values, was about 30 and 17 fold higher than that of mitragynine and morphine, respectively. Taken together with this potency, it is suggested that the opioid effect of *Mitragyna speciosa* is based on the activity of 7-hydroxymitragynine.

Opioid effect of mitragynine-related alkaloids

The discovery of the potent opioid effects of mitragynine and 7-hydroxyitragynine, prompted us to embark on the synthesis of novel lead compounds based on the mitragynine skeleton. We initially directed our attention to a methoxy group at the C9 position on the indole ring in mitragynine, because it is a structural characteristic of *Mitragyna* alkaloids, compared with common Corynanthe-type indole alkaloids isolated from other plants (Lounasmaa et al., 1994). It is interesting that a transformation of the substituent at C9, i.e., from OMe to H, led to a shift of intrinsic activity from a full agonist to an antagonist of opioid receptors. Thus, it was found that the functional group at C9 of mitragynine-related compounds manages the relative inhibitory activity, which means the intrinsic activity on opioid receptors. The introduction of an acetoxy group at C9 on the indole ring (9-acetoxymitragynine) led to marked reduction of both intrinsic activity and potency compared with those of mitragynine. The 9-demethyl analogue of mitragynine, 9-hydroxycorynantheidine, inhibited electrically-induced twitch contraction, but its maximum inhibition was about 50%, lower than that of mitragynine. Therefore, it is speculated that 9-hydroxycorynantheidine may possess partial agonist properties. 9-Methoxymethylcorynantheidine did not show any opioid agonistic activities. The present results demonstrate that the intrinsic activities of the compounds on opioid receptors are determined by the functional groups at the C9 position, and that a methoxy group at the C9 position is the most suitable functional group for pharmacophore binding to opioid receptors.

7-Hydroxymitragynine, a minor constituent of *Mitragyna speciosa*, was found to exhibit high potency toward opioid receptors. The intrinsic activity of 7-hydroxymitragynine suggests its full agonistic effect on opioid receptors. The introduction of a hydroxyl group at the C7 position led to a higher potency compared with mitragynine. Therefore, we directed our attention to the transformation of the substituent at the C7 position. The introduction of a methoxy, ethoxy, or acetoxy group at the C7 position led to a marked reduction in both intrinsic activity and potency toward opioid receptors. These results suggest that the hydroxyl group at the C7 position in the mitragynine skeleton is necessary for the increased potency toward opioid receptors.

In the course of our study, we investigated the constituents of *Mitragyna speciosa* and

semi-synthetic compounds derived for mitragynine. Among them, we found two interesting compounds. One is 7-hydroxymitragynine, which showed the most potent effect in the constituent in of *Mitragyna speciosa* and mitragynine-related compounds. The other is 9-hydroxycorynantheidine, which possesses partial agonist properties. In the search for alternative analgesics for morphine, opioids showing a partial agonist profile have yielded good results. For example, buprenorphine, a partial opioid agonist, is used clinically to treat pain, and more recently, it has been used as an alternative to methadone in maintenance and detoxification of heroin addicts (Bickel et al., 1988; Kosten and Kleber, 1988). In the next chapter, we investigate the full and partial agonist characters of 7-hydroxymitragynine and 9-hydroxycorynantheidine, respectively.

Summary

In the present chapter, we described the opioid effects of constituents isolated from *Mitragyna speciosa* and semi-synthetic compounds derived from mitragynine. Among them, 7-hydroxymitragynine showed the most potent opioid effect, which was 17 fold more potent than that of morphine. 9-Hydroxycorynantheidine, a 9-demethyl analogue of mitragynine, showed a partial agonistic effect on opioid receptors in guinea-pig ileum.

Part II. Elucidation of opioid effect of 7-hydroxymitragynine and 9-hydroxycorynanthidine by *in vitro* assays

1. Introduction

In chapter I, we surveyed opioid activity of constituents isolated from *Mitragyna speciosa* and found that a minor constituent 7-hydroxymitragynine exhibited about 17 fold higher potency than morphine in the guinea-pig ileum test. It was also found that the functional group at C9 of mitragynine-related compounds controls the maximum activity, which means the intrinsic activity on opioid receptors. The 9-demethyl analogue of mitragynine, 9-hydroxycorynantheidine, inhibited electrically induced twitch contraction in guinea-pig ileum, but its maximum inhibition was about 50%, lower than that of mitragynine. Therefore, it is speculated that 9-hydroxycorynantheidine may possess partial agonist properties (Takayama et al., 2002). However, it has not yet been determined whether 9-hydroxycorynantheidine is a partial agonist on opioid receptors.

In the present chapter, we examined the partial agonistic character of 9-hydroxymitragynine and involvement of the opioid receptor subtypes on the inhibitory effect of 7-hydroxymitragynine and 9-hydroxycorynantheidine in isolated guinea-pig ileum, mouse vas deferens contraction and receptor binding assays.

2. Materials and methods

Animals

All experiments were performed in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data, and animal discomfort was kept to the minimum. Male albino guinea pigs (320–540 g, Takasugi Lab. Animals, Japan) and male ddY mice (30–45 g, SLC, Japan) were killed by CO_2 inhalation.

Isolation of guinea-pig ileum

The guinea-pig ileum was dissected and placed in Krebs-Henseleit solution (mM): NaCl, 112.08; KCl, 5.90; $CaCl_2$, 1.97; $MgCl_2$, 1.18; NaH_2PO_4, 1.22; $NaHCO_3$, 25.00 and glucose, 11.49. The ileum was placed under 1 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37ºC and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a needle 5 mm in length) electrode. After 60 min equilibration in Krebs-Henseleit solution, the ileum was transmurally stimulated (Cox and Weinstock, 1966) with monophasic pulses (0.2 Hz and 0.1 ms duration) by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Contractions were isotonically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by transmural stimulation elicited through the ring electrodes were examined. At the start of each experiment, a maximum response to acetylcholine (3 μM) in each tissue was obtained to check its stability. The mean amplitude of the electrically stimulated contraction was about 30% of the maximal response to acetylcholine (3 μM). The electrically induced twitch contraction was almost abolished by tetrodotoxin (1 μM) and atropine (0.1 μM), as described previously (Watanabe et al., 1997). Thus, the electrical stimulation induced cholinergic contraction in guinea-pig ileum (Brookes et al., 1991). The height of the twitch response to transmural stimulation was measured before and after the drug challenge. All concentration-response curves were constructed in a cumulative manner. Contraction (%) is expressed as a percentage of the twitch response to the transmural stimulation before the drug challenge. The apparent agonist efficacies (intrinsic activity) were determined by comparing the maximum effect of mitragynine (intrinsic activity = 1.00).

β-Funaltorexamine hydrochloride (β-FNA) exhibits irreversible μ-opioid antagonistic and short-lived reversible agonistic profiles (Portoghese et al., 1980; Takemori et al., 1981). To investigate the effects of test compounds on μ-opioid receptors, the ileum was preincubated with β-FNA, a selective μ-opioid receptor antagonist, at 10, 30 or 100 nM for 30 min, and then it was washed 20

times with Krebs-Henseleit solution. In addition, it was washed the again at 15 min intervals for 60 min to remove the opioid receptor agonistic action of β-FNA (Ozaki et al., 1994).

Isolation of mouse vas deferens

The mouse vas deferens was dissected and placed in eliminating $MgCl_2$ from Krebs-Henseleit solution. The tissues were placed under 0.2 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37ºC and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a needle 5 mm in length) electrode. After 60 min equilibration in Krebs-Henseleit solution, the tissues were transmurally stimulated with a train of 10 pulses, 0.5 msec duration, 2 msec interval by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan) every 1 min. Contractions were isotonically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by transmural stimulation elicited through the ring electrodes were examined. At the start of each experiment, a maximum response to norepinephrine (30 μM) with 0.1 mM ascorbic acid in each tissue was obtained to check its stability. All concentration-response curves were constructed in a cumulative manner. The height of the twitch response to transmural stimulation was measured before and after the drug challenge. Contraction (%) is expressed as a percentage of the twitch response to the transmural stimulation before the drug challenge.

Receptor binding assay

The whole male guinea-pig brain (excluding the cerebellum) was quickly removed, weighed, placed in ice cold 50 mM Tris-HCl buffer, pH 7.4, and frozen immediately. Frozen brains were stored at −70ºC until the assay. For each experiment, frozen brains from two animals were thawed and homogenized with a Polytron homogenizer (PT 10-35, Kinematica, Littau, Switzerland) for 60 sec in 50 mM Tris-HCl (pH 7.4) and centrifuged at 49,000 g for 10 min (Childers et al., 1979). The pellet

was re-homogenized and centrifuged again. For the binding assays, membrane fractions were suspended in the assay buffer. The protein concentration was measured by using a DC-protein assay kit (Bio-Rad, Richmond, VA, USA).

Saturation-binding isotherms were produced by incubating membrane proteins with radiolabeled compounds in different concentrations. Using the above solution, 0.1 ml aliquots of protein were added to 0.9 ml of solutions of the labeled assay sample with unlabeled competing ligands, which were dissolved in 50 mM Tris-HCl, pH 7.4, assay buffer, in appropriate concentrations. The assay solution contained one of the followings; 3 nM of [^{3}H][D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin ([^{3}H]DAMGO), 3 nM of [^{3}H](5α,7α,8β)-(+)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro [4.5]dec-8-yl]-benzeneacetamide ([^{3}H]U69593) or 1 nM of [^{3}H][D-Pen^2, D-Pen^5]-enkephalin ([^{3}H]DPDPE). The incubation periods were 3, 4 and 1 hr for [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69593, respectively, at 25ºC. The reaction was terminated by rapid filtration under reduced pressure through glass fiber filters (Whatman GF/B, presoaked in 0.3% polyethyleneimine), followed by the addition of 4 ml of ice-cold Tris-HCl buffer. Filters were further washed with 4 ml ice-cold buffer and allowed to dry. The radioactivity bound to the filters was measured by liquid scintillation spectrometry (Aloka LSC-5100, Tokyo, Japan). Nonspecific binding for [^{3}H]DAMGO, [^{3}H]DPDPE or [^{3}H]U69593 was determined in the presence of 1 μM unlabeled DAMGO, naltrindole hydrochloride and U69593, respectively. All values were presented as the mean ± S.E.M. The apparent dissociation constant (K_D) and maximum binding site density (B_{max}) for radioligands were estimated by Scatchard analysis of the saturation. The ability of unlabeled drugs to inhibit specific radioligand binding was expressed as the IC_{50} value, which was the molar concentration of the unlabeled drug necessary to displace 50% of the specific binding. Inhibition constants (Ki) of unlabeled compounds were calculated as described by Cheng and Prusoff (1973). Relative affinities (%) of 7-hydroxymitragynine and 9-hydroxycorynantheidine for μ-, δ- and κ-opioid receptors were calculated according to the following equations:

$$\text{Relative affinity (\%)} = \frac{\text{Ka for } \mu, \delta, \kappa}{\text{Ka for } \mu + \text{Ka for } \delta + \text{Ka for } \kappa} \times 100\ (\%)$$

(Ka = 1 / Ki)

Drugs

The drugs used in this study were acetylcholine chloride (Dai-ichi Pharmaceutical Co., Tokyo, Japan), norepinephrine bitartarate (Wako, Osaka, Japan), DPDPE (Bachem, Torrance, CA), naloxone hydrochloride, DAMGO, U69593, naltrindole hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), β-funaltorexamine hydrochloride (Research Biochemicals, Natick, MA, USA) and [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69593 (NEN Life Science Products Inc., Boston, MA, USA). Mitragynine was isolated from the extract of leaves of *Mitragyna speciosa*. 7-Hydroxymitragynine and 9-hydroxycorynantheidine were synthesized from mitragynine as described previously (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).

Mitragynine, 7-hydroxymitragynine, and 9-hydroxycorynantheidine were first dissolved in 100% dimethylsulfoxide to yield a 10 mM solution and then subsequently diluted with distilled water. β-Funaltorexamine hydrochloride were first dissolved in 100% dimethylsulfoxide to yield a 1 mM solution, and then subsequently diluted with distilled water. Other drugs were dissolved in distilled water.

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with 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.

3. Results

Effect of 7-hydroxymitragynine and 9-hydroxycorynantheidine on electrically induced contraction in guinea-pig ileum

The inhibitory effects of morphine, mitragynine, 7-hydroxymitragynine, and 9-hydroxycorynantheidine on twitch contraction induced by electrical stimulation in guinea-pig ileum are shown in Figure 1A. The addition of 7-hydroxymitragynine inhibited the electrically stimulated twitch contraction in a concentration-dependent manner as mitragynine and morphine did. Typical recording of the effect of 7-hydroxymitragynine is shown in Figure 1B. The pD_2 values were 7.78 ± 0.08 for 7-hydroxymitragynine, 6.50 ± 0.06 for mitragynine and 7.02 ± 0.08 for morphine. Consequently, 7-hydroxymitragynine exhibits about 13 and 46 fold higher potency than morphine and mitragynine, respectively. Naloxone reversed the inhibitory effect of 300 nM 7-hydroxymitragynine (control, 32.8 ± 5.3%; naloxone 10 nM, 51.7 ± 10.2%; naloxone 300 nM, 108.0 ± 5.3%, $P<0.001$ vs. control) as well as that of morphine (control, 22.3 ± 7.8%; naloxone 10 nM, 37.0 ± 9.8%; naloxone 300 nM, 133.0 ± 13.5%, $P < 0.001$ vs. control, Data represent mean ± S.E.M. of five animals). 9-Hydroxycorynantheidine, the 9-demethyl analogue of mitragynine, inhibited the electrically stimulated ileum contraction, but its maximum inhibition (intrinsic activity = 0.56) was lower than that of mitragynine. Naloxone reversed the inhibitory effect of 3 μM 9-hydroxycorynantheidine (control, 49.2 ± 5.0%; naloxone 10 nM, 60.9 ± 6.8%; naloxone 300 nM, 110.6 ± 4.1%, $P < 0.001$ vs. control, Data represent mean ± S.E.M. of five animals). 7-Hydroxymitragynine (300 nM) and 9-hydroxycorynantheidine (3 μM) did not affect the concentration-response curve for acetylcholine in the ileum (data not shown). These results suggest that both 7-hydroxymitragynine and 9-hydroxycorynantheidine have opioid agonistic activity in the guinea-pig ileum test.

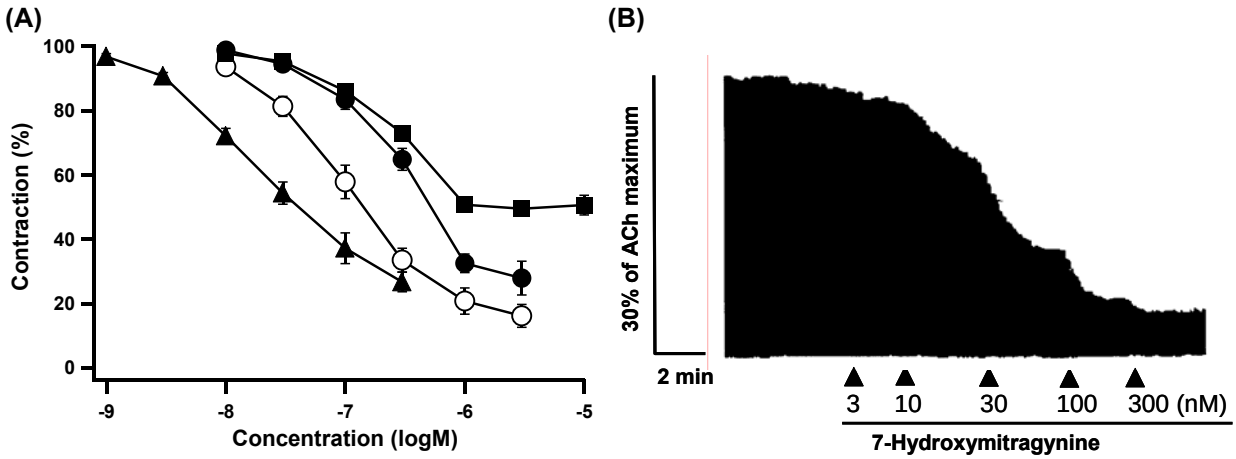


Figure 1 (A) Concentration-response curves for inhibitory effects of morphine (○), mitragynine (●), 7-hydroxymitragynine (▲) and 9-hydroxycorynantheidine (■) on electrical stimulation-induced contraction in guinea-pig ileum. Each value is expressed as inhibition % of the transmurally stimulated twitch contraction before the addition of samples. Data represent mean ± S.E.M. of five animals. (B) Typical recording of the effect of 7-hydroxymitragynine on electrical stimulation-induced in the guinea-pig ileum.

Involvement of μ-opioid receptor subtypes in the opioid effect of 7-hydroxymitragynine and 9-hydroxycorynantheidine

The guinea-pig ileum tissue contains predominantly μ-and κ-opioid receptors, while mouse vas deferens includes δ-opioid receptors. We investigated the involvement of the μ- and κ- opioid receptors in the effect of 7-hydroxymitragynine and 9-hydroxycorynantheidine using guinea-pig ileum and mouse vas deferens. The pA_2 values for naloxone in the response curves for DAMGO, U69593, 7-hydroxymitragynine and 9-hydroxycorynantheidine were compared in guinea-pig ileum test (Table 1). In the absence of naloxone, 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO, and U69593 inhibited the contraction. The concentration-response curves for 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO, and U69593 were shifted to the right in the presence of naloxone (data not shown). The slope factors for 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO, and U69593 were not significantly different from a unity, suggesting their competitive inhibition. The pA_2 values of naloxone were 8.95 ± 0.30 for 7-hydroxymitragynine, 8.69 ± 0.31 for 9-hydroxycorynantheidine, 8.77 ± 0.35 for DAMGO, and 7.50 ± 0.36 for U69593.

Table 1 pD_2 values for inhibition of electrically stimulated contraction by 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO and U69593 in guinea-pig ileum, and pA_2 values of naloxone against 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO and U69593

	pD_2	pA_2	Slope
7-Hydroxymitragynine	7.78 ± 0.08	8.95 ± 0.30	0.91 ± 0.20
9-Hydroxycorynantheidine	6.56 ± 0.07	8.69 ± 0.31	0.93 ± 0.16
DAMGO	7.83 ± 0.07	8.77 ± 0.35	1.18 ± 0.18
U69593	9.01 ± 0.12	7.50 ± 0.36	1.19 ± 0.09

pD_2 values are the negative logarithm of the IC_{50} values. The pA_2 values are calculated from parallel shifts of the curves for the agonists. Data are expressed as the mean ± S.E.M. of five animals.

To investigate the involvement of δ-receptor in the opioid effect of 7-hydroxymitragynine and 9-hydroxycorynantheidine, compounds were tested in the electrically stimulated mouse vas deferens assays using δ-opioid selective antagonist (Table 2). Naltrindole (30 nM), a δ-opioid receptor antagonist, completely reversed the inhibitory effect of DPDPE, but did not reverse the effect of 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO, and U69593.

Table 2 Effect of naltrindole on twitch contraction inhibited by 7-hydroxymitragynine, 9-hydroxycorynantheidine, DAMGO and U69593 in mouse vas deferens

Compound (Concentration)	Contraction (%) Inhibited by Compounds	Contraction (%) Reversed by naltrindole	
		3 nM	30 nM
7-Hydroxymitragynine (300 nM)	7.8 ± 1.5	8.4 ± 1.9	18.9 ± 2.9 [a]
9-Hydroxycorynantheidine(10 μM)	57.4 ± 8.4	57.7 ± 8.8	56.5 ± 8.3
DPDPE (100 nM)	12.1 ± 3.2	42.5 ± 8.0 [b]	83.8 ± 2.9 [c]
DAMGO (300 nM)	11.9 ± 2.1	13.8 ± 3.3	19.4 ± 3.6
U69593 (1 μM)	19.1 ± 4.8	21.4 ± 5.2	24.0 ± 6.9

Each value represents mean ± S.E.M. of five animals. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, significantly different from the values before the addition of naltrindole.

Partial agonistic effect of 9-hydroxycorynantheidine on μ-opioid receptors in guinea-pig ileum

To determine the partial agonistic activity of 9-hydroxycorynantheidine on μ-opioid receptors in the guinea-pig ileum, selective agonist and antagonist were used. 9-Hydroxycorynantheidine (30–300 nM) slightly shifted the concentration-response curve for DAMGO to the right, and the pA_2 value was 7.12 ± 0.31 (Figure 2). The slope factor for DAMGO (1.36 ± 0.48) was not significantly different from unity.

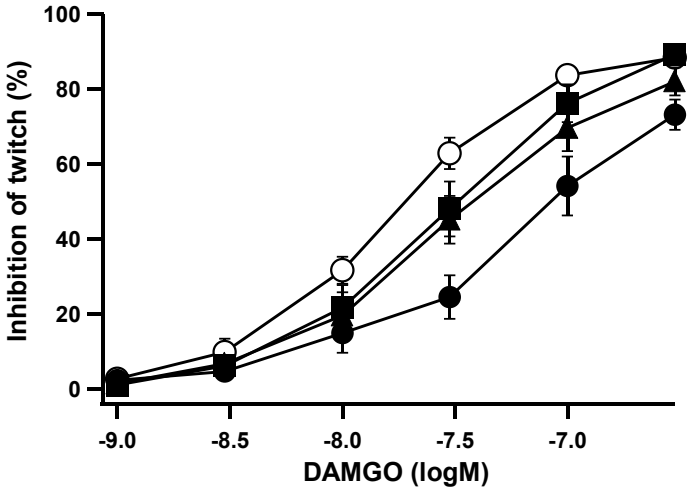


Figure 2 Concentration-response curves for DAMGO on electrical stimulation-induced contraction of guinea-pig ileum in the absence (○) or presence of 9-hydroxycorynantheidine (30 nM, ■; 100 nM, ▲; 300 nM, ●). Responses are expressed as inhibition % of the twitch contraction before agonist addition. Data represent mean ± S.E.M. of five animals.

The agonistic effect of 9-hydroxycorynantheidine was evaluated by using the μ-opioid-selective and irreversible antagonist β-FNA (Figure 3). Pretreatment with β-FNA (10–100 nM) shifted the concentration-response curve for DAMGO to the right in a competitive manner without affecting the maximum response. On the other hand, pretreatment with β-FNA (10–100 nM) did not shift the curve of 9-hydroxycorynantheidine and decreased the maximum response to 34%.

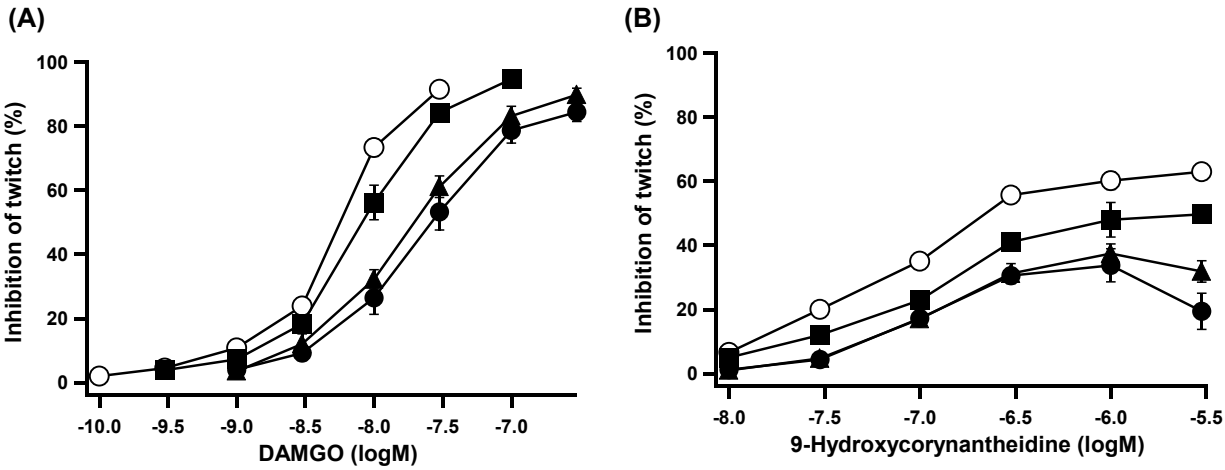


Figure 3 Concentration-response curves for (A) DAMGO and (B) 9-hydroxycorynantheidine on electrical stimulation-induced contraction in guinea-pig ileum in the absence (○) or the presence of β-funaltorexamine hydrochloride (β-FNA) (10 nM, ■; 30 nM, ▲; 100 nM, ●). Responses are expressed as inhibition % of the twitch contraction before agonist addition. Data represent mean ± S.E.M. of five animals.

Effect of 7-hydroxymitragynine and 9-hydroxycorynantheidine on opioid-receptor binding in brain

Competition binding assays revealed that both 7-hydroxymitragynine and 9-hydroxycorynantheidine bound to opioid receptors in homogenates of guinea-pig brain membrane (Table 3). The affinities of the compound for three opioid receptor types were determined by evaluating the inhibition of binding of ligands to μ-, δ- and κ-opioid receptors. Specific bindings of these radioligands for the opioid receptor types were saturable, and Scatchard plots were linear. The K_D values of [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69593 were 1.07 ± 0.06, 0.66 ± 0.05 and 0.87 ± 0.05 nM, respectively. Further, their B_{max} values were 88.2 ± 15, 41.2 ± 0.74 and 78.5 ± 9.8 fmol/mg protein, respectively.

Figure 4 shows displacement curves for the specific binding of [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69593 with various concentrations of 7-hydroxymitragynine and 9-hydroxycorynantheidine. DAMGO, 7-hydroxymitragynine, and 9-hydroxycorynantheidine bound preferentially to μ-opioid receptors with pKi values of 8.73 ± 0.04, 8.01 ± 0.03, and 7.92 ± 0.05, respectively. The relative affinities of 7-hydroxymitragynine for μ-, δ-, and κ-opioid receptors were 89.8%, 5.6%, and 4.6%, respectively. The affinities of 9-hydroxycorynantheidine were 99.6%, < 0.1% and 0.4%, respectively.

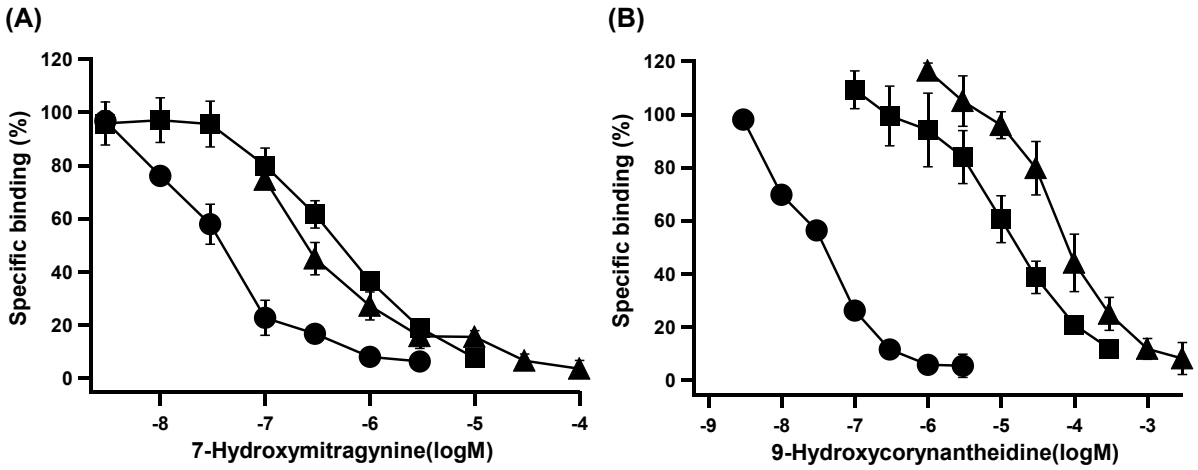


Figure 4 Displacement curves for (A) 7-hydroxymitragynine and (B) 9-hydroxycorynantheidine on specific binding of [^{3}H]DAMGO (●), [^{3}H]DPDPE (▲) and [^{3}H]U69593 (■) in guinea-pig brain homogenates. Each value is expressed as a percentage of the specific binding in the absence of 9-hydroxycorynantheidine. Data represent mean ± S.E.M. of five independent experiments performed in triplicate.

Table 3 Binding affinities (pKi) of 7-hydroxymitragynine and 9-hydroxycorynantheidine to μ-, δ- and κ-opioid receptors in homogenates of guinea-pig brain

	[^{3}H]DAMGO	[^{3}H]DPDPE	[^{3}H]U69593
7-Hydroxymitragynine	8.01 ± 0.03	6.84 ± 0.12	6.71 ± 0.11
9-Hydroxycorynantheidine	7.92 ± 0.05	4.51 ± 0.15	5.53 ± 0.15
DAMGO	8.73 ± 0.04	ND	ND
Naltrindole	ND	8.61 ± 0.01	ND
U69593	ND	ND	8.77 ± 0.03

The values are expressed as the mean ± S.E.M. of five separate displacement curves, each assayed in triplicate. The μ-binding sites were labeled with [^{3}H]DAMGO (3 nM), δ-sites with [^{3}H]DPDPE (1 nM) and κ-sites with [^{3}H]U69593 (3 nM). ND: not determined.

4. Discussion

We isolated a new compound, 7-hydroxymitragynine, as a minor constituent of the Thai medicinal herb *Mitragyna speciosa* (Ponglux et al., 1994). In the present study, we investigated its opioid effects in an isolated ileum contraction test, a receptor binding assay, and found it to be a potent μ-opioid agonist.

Involvement of the opioid receptors on the effect of 7- hydroxymitragynine

The guinea-pig ileum contains populations of functional μ- and κ-opioid receptors (Lord et al., 1977; Chavkin and Goldstein, 1981). The mouse vas deferens contains populations of functional δ-opioid receptors (Hughes et al., 1975). The present chapter showed that 7-hydroxymitragynine exhibited inhibitory action on the electrically evoked contractions in the guinea-pig ileum. We compared the pA_2 values of naloxone on opioid effects of 7-hydroxymitragynine, DAMGO, and U69593. The rightward shift of the concentration response curves for 7-hydroxymitragynine in the presence of naloxone confirms the opioid effect of 7-hydroxymitragynine. The pA_2 values of the opioid antagonist naloxone against the inhibitory action of μ selective agonist DAMGO and κ selective agonist U69593 represent the affinity of naloxone for μ- and κ-opioid receptors, respectively. The pA_2 value of naloxone against 7-hydroxymitragynine was very similar to that against DAMGO, but clearly different from that against U69593. These results suggested that 7-hydroxymitragynine predominantly acts on μ-opioid receptor. To investigate the involvement of δ-opioid receptor in the effect of 7-hydroxymitragynine, the mouse vas deferens was used. In the mouse vas deferens, 7-hydroxymitragynine inhibited the electrically induced contraction but this inhibitory effect did not antagonized by the δ-opioid receptor antagonist naltrindole. On the other hand, the inhibitory effect of δ-opioid receptor agonist DPDPE completely reversed by naltrindole. Taken together, 7-hydroxymitragynine induces the opioid effect mainly through the activation of μ-receptors.

Guinea-pig brain homogenates are commonly used as means of assessing the multiple opioid receptor binding spectra of narcotic analgesics. A close correlation between *in vitro* functional systems and opioid receptor binding in the brain has also been suggested (Pert and Snyder, 1973; Lord et al., 1977). Competition binding assays revealed that 7-hydroxymitragynine bound to opioid receptors in homogenates of guinea-pig brain membrane. Its affinities for three opioid receptor types were determined by evaluating the inhibition of binding of ligands to μ-, δ- and κ-opioid receptors. As a result, 7-hydroxymitragynine interacted with all three opioid sites, but bound preferentially to μ-opioid receptors. Taken together, the *in vitro* results demonstrated that 7-hydroxymitragynine is a

full agonist for μ-opioid receptors.

Involvement of the opioid receptors on the effect of 9-hydroxycorynantheidine

The opioid agonistic activities of the constituents of *Mitragyna speciosa* and semisynthetic compounds were evaluated using twitch contraction induced by electrical stimulation. In the course of investigating the structure-activity relationship, it was found that the functional group at C9 in mitragynine-related compounds determines its maximum activity, which means intrinsic activity on opioid receptors. A partial agonist binds to the same active site as the agonist, but elicits only a partial biologic response. Therefore, a partial agonist has a lower intrinsic activity than a full agonist. Indeed, 9-hydroxycorynantheidine behaved as a partial agonist while mitragynine behaved as a full agonist on opioid receptors in guinea-pig ileum. The inhibitory effect of 9-hydroxycorynantheidine was antagonized by the opioid receptor antagonist naloxone in the guinea-pig ileum, suggesting involvement of opioid receptors on the action of 9-hydroxycorynantheidine.

Next, we compared the pA_2 values of the opioid antagonist naloxone against the opioid effects of 9-hydroxycorynantheidine, DAMGO, and U69593. The rightward shift of the concentration response curves for 9-hydroxycorynantheidine in the presence of naloxone confirms the opioid effect of 9-hydroxycorynantheidine. The pA_2 values of naloxone against the inhibitory action of μ-selective agonist DAMGO and κ-selective agonist U69593 represent the affinity of naloxone for μ- and κ-opioid receptors, respectively. The pA_2 value of naloxone against 9-hydroxycorynantheidine was very similar to that against DAMGO, but clearly different from that against U69593. Therefore, 9-hydroxycorynantheidine is thought to act not on κ-opioid receptors, but on μ-opioid receptors. In the mouse vas deferens, 9-hydroxycorynantheidine inhibited the electrically induced contraction but this inhibitory effect did not antagonized by the δ-opioid receptor antagonist naltrindole. It is suggested that 9-hydroxycorynantheidine does not act on δ-opioid receptors. Taken together, 9-hydroxycorynantheidine inhibited the electrically stimulated contraction selectively through the μ-opioid receptors.

Receptor binding assays revealed that 9-hydroxycorynantheidine binds to opioid receptors in

homogenates of guinea-pig brain membrane. Its affinities for three opioid receptor types were determined by evaluating the inhibition of binding of ligands to μ-, δ- and κ-opioid receptors. The estimated affinity of 9-hydroxycorynantheidine for μ-opioid receptors is approximately 2600 and 250 times greater than that for δ- and κ-opioid receptors, respectively. As a result, 9-hydroxycorynantheidine had the high affinity and selectivity for μ-opioid receptors.

The results obtained in the above two assay systems were in close agreement on the involvement of μ-opioid receptors. In general, partial agonists have not only agonistic but also antagonistic effects. To determine the μ-opioid partial agonistic properties of 9-hydroxycorynantheidine, we investigated the agonistic and antagonistic effect of 9-hydroxycorynantheidine in guinea-pig ileum. 9-Hydroxycorynantheidine shifted the concentration-response curves for μ-selective agonist DAMGO slightly to the right. Logically, a partial agonist antagonizes the pharmacological effect of a full agonist, which acts on the same receptor, at the concentration that shows maximal response. Further proof for its involvement in the μ-opioid receptor was obtained when ileum was pretreated with the irreversible μ-opioid receptor antagonist β-FNA. It is widely accepted that a full maximum response was elicited by a full agonist at very low concentrations, which can only occupy certain fractions among in all specific receptors present. Those receptors that are unoccupied when a full maximum response is already elicited by an agonist are termed “spare receptors”. Drugs with high intrinsic activity require fewer drug–receptor interactions than drugs with low intrinsic activity to produce a maximal effect leading to the concept of spare receptors (Furchgott, 1966). The irreversible antagonist β-FNA, which is used to titrate away spare receptors, shifted the concentration-response curves for DAMGO to right in a competitive manner without affecting the maximum response; on the other hand, β-FNA did not shift the curve of 9-hydroxycoynantheidine to the right and decreased the maximum response at the same concentration range as an antagonist. In general, full agonists do not need to bind spare receptors for their maximum effect, and thus full agonists can induce the maximum effect in the presence of some concentration of an irreversible antagonist, but partial agonist needs to bind all specific receptors inducing spare receptors to elicit maximum response, and the maximum response is reduced by the irreversible antagonist. These results demonstrate that 9-hydroxycorynantheidine has partial agonist properties in the guinea-pig ileum and that its activity is due to μ-opioid receptor

activation.

Summary

7-Hydroxymitragynine and 9-hydroxycorynantheidine have selectivity for μ-opioid receptors in isolated guinea-pig ileum, mouse vas deferens contraction and receptor binding assays. 7-Hydroxymitragynine has full agonist and 9-hydroxycorynantheidine has partial agonist properties on μ-opioid receptors *in vitro* assays.

Part III. Antinociceptive and side effects of 7-hydroxymitragynine in mice: Discovery of a potent and orally active opioid analgesic

1. Introduction

In our laboratory, pharmacological studies were conducted for the characterization of the antinociceptive effect of mitragynine and the extract of *Mitragyna speciosa* on chemical, pressure, and thermal-stimulus pain tests in mice. Mitragynine and the extract showed antinociceptive effects on these tests in a dose-dependent manner, but the effects were much less potent than that of morphine. We studied the opioid agonistic effects of the constituents of *Mitragyna speciosa* using *in vitro* assays. Among them, 7-hydroxymitragynine showed most potent opioid effect which was 17 fold more potent than that of morphine and 9-hydroxycorynantheidine showed partial agonistic effect on opioid receptors. Opioid effects of 7-hydroxymitragynine and 9-hydroxycorynantheidine are due to μ-opioid receptor activation *in vitro* assays. However, the antinociceptive effects of 7-hydroxymitragynine and 9-hydroxycorynantheidine are not investigated *in vivo* experiments.

μ-Opioid agonists represent the major class of strong analgesics, such as morphine, used clinically. Morphine plays an important role as a pain-relieving agent, but it has a number of side effects, e.g., respiratory depression, nausea, vomiting, constipation, tolerance, and dependence. It is well known that chronic administration of opioids such as morphine leads to the development of tolerance and dependence (Pasternak, 1993). Constipation becomes a major problem during chronic opioid administration (Schug et al., 1992; McQuay et al., 1999; Portenoy et al., 1996), and relief from the adverse gastrointestinal effects markedly enhances the quality of life for patients. In the case of morphine, the dose required for its analgesic effect is much higher than that required for its constipating effects; thus, when morphine is used for analgesia, constipation is not negligible (Megens et al., 1998). 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 chapter, we investigated the antinociceptive effect of 7-hydroxymitragynine and

9-hydroxycorynantheidine *in vivo*, comparing that of morphine. We evaluated the effect of subcutaneous (s.c.) and oral (p.o.) administration of 7-hydroxymitragynine by using acute thermal pain tests in mice. Furthermore, we evaluated the inhibitory effect of gastrointestinal transit, development of tolerance, cross-tolerance to morphine, and naloxone-precipitated withdrawal signs in mice chronically treated with 7-hydroxymitragynine.

2. Materials and methods

Animals

Male ddY-strain mice (Japan SLC, Hamamatsu, Japan) weighing 25–32 g was used. Animals were housed in a temperature-controlled room at 24ºC with lights on from 07:00–19:00 and had free access to food and water. All experiments were performed in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data, and animal discomfort was kept to the minimum.

Antinociceptive activity

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 treatment with drugs, the nociceptive threshold was measured three times, and the mean of the reaction time was used as the pre-drug latency for each mouse. A cut-off time of 10 sec was used to prevent tissue damage.

Hot-plate test

Animals were placed on an electrically heated plate at 55 ± 0.2ºC, and the latency period until nociceptive responses such as licking, shaking the legs, or jumping was measured. Prior to treatment with drugs, the nociceptive threshold was measured three times, and the mean reaction time was used as the pre-drug latency for each mouse. The cut-off time of 30 sec was used to prevent tissue damage.

Antinociception in tail-flick and hot-plate tests was quantified using the percentage of maximum possible effect (% MPE) developed by Harris and Pierson (1964) and calculated as: % MPE = [(test – control) / (cut-off time – control)] × 100.

Gastrointestinal transit

Mice were fasted, with water available ad libitum, for 18 h before the experiments. Fifteen minutes after s.c. injection of 7-hydroxymitragynine, morphine, vehicle, or saline, a charcoal meal (an aqueous suspension of 10% charcoal and 5% gum Arabic) was orally administered at a volume of 0.25 ml. Thirty minutes after administration of the charcoal meal, the animal was sacrificed by cervical dislocation, and the small intestine from the pylorus to the cecum was carefully removed. Both the length from the pylorus to the cecum and the farthest distance to which the charcoal meal had traveled were measured. For each animal, the percentage of gastrointestinal transit (GIT) was calculated as the percentage of distance traveled by the charcoal meal relative to the total length of the small intestine. The inhibition of gastrointestinal transit (%) was calculated as: Inhibition of gastrointestinal transit (%) = [(saline or vehicle GIT – drug GIT) / (saline or vehicle GIT)] × 100.

Development of tolerance

Morphine or 7-hydroxymitragynine tolerance was produced by twice daily injection of morphine (8 mg/kg, s.c.) or 7-hydroxymitragynine (2 mg/kg, s.c.) for 5 consecutive days. The effect of an

agonist was measured daily 15 and 30 min after the first administration of 7-hydroxymitragynine and morphine, respectively. The development of tolerance was defined as a significant reduction of the analgesic effect of the agonist compared with the effect produced by the treatment of the first day.

Determination of cross-tolerance

Animals were pretreated with morphine (8 mg/kg, s.c.), 7-hydroxymitragynine (2 mg/kg, s.c.), saline or vehicle by administration twice per day for the first 5 days. On day 6, animals tolerant to morphine or non-tolerant (i.e., treated with saline for 5 days) received 7-hydroxymitragynine (2 mg/kg, s.c.), and the antinociceptive effects were evaluated 15 min later by the tail-flick test. Animals tolerant to 7-hydroxymitragynine or non-tolerant (i.e., treated with vehicle for 5 days) received morphine (8 mg/kg, s.c.), and the antinociceptive effects were evaluated 30 min later by the tail-flick test.

Naloxone-induced withdrawal symptoms

Morphine or 7-hydroxymitragynine was injected s.c. daily at 9:00 AM and 7:00 PM according to the schedule reported previously (Suzuki et al., 1995; Kamei et al., 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 AM and 7:00 PM were: 1st day (8, 15), 2nd day (20, 25), 3rd day (30, 35), 4th day (40, 45), 5th day (45 at 9:00 AM 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 cylinder (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 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 Ind., Osaka, Japan), naloxone hydrochloride (NX; MP Biomedicals, Irvine, CA), naltrindole hydrochloride (NTI), nor-binaltorphimine dihydrochloride (norBNI), naloxonazine dihydrochloride (NLZ), naloxone methiodide (NX-M) (Sigma Chemical Co., St. Louis, MO, USA) and β-funaltorexamine hydrochloride (β-FNA; Tocris-Cookson, Bristol, UK). Mitragynine was isolated from the extract of the leaves of *Mitragyna speciosa* as described previously (Ponglux et al., 1994), and total synthesis of mitragynine was also established (Takayama et al., 1995). 7-Hydroxymitragynine and 9-hydroxycorynantheidin were synthesized from mitragynine as described previously (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).

For s.c. administration, 7-hydroxymitragynine was dissolved in phosphate-buffered saline (pH, 5.5). Mitragynine and 9-hydroxycorynantheidine were first dissolved in 100% dimethylsulfoxide and then subsequently diluted with 0.5% carboxyl methylcellulose. The final concentration of dimethylsulfoxide was 4.8%. Other drugs were dissolved in saline. For p.o. administration, 7-hydroxymitragynine was dissolved in 10 mM phosphate-buffer (pH, 5.5). Morphine was dissolved in distilled water. All the drugs were administered using a volume of 0.1 ml/10 g body weight.

The opiate antagonists, NX-M (3 mg/kg), NX (2 mg/kg) and NTI (3 mg/kg), norBNI (20 mg/kg), and NLZ (35 mg/kg) and β-FNA (40 mg/kg), were administered s.c. 15 min, 30 min, 3 h, and 24 h, respectively, before 7-hydroxymitragynine or morphine injection (s.c.).

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with 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. ED_{50} values and 95% confidence limits were determined using the Litchfield-Wilcoxon method (Litchfield and Wilcoxon, 1949).

3. Results

Antinociceptive effect of subcutaneously administered 7-hydroxymitragynine and 9-hydroxycorynantheidine in mice

7-Hydroxymitragynine (0.25–2 mg/kg, s.c.) induced dose-related antinociceptive responses in the tail-flick and hot-plate tests (Figure 2). The effect peaked at 15 and 7.5 min after injection in the tail-flick and hot-plate tests, respectively. The ED_{50} values (95% confidence limits) for 7-hydroxymitragynine was 0.80 mg/kg (0.48–1.33) and 0.93 mg/kg (0.59–1.45) in the tail-flick and the hot-plate tests, respectively. The vehicle did not show any antinociceptive activity in either test.

Morphine (1.25–8 mg/kg, s.c.) produced dose-related antinociceptive response with a peak effect at 30 min in both tests (data not shown). The ED_{50} values (95% confidence limits) for morphine were 4.57 mg/kg (3.12–6.69) and 4.08 mg/kg (2.75–6.06) in the tail-flick and hot-plate tests, respectively. Compared to morphine on mg/kg (μmol/kg) basis, 7-hydroxymitragynine was 5.7 (6.3) and 4.4 (4.9) times more potent in the tail-flick and hot-plate tests, respectively (Figure 6A, B). 7-Hydroxymitragynine affected behavioral responses: 2 mg/kg of 7-hydroxymitragynine elicited an increase spontaneous locomotor activity and Straub tail, as 8 mg/kg of morphine did (data not shown).

9-Hydroxymitragynine had no measurable antinociceptive effect after s.c. administration at doses up to 100 mg/kg (Figure 3A). Mitragynine (60 mg/kg) produced only 30 % MPE value at 60 min after s.c. administration (Figure 3B).

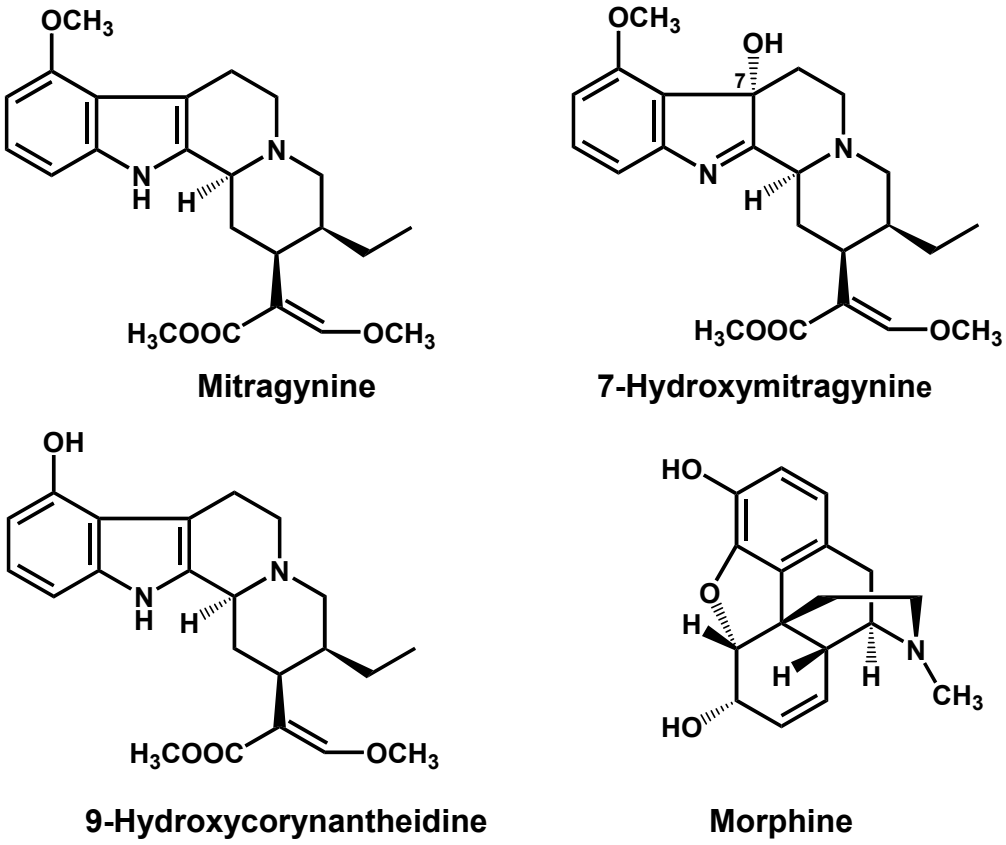


Figure 1 Chemical structures of mitragynine, 7-hydroxymitragynine, 9-hydroxycorynantheidine, and morphine

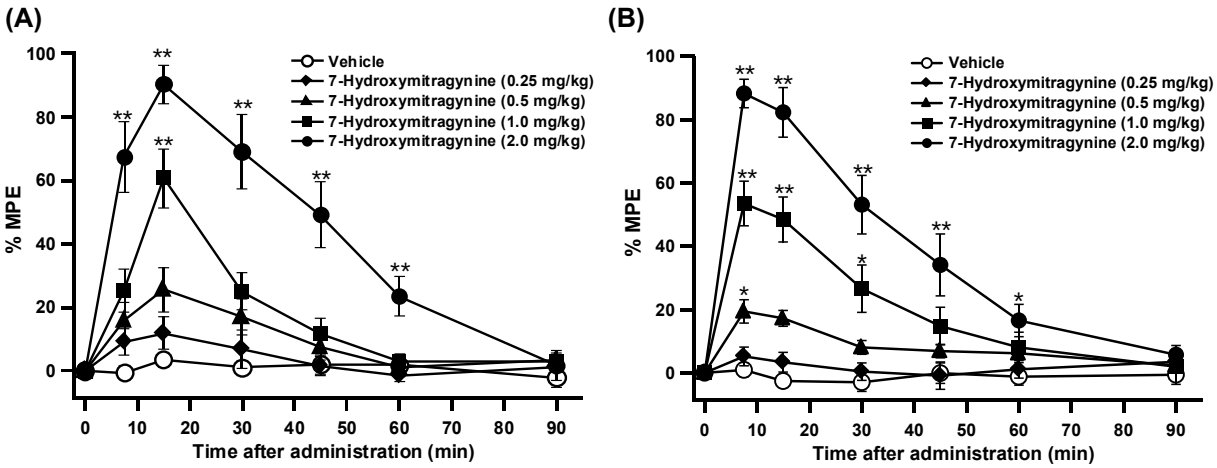


Figure 2 Time course of the antinociceptive effects produced by s.c. administration of 7-hydroxymitragynine (0.25–2.0 mg/kg) in the tail-flick test (A) and hot-plate test (B) in mice. Each value represents mean ± S.E.M. of data obtained from seven or eight mice. * $P < 0.05$; ** $P < 0.01$, versus the vehicle group.

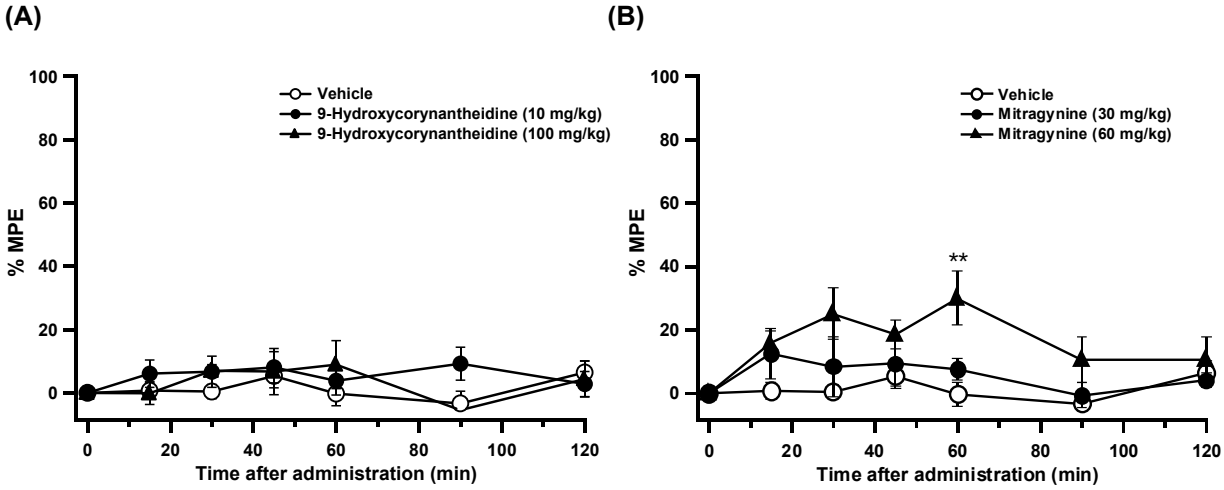


Figure 3 Time course of the antinociceptive effects produced by s.c. administration of (A) 9-hydroxycorynantheidine (10, 100 mg/kg) and (B) mitragynine (30, 60 mg/kg) in the tail-flick test in mice. Each value represents mean ± S.E.M. of six mice. ** $P < 0.01$, versus the vehicle group.

Characterization of the antinociception induced by subcutaneously administered 7-hydroxymitragynine in mice

In order to determine the opioid receptor type selectivity of 7-hydroxymitragynine antinociception, mice were pretreated with selective opioid receptor antagonists (Figure 4). In the tail-flick test, the antinociceptive effect of 7-hydroxymitragynine was significantly blocked by the non-selective opioid antagonist naloxone, the irreversible μ_1/μ_2-opioid receptor selective antagonist

β-FNA, and the μ_1-opioid receptor selective antagonist NLZ. The selective δ-antagonist NTI and the selective κ-antagonist norBNI were ineffective against 7-hydroxymitragynine-induced antinociception. In the hot-plate test, the effect of 7-hydroxymitragynine was completely blocked by naloxone and β-FNA, and partially (38%) blocked by NTI. The κ-opioid receptor antagonist norBNI was ineffective against 7-hydroxymitragynine-induced antinociception. When these opioid antagonists were administered s.c. alone at the doses used in the present study, they did not produce any changes in the tail-flick and the hot-plate test results (data not shown).

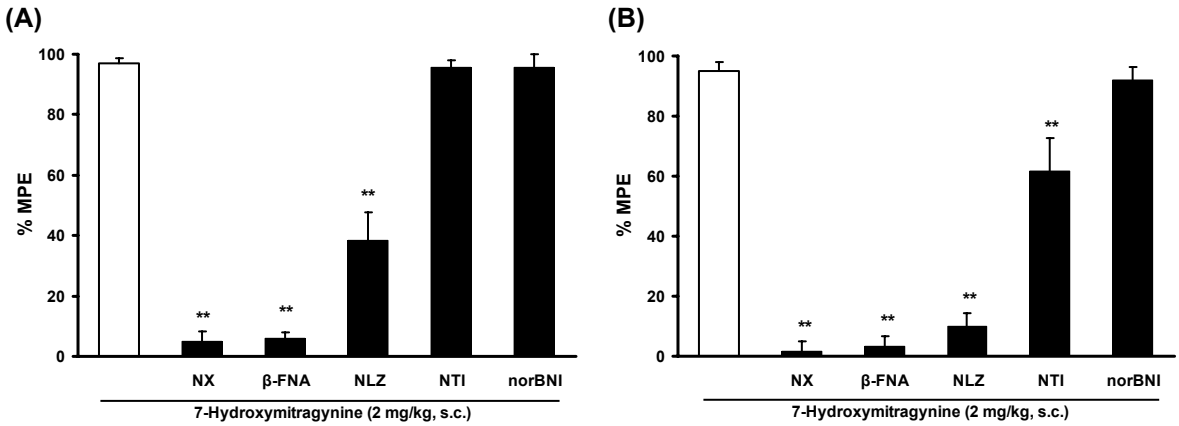


Figure 4 Effects of opioid receptor antagonists on the antinociception by 7-hydroxymitragynine (2 mg/kg) after s.c. administration. The antinociceptive effect of 7-hydroxymitragynine was determined in the mice tail-flick test (A) and the hot-plate test (B) after s.c. administration of the following antagonists: naloxone (NX; 2 mg/kg), β-funaltrexamine (β-FNA; 40 mg/kg), naloxonazine (NLZ; 35 mg/kg), naltrindole (NTI; 3 mg/kg), and nor-binaltorphimine (norBNI; 20 mg/kg). Measurements were performed 15 and 7.5 min after s.c. administration of 7-hydroxymitragynine in the tail-flick and hot-plate tests, respectively. Each value represents mean ± S.E.M. of seven or eight mice. The asterisk (*) donates values that were significantly different from 7-hydroxymitragynine treated mice by a Bonferroni test (**, $P < 0.01$).

Effect of 7-hydroxymitragynine on gastrointestinal transit

The effect of 7-hydroxymitragynine on the passage of a charcoal meal was examined. 7-Hydroxymintragynine (0.25–4 mg/kg, s.c.) and morphine (0.5–8 mg/kg, s.c.) dose-dependently and significantly inhibited gastrointestinal transit (Figure 5A, C). The ED_{50} values (95% confidence limits) for 7-hydroxymitragynine and morphine were 1.19 mg/kg (0.54–2.63) and 1.07 mg/kg

(0.40–2.86), respectively (Table 1).

The inhibitory effects of 7-hydroxymitragynine and morphine on gastrointestinal transit were similar, and were significantly antagonized by pretreatment with the μ_1/μ_2-opioid receptor selective antagonist β-FNA (40 mg/kg). The μ_1-opioid receptor antagonist NLZ (35 mg/kg) slightly blocked the effects of 7-hydroxymitragynine and morphine. The peripheral opioid receptor antagonist naloxone methiodide (NX-M) slightly blocked the effect of 7-hydroxymitragynine and significantly blocked the effect of morphine (Figure 5B, D). No change in the gastrointestinal transit was observed when each antagonist was administered alone (data not shown).

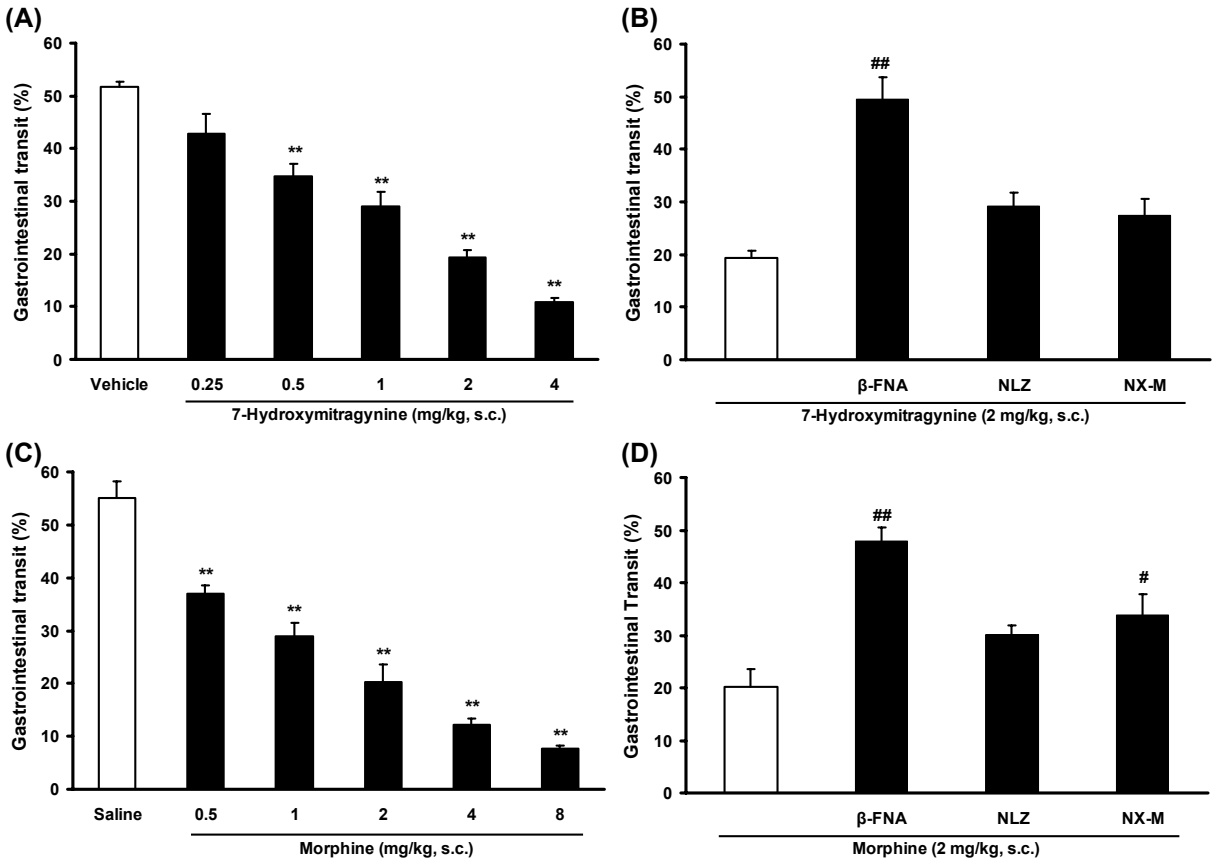


Figure 5 Effects of 7-hydroxymitragynine and morphine on gastrointestinal transit of a charcoal meal in mice. Each drug was administered s.c. 15 min before oral administration of charcoal meal. Gastrointestinal transit was determined at 30 min after administration of the charcoal meal. Inhibition of gastrointestinal transit by 7-hydroxymitragynine (A) and morphine (C). Antagonism of the antitransit effect of a single dose (2 mg/kg, s.c.) of 7-hydroxymitragynine (B) and morphine (D) by the following antagonists: β-funaltrexamine (β-FNA; 40 mg/kg), naloxonazine (NLZ; 35 mg/kg), and naloxone methiodide (NX-M; 3 mg/kg). Each value represents mean ± S.E.M. of six or seven mice. The asterisk (*) donates values that were significantly different from saline- or vehicle-treated mice by a Bonferroni test (**, $P < 0.01$). The # donates values that were significantly different from agonist alone treated mice by Bonferroni test ($^{\#}$, $P < 0.05$, $^{\#\#}$, $P < 0.01$).

Table 1 Antinociceptive and inhibitory effects on gastrointestinal transit (IGIT) of morphine and 7-hydroxymitragynine in mice

Compound	Tail-flick (TF) ED_{50}	Hot-plate (HP) ED_{50}	IGIT ED_{50}	TF/IGIT	HP/IGIT
Morphine	4.57 (3.12–6.69)	4.08 (2.75–6.06)	1.07 (0.40–2.86)	4.27	3.81
7-Hydroxymitragynine	0.80 (0.48–1.33)	0.93 (0.59–1.45)	1.19 (0.54–2.63)	0.67	0.78

ED_{50} value represent effective dose (mg/kg) 50% (95% confidence limits).

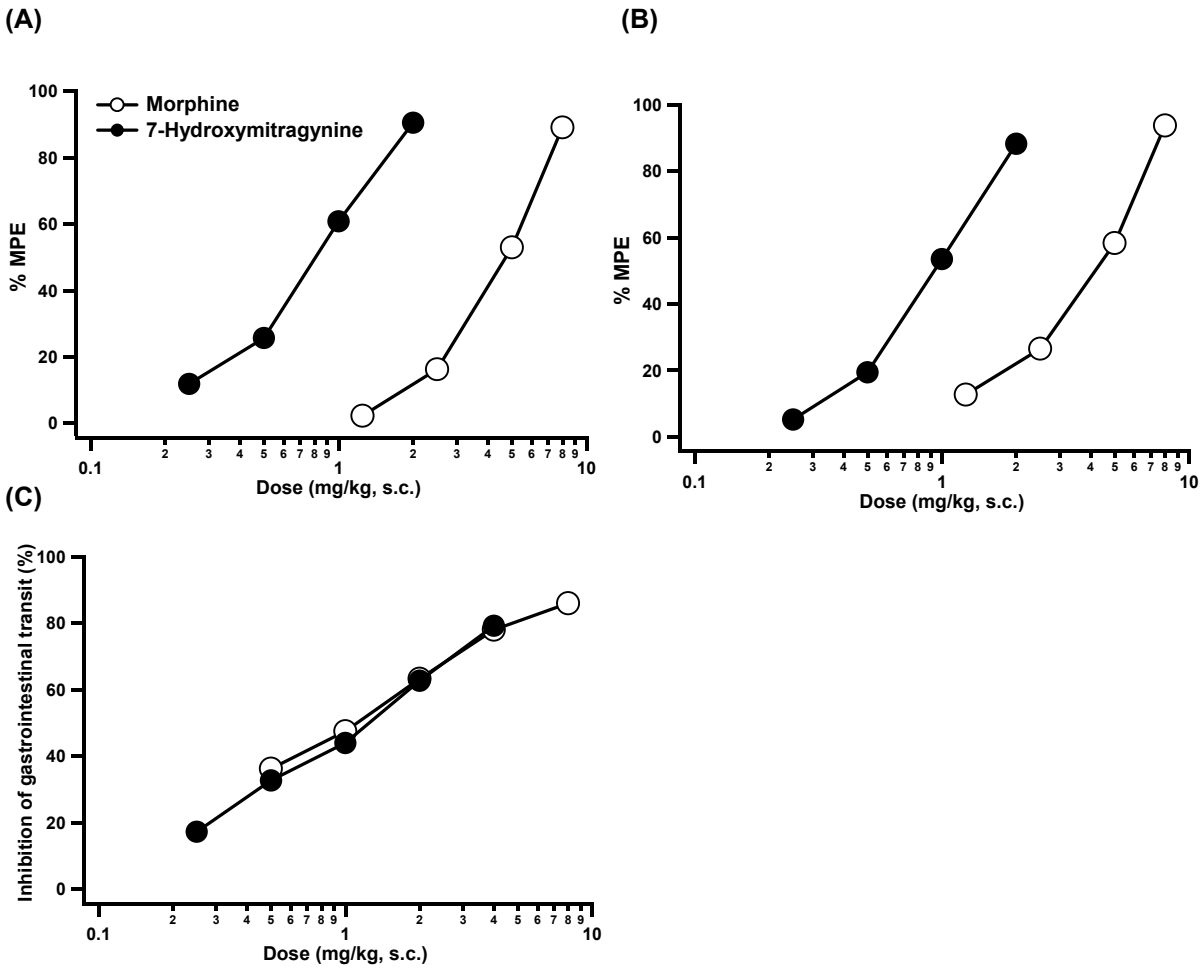


Figure 6 Dose-response curves of antinociceptive effect and inhibitory effect on gastrointestinal transit of subcutaneous administration of morphine and 7-hydroxymitragynine in (A) tail-flick test, (B) hot-plate test, and (C) gastrointestinal transit.

Development of tolerance and cross 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 (2 mg/kg, s.c., twice daily) or morphine (8 mg/kg, s.c., twice daily), are shown in Figure 7. The repeated administration of morphine and 7-hydroxymitagynine produced a development of tolerance. The animals pretreated with 7-hydroxymitragynine (2 mg/kg, s.c., twice daily for 5 days) exhibited significant and complete tolerance to the antinociceptive effects induced by 7-hydroxymitragynine (Figure 7), and showed cross-tolerance to morphine (Figure 8). Vehicle did not affect the antinociceptive responses. As was seen in the 7-hydroxymitragynine-pretreated group, the animals pretreated with morphine (8 mg/kg, s.c., twice daily for 5 days) showed cross-tolerance to 7-hydroxymitragynine.

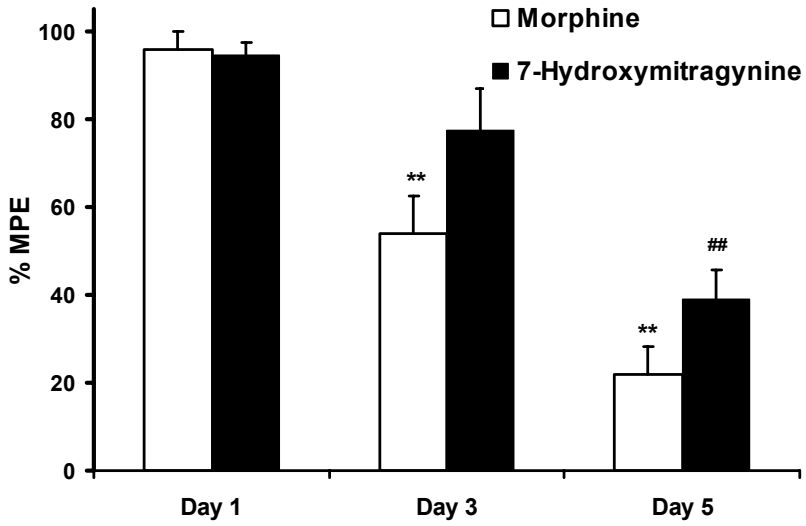


Figure 7 Development of tolerance to the antinociceptive activities of morphine (8 mg/kg, s.c.) and 7-hydroxymitragynine (2 mg/kg, s.c.) administered twice daily in mouse tail-flick test. Each point represents the mean ± S.E.M. of seven or eight mice. $^{\#\#}$ $P < 0.01$, versus the antinociceptive activities on the first day of 7-hydroxymitragynine administration. ** $P < 0.01$ versus the antinociceptive activities on the first day of morphine.

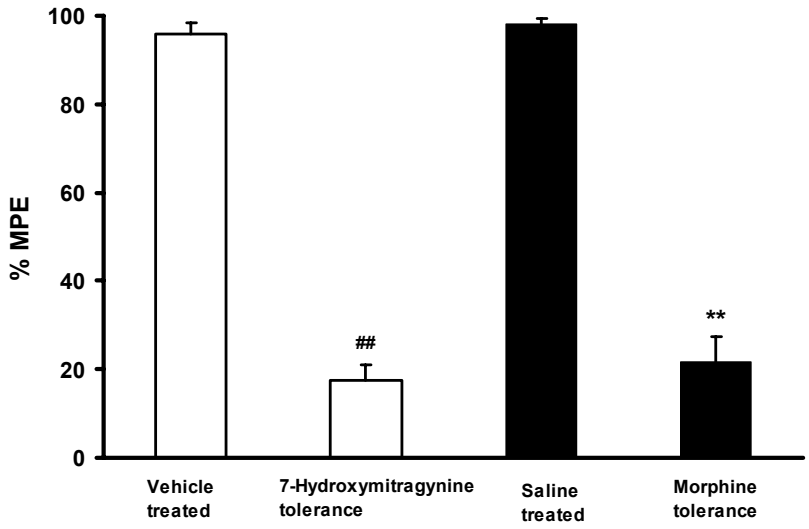


Figure 8 Cross-tolerance between morphine and 7-hydroxymitragynine. Groups of seven or eight mice received vehicle, 7-hydroxymitragynine (2 mg/kg, s.c.), saline or morphine (8 mg/kg, s.c.) twice daily for 5 days. On day six, morphine (8 mg/kg, s.c., open column) or 7-hydroxymitragynine (2 mg/kg, s.c., solid column) was administered to each mice. Each column represents the mean ± S.E.M. of eight mice. $^{\#\#}$ $P < 0.01$ versus the vehicle-treated group. ** $P < 0.01$, versus the saline-treated group.

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

Morphine-dependent mice, which were treated chronically 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 2).

Table 2 Naloxone-precipitated withdrawal responses in morphine- and 7-hydroxymitragynine-dependent mice

	Positive mice / total mice		
Withdrawal signs	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 numbers of total animals. Test drugs were injected 30 min before naloxone administration (3 mg/kg, s.c.).

Antinociceptive effect of orally administered 7-hydroxymitragynine in mice

7-Hydroxymitragynine (1–8 mg/kg, p.o.) induced dose-related antinociceptive response in the tail-flick and the hot-plate tests (Figure 9). The effect peaked at 15 and 7.5–15 min after injection in the tail-flick and the hot-plate tests, respectively. The ED_{50} values (95% confidence limits) for 7-hydroxymitragynine was 4.43 mg/kg (1.57–6.93) and 2.23 mg/kg (1.38–3.60) in the tail-flick and the hot-plate test, respectively. Vehicle did not show any antinociceptive activity in the both tests.

Morphine (25–100 mg/kg, p.o.) produced dose-related antinociceptive response with a peak effect at 60 and 30 min after injection in the tail-flick and the hot-plate tests, respectively. (data not shown). The ED_{50} values (95% confidence limits) for morphine was 63.0 mg/kg (37.2–106.8) and 48.2 mg/kg (27.5–84.5) in the tail-flick and the hot-plate test, respectively. Compared to morphine on mg/kg (μmol/kg) base, 7-hydroxymitragynine was 14.2 (15.7) and 21.6 (23.9) times more potent in the tail-flick and hot-plate test, respectively (Table 3 and Figure 10A, B).

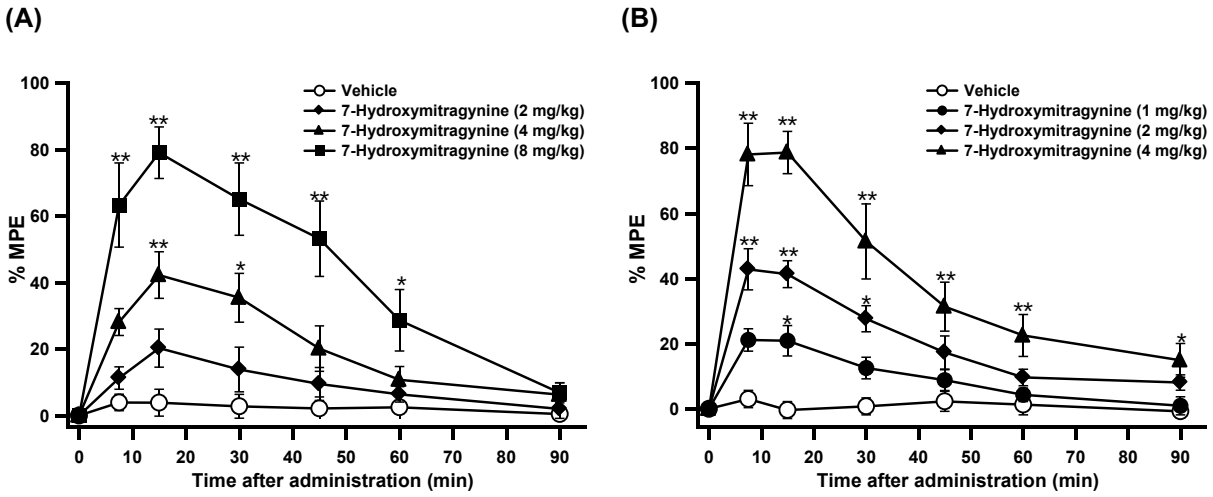


Figure 9 Time course of the antinociceptive effects produced by oral administration of 7-hydroxymitragynine (1–8 mg/kg) in the tail-flick test (A) and hot-plate test (B) in mice. Each value represents mean ± S.E.M. of seven or eight mice. * $P < 0.05$; ** $P < 0.01$, versus the vehicle group.

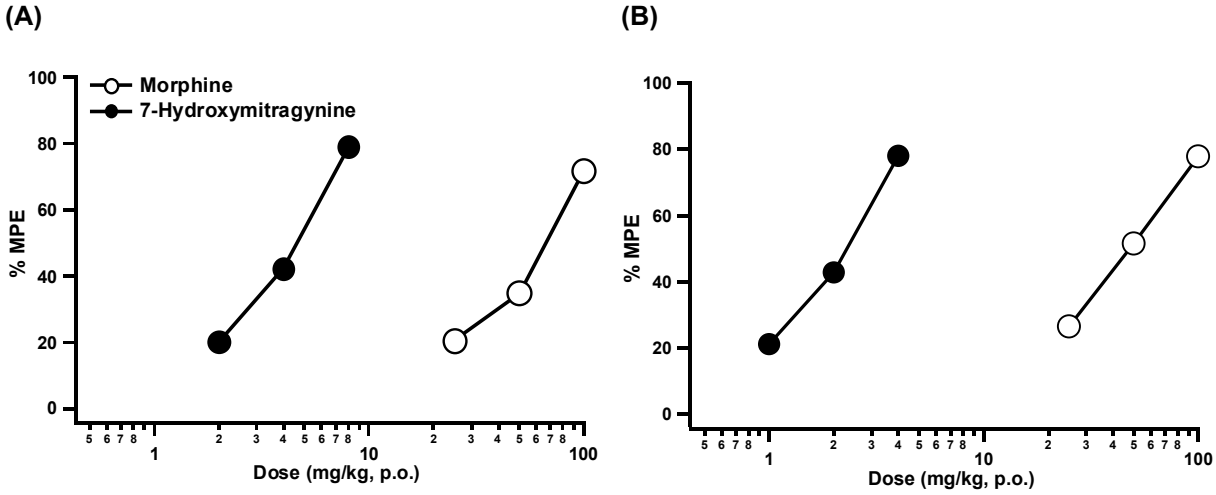


Figure 10 Antinociceptive potency of morphine and 7-hydroxymitragynine in mice. Dose-response curves of morphine and 7-hydroxymitragynine after oral administration: (A) tail-flick test, (B) hot-plate test.

Table 3 Antinociceptive effect (ED_{50}) of morphine and 7-hydroxymitragynine after s.c. or p.o. administration in mice tail-flick and hot-plate tests

Compound	Tail-flick			Hot-plate		
	ED_{50} (s.c.)	ED_{50} (p.o.)	p.o./s.c.	ED_{50} (s.c.)	ED_{50} (p.o.)	p.o./s.c.
Morphine	4.57	63.0	13.8	4.08	48.2	11.8
7-Hydroxymitragynine	0.80	4.43	5.54	0.93	2.23	2.40

ED_{50} value represent effective dose (mg/kg) 50%.

Computational superposition of morphine and 7-hydroxymitragynine

We explored the structural similarity between morphine and 7-hydroxymitragynine using molecular modeling techniques (Figure 11). 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.

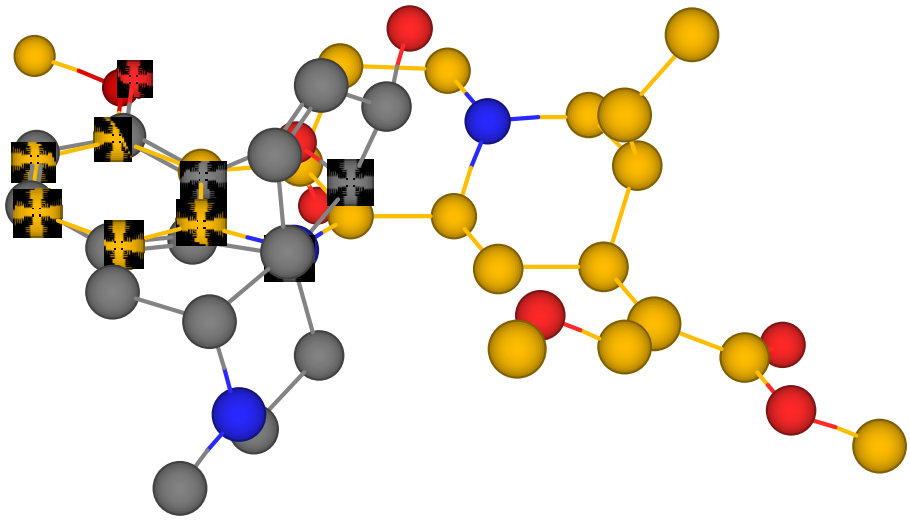


Figure 11 Overlay of the low-energy conformation of 7-hydroxymitragynine (yellow) and morphine (gray). Hydrogen atoms are omitted. Red and blue balls represent oxygen and nitrogen atoms, respectively.

4. Discussion

Antinociceptive effect of subcutaneously administered 7-hydroxymitragynine in mice and involvement of opioid receptors

To evaluate the antinociceptive effect of the 7-hydroxymitragynine, acute thermal pain (tail-flick and hot-plate) tests were performed. The tail-flick test was used to study possible involvement of spinal opioid receptors, whereas the hot-plate test was used to study possible involvement of

supraspinal receptors. 7-Hydroxymitragynine produced potent dose-dependent antinociceptive effects about 5.7 and 4.4 times more potent than morphine in the tail-flick and hot-plate tests, respectively. The antonociceptive effect of 7-hydroxymitragynine in the tail-flick and hot-plate tests peaked at 15 and 7.5 min, respectively, after s.c. administration, while the effect of morphine peaked at 30 min after administration in both tests. The higher potency and rapider effect of 7-hydroxymitragynine than morphine, may be a result of its high lipophilicity, and its ease in penetrating the blood-brain barrier. Indeed, it has been shown that analgesics with high lipophilicity, such as fentanyl, rapidly penetrate the blood-brain barrier, and thus fentanyl produces more potent and rapid antinociception than morphine does (Narita et al., 2002). In contrast, 9-hydroxymitragynine showed no measurable antinociceptive effect after s.c. administration at high doses (100 mg/kg). This may be due to the antagonistic effect of 9-hydroxymitragynine. In guinea-pig ileum, 9-hydroxycorynantheidine showed antagonistic effect to the μ-opioid agonistic effect of DAMGO (Matsumoto et al., 2006).

Selective antagonists were employed in order to clarify the involvement of the opioid receptor subtypes in the antinociceptive effect of 7-hydroxymitragynine. μ-Opioid receptors are divided into two distinct subtypes that mediate antinociception at the spinal and supraspinal levels: the μ_1-opioid receptor being important for supraspinal antinociception, whereas μ_2-opioid receptor is involved in spinal antinociception (Ling and Pasternak, 1983; Bodnar et al., 1988; Paul et al., 1989). To investigate the relative involvement of μ_1- and μ_2-opioid receptors in spinal and supraspinal antinociception of 7-hydroxymitragynine, the μ_1/μ_2-opioid receptor antagonist β-FNA and the μ_1-opioid antagonist naloxonazine were used. It was found that the antinociceptive effects of 7-hydroxymitragynine are mediated primarily through the μ-opioid receptors because the μ_1/μ_2-opioid receptor antagonist β-FNA almost completely blocked the effect in the tail-flick and hot-plate tests. In addition, naloxonazine has been shown to preferentially block μ_1-opioid receptors rather than μ_2-opioid receptors (Sakurada et al., 1999). Naloxonazine significantly blocked the antinociceptive effect of 7-hydroxymitragynine in the tail-flick and hot-plate tests, suggesting that the antinociception induced by 7-hydroxymitragynine is highly involved in the μ_1-receptors. However, it was also found that the effect of 7-hydroxymitragynine was partially blocked by the δ-selective antagonist naltrindole in the hot-plate test, suggesting partial involvement of the supraspinal δ-opioid receptors. In addition,

Thongpradichote et al. (1988) revealed that mitragynine, which is a main constituent of *Mitragyna speciosa* and has structural similarities to 7-hydroxymitragynine, has an antinociceptive activity through the supraspinal μ- and δ-opioid receptors. These results suggest that the supraspinal δ-opioid receptors are involved in the antinociceptive effect of 7-hydroxymitragynine.

Evaluation of gastrointestinal transit

Opioids are well known to inhibit gastrointestinal transit. In the case of morphine, the dose required for its analgesic effect is much higher than required for its constipating effects. We investigated the inhibition of gastrointestinal transit to evaluate the inhibitory effect of 7-hydroxymitragynine on gastrointestinal transit and its antinociceptive effect in comparison with morphine. 7-Hydroxymitragynine inhibited gastrointestinal transit in a dose-dependent manner, as morphine did. The ratios of ED_{50} values for the antinociceptive effect in the tail-flick or hot-plate test and inhibitory effect on gastrointestinal transit (IGIT) are shown in Table 1. The IGIT ED_{50} value of 7-hydroxymitragynine was larger than that of its antinociceptive ED_{50}. On the other hand, morphine significantly inhibited gastrointestinal transit at much smaller doses than its antinociceptive doses. The IGIT ED_{50} of morphine was about 4.3 and 3.8 times lower than those of its tail-flick ED_{50} and hot-plate ED_{50} values, respectively. These results suggest that 7-hydroxymitragynine induces constipation less potently than morphine at the equi-antinociceptive doses.

It appears that among opioid receptors the μ-opioid receptors play a prominent role in morphine-induced constipation (Roy et al., 1998). We investigated the pharmacological properties of the 7-hydroxymitragynine on the gastrointestinal transit. The inhibitory effect of 7-hydorxymitragynine and morphine are markedly blocked by β-FNA, indicating that their effects are mediated by μ-opioid receptors. It is well known that the inhibitory effects on the gut after systemic administration of morphine are mediated by opioid receptors located at central and peripheral sites (Goldberg et al., 1998; Shook et al., 1987). We investigated the effect of 7-hydroxymitragynine using centrally and peripherally acting antagonists. The inhibitory effects of 7-hydroxymitragynine and morphine were slightly blocked by the centrally acting μ_1-antagonist naloxonazine. We also

investigated the peripheral component using naloxone methiodide, which has restricted access to the central nervous system (Lewanowitsch and Irvine, 2002). Naloxone methiodide slightly blocked the effects of 7-hydroxymitragynine, although it moderately and significantly blocked the effects of morphine. These results suggest that 7-hydroxymitragynine inhibits gastrointestinal propulsive activity through central and peripheral opioid receptors. These findings let us speculate that 7-hydroxymitragynine interacts less with the peripheral opioid receptors than morphine in the inhibition of the gastrointestinal transit.

Evaluation of tolerance and cross-tolerance and physical dependence

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-hydroxymitragynine and morphine are induced mainly through the activation of μ-opioid receptors in mouse tail-flick tests. 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.

As is generally accepted, the potent and repeated stimulation by 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 was mainly mediated by μ-opioid receptors in the mouse tail-flick

and hot-plate test. Furthermore, the mice rendered tolerant to 7-hydroxymitragynine clearly displayed cross-tolerance to morphine antinociception in the tail-flick test. These results possibly show similarities between naloxone-precipitated withdrawal in morphine and 7-hydroxymitragynine dependent mice.

Antinociceptive effect of orally administered 7-hydroxymitragynine

Natives of Thailand and Malaysia use the leaves of the *Mitragyna speciosa* in fresh or dried forms, and they further prepare syrup by evaporating a solution made from dried leaves. The leaves are very effective when taken orally (chewed, or the syrup was drunk after dissolving it in hot water). Macko et al. (1972) reported that the oral administration of mitragynine was more effective than its subcutaneous administration. This suggested that there may be orally active compounds in *Mitragyna speciosa*. When given subcutaneously, 7-hydroxymitragynine produced antinociceptive effect in mice tail-flick and hot-plate tests. Their effects were about 5.7 and 4.4 times more potent than that of morphine in the tail-flick and the hot-plate tests, respectively. Thus, we investigated the antinociceptive effect of 7-hydroxymitragynine via oral route, due to the traditional usage of *Mitragyna speciosa* and clinical relevance of this route to administration for human patients.

7-Hydroxymitragynine produced dose-dependent and potent antinociceptive effects in the tail-flick and the hot-plate tests. It was about 14.2 and 21.6 times more potent than that of morphine after p.o. administration in the tail-flick and the hot-plate tests, respectively. Interestingly, 7-hydroxymitragynine had a favorable bioavailability (oral / subcutaneous dose ratio). Ratios of p.o. to s.c. potencies of 7-hydroxymitragynine in the tail-flick and the hot-plate tests were 5.54 and 2.20, respectively. On the other hand, ratios of morphine in the tail-flick and hot-plate tests were 13.8 and 11.8, respectively. These results obtained in this study, suggest that 7-hydroxymitragynine may be a therapeutic useful analgesic and support that the traditional use of *Mitragyna speciosa* per oral administration.

Structural similarity between 7-hydroxymitragynine and morphine

Next, we investigated structural similarities between morphine and 7-hydroxymitragynine using molecular modeling techniques. As shown in Figure 11, we cannot superimpose all three functional groups, i.e., a nitrogen atom, a benzene residue and an 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.

Summary

7-Hydroxymitragynine acts predominantly on μ-opioid receptors, especially on central μ-opioid receptors, leading to antinociception. Antinociceptive effect of subcutaneously administered 7-hydroxymitragynine was about 4–6 times more potent than that of morphine. Especially in oral administration, 7-hydroxymitragynine showed 14–22 times more potent. Antinociceptive tolerance to 7-hydroxymitragynine was developed as was seen with morphine. Cross-tolerance to morphine was induced in mice rendered tolerant to 7-hydroxymitragynine and vice versa. On the gastrointestinal transit study, 7-hydroxymitragynine was less constipating than morphine at the equi-antinociceptive doses. Taken together, 7-hydoxymitragynine has promising characteristic as a novel analgesic because of its unique structure and strong potency.

Part IV. Effects of mitragynine on isolated tissues

1. Introduction

From the leaves of *Mitragyna speciosa*, mitragynine was obtained as the major constituent. We have studied the pharmacological effects of mitragynine on electrically induced contraction in the guinea-pig ileum and radioligand binding assay, and found that mitragynine acts on opioid receptors (Watanabe et al., 1997; Yamamoto et al., 1999; Takayama et al., 2002). It was expected that mitragynine would exhibit opioid effects in the mouse vas deferens, since opioid receptors are also present in this tissue. But in fact, the inhibitory effect of mitragynine on neurogenic contraction of the mouse vas deferens was not influenced by naloxone, and its inhibitory effect can not be explained only by its opioid effect. It seems that other mechanisms besides stimulation of opioid receptors are involved in mitragynine action in its smooth muscle.

In the present chapter, we investigated the effects of mitragynine on neurogenic contraction in various smooth muscle preparations (guinea-pig ileum, mouse vas deferens and guinea-pig vas deferens). Neuronal Ca^{2+} channels play an essential role in neurogenic contraction of the vas deferens. Therefore, we investigated the effect of mitragynine on the cytosolic Ca^{2+} level in cultured neuroblastoma cells.

2. Materials and methods

Animals

All experiments were performed in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data, and animal discomfort was kept to the minimum. Male albino guinea pigs (320–540 g, Takasugi Lab. Animals, Japan) and male ddY mice (25–40 g, SLC, Japan) were killed by CO_2 inhalation.

Isolation of guinea-pig ileum

The guinea-pig ileum was dissected and placed in Krebs-Henseleit solution (mM): NaCl, 112.08; KCl, 5.90; $CaCl_2$, 1.97; $MgCl_2$, 1.18; NaH_2PO_4, 1.22; $NaHCO_3$, 25.00 and glucose, 11.49. The ileum was placed under 1 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37ºC and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a needle 5 mm in length) electrode. After 60 min equilibration in Krebs-Henseleit solution, the ileum was transmurally stimulated (Cox and Weinstock, 1966) with monophasic pulses (0.2 Hz and 0.1 ms duration) by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Contractions were isotonically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by transmural stimulation elicited through the ring electrodes were examined. At the start of each experiment, a maximum response to acetylcholine (3 μM) in each tissue was obtained to check its stability. The mean amplitude of the electrically stimulated contraction was about 30% of the maximal response to acetylcholine (3 μM). The height of the twitch response to transmural stimulation was measured before and after the drug challenge. Contraction (%) is expressed as a percentage of the twitch response to the transmural stimulation before the drug challenge.

Isolation of mouse vas deferens

The mouse vas deferens was dissected and placed in eliminating $MgCl_2$ from Krebs-Henseleit solution. The tissues were placed under 0.2 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37ºC and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a needle 5 mm in length) electrode. After 60 min equilibration in Krebs-Henseleit solution, the tissues were transmurally stimulated with a train of 10 pulses, 1.5 msec duration by a stimulator

(SEN-7203, Nihon Kohden, Tokyo, Japan) every 30 sec. Contractions were isotonically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by transmural stimulation elicited through the ring electrodes were examined. The height of the twitch response to transmural stimulation was measured before and after the drug challenge. Contraction (%) is expressed as a percentage of the twitch response to the transmural stimulation before the drug challenge.

Isolation of guinea-pig vas deferens

The epidydimal portion of the vas deferens was dissected from guinea pigs, and placed in Krebs-Henseleit solution of the following composition (mM): NaCl, 112; KCl, 5.9; $CaCl_2$, 2; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25 and glucose, 11; EDTA, 0.03 (pH 7.4). A segment of the vas deferens(10−15 mm long) was placed in a 5-ml organ bath containing the nutrient solution and suspended from an isometric transducer (Toyo Boldwin, T-7-8-240, Orientec, Japan) under a load of 0.5 g. Contractions of the preparation were amplified by DC strain amplifier (San-ei 6M92) and recorded on a pen-writing recorder (Hitachi, Mod 056). The nutrient solution was maintained at 37˚C and saturated with 95% O_2 and 5% CO_2. Tissues were transmurally stimulated by a needle-ring platinum electrode. The needle electrode was vertically positioned and inserted in the lower end, and the ring electrode was positioned at the upper end of the preparation. Square-wave pulses (10 Hz, 0.3 msec duration, 50 V) were delivered to the guinea-pig vas deferens every 1 min for 10 sec. During electrical stimulation, mitragynine was cumulatively administered to the bath fluid. The height of the twitch response to transmural stimulation was measured before and after the drug challenge. Contraction (%) is expressed as a percentage of the twitch response to the transmural stimulation before the drug challenge.

Neuroblastoma cell culture

Mouse neuroblastoma cells (N1E-115) were cultured in Dulbecco's modified Eagle's medium

(GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum at 37˚C in a humidified atmosphere of 5% CO_2 in air. After mechanical agitation, 3×10^4 cells were removed to 35 mm tissue culture dishes containing 4 ml of the medium. After cell attachment, the dish was mounted on the stage of an inverted phase-contrast microscope (Nikon, Tokyo, Japan). These cells expressed predominately T channel currents (Pang et al., 1990). In experiments where L channels were specifically sought, the cells were grown and maintained at confluence for 3–4 weeks under the same culture conditions with the addition of 2% dimethylsulfoxide. These cells expressed predominately long-lasting (L)-channel currents (Pang et al., 1990). The transient (T)-channel component was very small, and, hence, the inward current measured was conducted predominately via L channels at a holding potential of –40 mV.

Ca^{2+} channel current recording in neuroblastoma cells

The whole-cell variation of the patch-clamp technique was used as described previously (Pang et al., 1990). The pipettes had a resistance of 2–15 MΩ. Membrane current recordings were made with an Axopatch-1B patch-clamp amplifier (Axon Instruments, Union City, CA, USA). All signals were filtered at 1 kHz and stored on diskettes by using a digital oscilloscope and its associated disk drive. Because the peak currents measured with 20 mM Ba^{2+} as the charge carrier were usually small (≈ 200 pA) and the series resistance was usually < 10 MΩ, the voltage error was < 2 mV. Hence, series resistance compensation was not usually employed. If the capacitive transient overlapped with the onset of the inward current or if the spatial voltage control was inadequate (i.e., N1E-115 cells with long neural outgrowths), the experimental data were rejected. The specified the current-voltage plots were constructed by using the peak values (corrected for leakage) from the original records for T-type or L-type Ca^{2+} channel currents. The holding membrane potential was fixed at –80 mV when the T-type Ca^{2+} channels were under investigation, while the holding membrane potential was fixed at –40 mV when the L-type Ca^{2+} channel currents were measured. Ba^{2+} currents through Ca^{2+} channels were elicited by 200 msec depolarization at intervals of 5 sec. Stable readings were first obtained at 5 min for every single-cell recording, and then the drugs were added to the bath solution. Experiments

were performed at room temperature to prolong cell survival and channel recording time. In the present neuroblastoma cells studies, stable recordings could be maintained for an average of 30 min. The bath solution contained (mM): Tris, 110; KCl, 5; CsCl, 5; 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 20; glucose, 30; $BaCl_2$, 20 and tetrodotoxin, 0.5 μM.

Cytosolic Ca^{2+} level ($[Ca^{2+}]i$) in neuroblastoma cells

$[Ca^{2+}]i$ was measured by using fura-2. Neuroblastoma cells (monolayer) grown on glass coverslips were incubated with 2 μM fura-2 acetoxymethyl ester for 1 h in a dark place at room temperature and then washed 3 times by using a solution containing (mM) NaCl, 145; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; NaH_2PO_4, 0.5; 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 10, glucose, 10 (pH 7.4) and maintained the same buffer. The glass coverslip attached with cells was transferred to a 1 ml Sykes-Moore chamber on the stage of an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan). The experiments were performed at room temperature. The cells loaded with fura-2 were excited at 340 and 380 nm, and the fluorescence of these cells was measured at 510 nm by using a fluorospectrometer (Spex, Edison, NJ, USA) coupled to an inverted microscope. The $[Ca^{2+}]i$ signal was calibrated as described by Grynkiewicz et al. (1985).

Drugs

The following drugs were used: α,β-methylene ATP, prazosin (Sigma, St. Louis, MO, USA), norepinephrine bitartarate (Wako, Osaka, Japan), hexamethonium chloride (Tokyo Kasei, Japan), tetrodotoxin (Sankyo, Japan), morphine (Takeda Chemical Industries, Osaka, Japan), fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA). Mitragynine was isolated from the extract of the leaves of Mitragyna speciosa as described previously (Ponglux et al., 1994), and total synthesis of mitragynine was also established (Takayama et al., 1995). The purity (>99%) of mitragynine was checked by HPLC and ^{1}H-NMR (500 MHz) analysis (Takayama et al., 2002). Mitragynine was first dissolved in 100% dimethylsulfoxide to yield a 1 mM solution, and then

subsequently diluted with distilled water. Other drugs were dissolved in distilled water.

Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with 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.

3. Results

Effect of mitragynine on electrically induced contraction in the guinea-pig ileum and mouse vas deferens

The effects of mitragynine and morphine on contraction evoked by single pulse electrical transmural stimulation were studied in the guinea-pig ileum (Figure 1). The mean amplitude of ileum contraction evoked by electrical stimulation was about 30% of the maximal response to ACh (3 μM). This contraction was abolished by tetrodotoxin (100 nM) and atropine (30 nM). However, hexamethonium (100 μM) did not affect the contraction (6 ± 3% inhibition).

The *in vitro* biological activities were evaluated using isolated guinea-pig ileum for μ-and κ-opioid receptors and mouse vas deferens for δ-opioid receptors. To investigate the involvement of the μ- and κ- opioid receptor in the effect of mitragynine, we compared the pA_2 values of naloxone in the response curves for mitragynine, morphine, DAMGO and U69593 in guinea-pig ileum (Table 1). Mitragynine inhibited the electrically stimulated contraction in a concentration-dependent manner as did morphine and their pD_2 values were 6.92 ± 0.05 and 7.67 ± 0.06. The concentration-response curves for mitragynine, morphine, DAMGO and U69593 were shifted to the right in the presence of naloxone (data not shown). The slope factors for mitragynine, morphine, DAMGO, and U69593 were

not significantly different from unity, suggesting the competitive inhibition. The pA_2 values of naloxone were 8.77 ± 0.12 for mitragynine, 8.61 ± 0.15 for morphine, 8.77 ± 0.35 for DAMGO, and 7.50 ± 0.36 for U69593.

Mitragynine also inhibited the electrically elicited mouse vas deferens contraction in a dose dependent manner as did morphine and δ selective agonist DPDPE, and their pD_2 values were 4.57 ± 0.14 for mitragynine, 5.85 ± 0.08 for morphine, and 8.53 ± 0.16 for DPDPE. The concentration-response curves for morphine and DPDPE were shifted to the right in the presence of naloxone or δ selective antagonist naltrindole (data not shown). On the other hand, the inhibitory effect of mitragynine on mouse vas deference was not affected by the above mentioned antagonists, even at a high dose of 10 μM (Table 2).

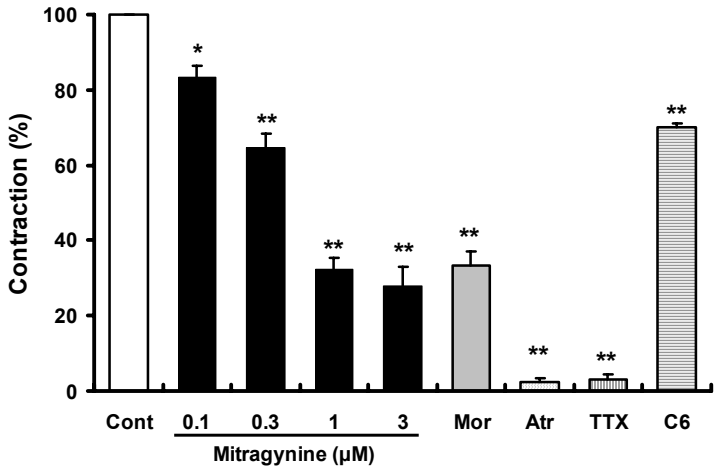


Figure 1 Effect of mitragynine on twitch response to electrical stimulation in guinea-pig ileum. Data are presented as the mean ± S.E.M. of values obtained from 5-6 guinea pigs. Contraction percentage is calculated by regarding the resting and electrically stimulated responses (Cont) as 0% and 100%, respectively. *P < 0.05, **P < 0.01, significantly different from the control group. MG: mitragynine 0.1–3 μM; Mor: morphine 0.3 μM; Atr: Atropine 30 nM; TTX: tetrodotoxin 100 nM; C6: hexamethonium 100 μM

Table 1 pD_2 values for inhibition of electrically stimulated contraction by mitragynine and morphine in guinea-pig ileum, and pA_2 values of naloxone inhibition of mitragynine and morphine

	pD_2	pA_2 (naloxone)	Slope
Mitragynine	6.92 ± 0.05	8.77 ± 0.12	0.93 ± 0.13
Morphine	7.67 ± 0.06	8.61 ± 0.15	1.14 ± 0.20
DAMGO	7.83 ± 0.07	8.77 ± 0.35	1.18 ± 0.18
U69593	9.01 ± 0.12	7.50 ± 0.36	1.19 ± 0.09

pD_2 values are the negative logarithm of the IC_{50} values. The pA_2 values are calculated from parallel shifts of the curves for the agonists. Data are expressed as the mean ± S.E.M. of five animals.

Table 2 pD_2 values for inhibition of electrically stimulated contraction by mitragynine, morphine and DPDPE in the mouse vas deferens, and pA_2 values of naloxone inhibition of mitragynine, morphine and DPDPE

	pD_2	pA_2 (naltrindole)	pA_2 (naloxone)
Mitragynine	4.57 ± 0.14	< 6	< 6
Morphine	5.85 ± 0.08	7.74 ± 0.20	8.14 ± 0.15
DPDPE	8.53 ± 0.16	9.48 ± 0.16	7.19 ± 0.11

pD_2 values are the negative logarithm of the IC_{50} values. The pA_2 values are calculated from parallel shifts of the curves for the agonists. Data are expressed as the mean ± S.E.M. of five animals.

Effect of mitragynine on electrically induced contraction in the guinea-pig vas deferens

Figure 2 shows the effect of mitragynine on electrically induced twitch response in the guinea-pig vas deferens. Electrical transmural stimulation of the vas deferens elicited twitch contractions of smooth muscle. This response was abolished by tetrodotoxin (100 nM), but was not inhibited by hexamethonium (100 μM). Prazosin (10 μM) and α,β-methylene ATP (10 μM) decreased the twitch response, and the combination of prazosin and α,β-methylene ATP completely inhibited the twitch response. Mitragynine (0.3–10 μM) inhibited the twitch response in a concentration-dependent manner. The inhibitory effect of mitragynine on electrically-elicited contraction of guinea-pig vas deferens was not restored by naloxone (100 μM) (data not shown). Morphine (1 μM) slightly inhibited the twitch response.

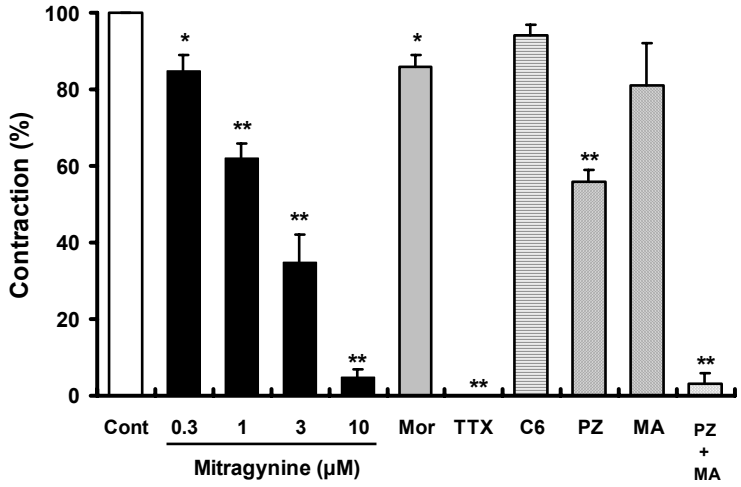


Figure 2 Effect of mitragynine on twitch response to electrical stimulation in guinea-pig vas deferens. Data are presented as the mean ± S.E.M. of values obtained from 4-6 guinea pigs. Contraction percentage is calculated by regarding the resting and electrically stimulated responses (Cont) as 0% and 100%, respectively. *P < 0.05, **P < 0.01, significantly different from the control group. MG: mitragynine 0.3–10 μM; Mor: morphine 1 μM; TTX: tetrodotoxin 100 nM; C6: hexamethonium 100 μM; PZ, prazosin 10 μM; MA: α,β-methylene ATP 10 μM.

Effect of mitragynine on norepinephrine-, ATP- and KCl-induced contraction in vas deferens

As summarized in Table 3, mitragynine (30 μM) failed to inhibit the contraction by norepinephrine or by ATP in guinea-pig vas deferens. In addition, mitragynine (30 μM) did not reduce KCl-induced contraction in the presence of tetrodotoxin, prazosin and α,β-methylene ATP. Norepinephrine- and ATP-induced contraction was abolished by pretreatment with prazosin and α,β-methylene ATP, respectively.

Table 3 Effect of mitragynine, prazosin and α,β-methylene ATP (α,β-Me ATP) on contractile response to norepinephrine (NE), ATP and KCl in guinea-pig vas deferens

Compound (Concentration)	Contraction (%)		
	NE (30 μM)	ATP (100 μM)	KCl (50 mM)
Mitragynine (30 μM)	112 ± 5	133 ± 12	109 ± 3 [a]
Prazosin (10 μM)	0 [b]	145 ± 13	–
α,β-Me ATP (10 μM)	124 ± 7 [a]	0 [b]	–

KCl-induced myogenic contraction was induced in the presence of tetrodotoxin (100 nM), prazosin (10 μM) and α,β-methylene ATP (10 μM). Mitragynine, prazosin and α,β-methylene ATP was added to the organ bath 5 min before the stimulation. The value of the maximum response to NE or ATP alone, or to KCl with tetrodotoxin, prazosin and α,β-methylene ATP was represented as 100%. Data are presented as the mean ± S.E.M. of values determined from 5–6 guinea pigs. [a] $P < 0.05$, [b] $P < 0.01$, significantly different from the corresponding control group.

Effect of mitragynine on T-type and L-type Ca^{2+} channel currents in neuroblastoma cells

We recorded two types of Ca^{2+} channel currents, which were transient (T) or long-lasting (L) inward Ba^{2+} currents. In the normal cells, step depolarization from a holding potential of –80 mV evoked transient inward Ba^{2+} currents. Complete inactivation of these inward currents occurred within the 200 msec test pulse.

Figure 3A shows that mitragynine (1 μM) inhibited the T-type Ca^{2+} channel currents. The currents were activated by depolarizing the cell from a holding potential of –80 mV to –20 mV. Records were obtained before and 5 min after the addition of mitragynine (1 μM). The peak inward Ba^{2+} current was measured as the maximum inward current change from the zero current level. Mitragynine produced a significant reduction in current amplitude in a concentration-dependent

manner (Figure 4A), but did not shift the I-V relationship along the voltage axis (Figure 3A).

In cells cultured with dimethylsulfoxide, L-type Ca^{2+} channel currents were most often recorded. The holding potential was set at –40 mV, and stepwise depolarization evoked long-lasting inward Ba^{2+} currents. During the 200 msec period of depolarizing pulse, the L-type Ca^{2+} channel currents were not inactivated. Mitragynine inhibited the L-type Ca^{2+} channel currents (Figure 3B). Figure 4B shows that mitragynine inhibited the L-type Ca^{2+} channel current without altering the channel kinetics.

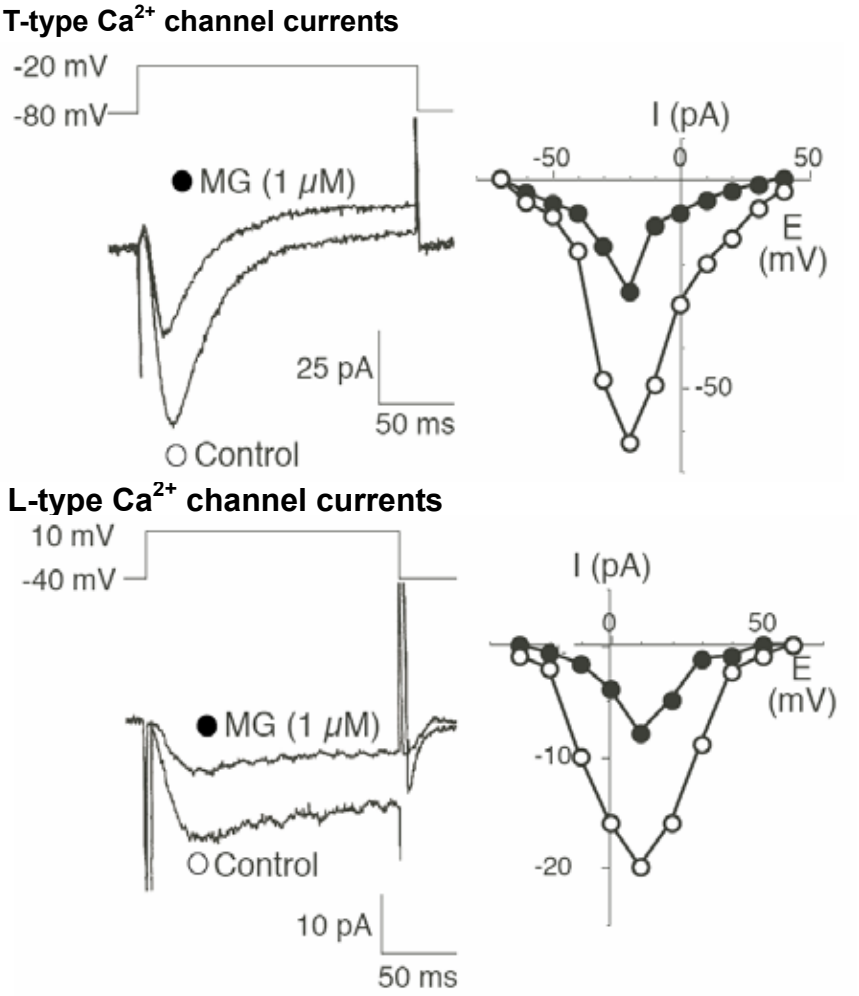


Figure 3 Typical recordings showing that effects of mitragynine on (A) T-type and (B) L-type Ca^{2+} channel currents in neuroblastoma cells. (A) Left: original current records at –20 mV. Test pulses of 200 msec were applied from a holding potential of –80 mV. The control inward current was determined before application of mitragynine. The current was inhibited at 5 min after application of mitragynine (1 μM). Right: curve of the current-voltage relationship. Peak current appears at test pulse of –20 mV. (B) Left: Original current records at 10 mV. Test pulses of 200 msec were applied from a holding potential of –40 mV. Right: curve of the current-voltage relationship. Peak current appears at test pulse of 10 mV.○: control; ●: mitragynine 1 μM.

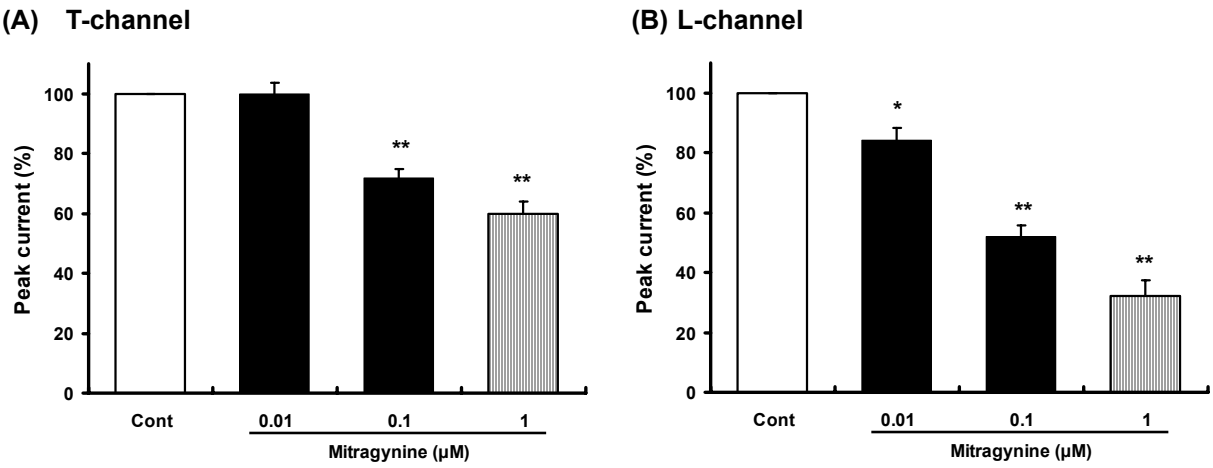


Figure 4 Effect of mitragynine on (A) T-type and (B) L-type Ca^{2+} channel currents in neuroblastoma cells. Data are presented as the mean ± S.E.M. of values determined from 4–6 experiments. Peak current (%) is calculated by regarding the resting and the stimulated responses (control) as 0% and 100%, respectively. *P < 0.05, **P < 0.01, significantly different from the corresponding control group (Cont).

Effect of mitragynine on KCl-induced cytosolic Ca^{2+} level ($[Ca^{2+}]i$) increase in neuroblastoma cells

In the presence of extracellular Ca^{2+} (1 mM), KCl (15 mM) depolarized the membrane and induced a rapid and phasic increase in $[Ca^{2+}]i$ in neuroblastoma cells. After the phasic increase, the response to KCl reached a plateau at a level above the basal value. Figure 5A shows the typical records of the KCl-induced $[Ca^{2+}]i$ increase before and after exposure to mitragynine (1 μM) and after washout of the drug. Mitragynine inhibited the KCl-induced $[Ca^{2+}]i$ increase, and this inhibition was abolished by washout. Figure 5B was constructed by using the net increase in $[Ca^{2+}]i$ at the peak response. The increase in intracellular Ca^{2+} by KCl was set to 100%. This inhibitory effect of mitragynine is dependent on the concentration used (10 nM–1μM).

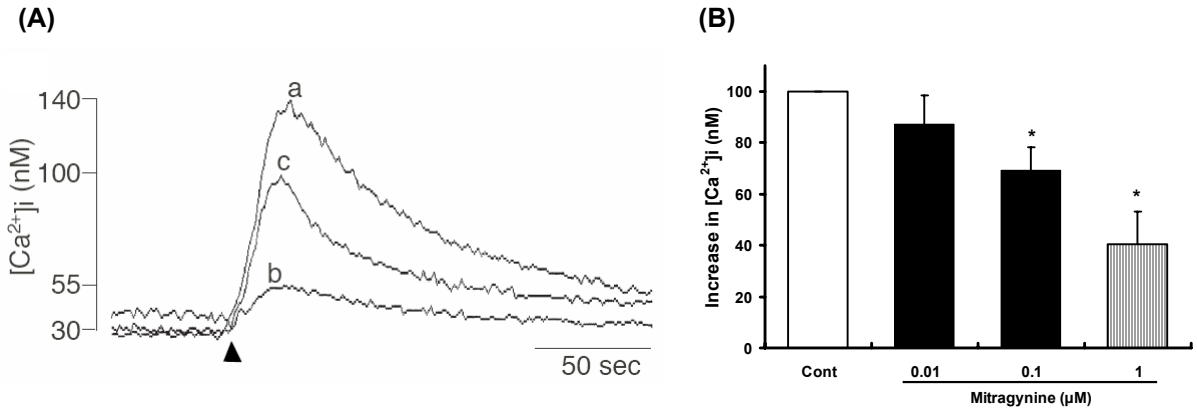


Figure 5 Effect of mitragynine on KCl-induced $[Ca^{2+}]i$ increase in neuroblastoma cells. (A) Three representative records before and after the administration of mitragynine (MG, 1 μM) and washout. The trace shown is a typical experiment showing the effect of (a) KCl alone, (b) KCl after incubation with mitragynine and (c) KCl after washout. These three tests were performed sequentially on the same cell preparation. ▲: Time when KCl (15 mM) was added. (B) Data are presented as the mean ± S.E.M. of values determined from 4–6 experiments. The $[Ca^{2+}]i$ increment is expressed as a percentage of the maximum response to KCl in each stimulation. *P < 0.05, significantly different from the control group.

4. Discussion

In the present study, we investigated opioid effects of mitragynine using various isolated tissue preparations. The electrically stimulated ileal preparation from guinea-pig was used as a model of the action of mitragynine. Mitragynine inhibited the electrically stimulated ileum contraction in a concentration-dependent manner as reported previously (Watanabe et al., 1997). The guinea-pig ileum contains populations of functional μ- and κ-opioid receptors (Lord et al., 1977; Chavkin and Goldstein, 1981). The inhibitory effect of mitragynine was antagonized by the opioid receptor antagonist naloxone. The pA_2 values of the opioid antagonist naloxone against the inhibitory action of μ selective agonist DAMGO and κ selective agonist U69593 represent the affinity of naloxone for μ- and κ-opioid receptors, respectively. The pA_2 value of naloxone against mitragynine was very similar to that against DAMGO and morphine but clearly different from that against U69593. It is well known that morphine inhibited the guinea-pig ileum contraction predominantly through μ-opioid receptors. These results suggested that μ-opioid receptors are involved in the action mitragynine on guinea-pig ileum.

In mouse vas deferens, mitragynine inhibited twitch contraction, but its effect was much smaller than that of morphine and δ-receptor selective agonist DPDPE. In contrast to morphine and DPDPE which were sensitive to naloxone and naltrindole, inhibitory effect of mitragynine was refractory to micromolar doses of naloxone or naltrindole. It was expected that inhibitory effect of mitragynine may be sensitive to naloxone in the mouse vas deference, since μ-receptors as well as δ- and κ-receptors are also present in this tissue, but in fact, the mitragynine effect was not influenced by either naloxone or naltrindole, even at high doses. It seems that other mechanisms besides opioid receptors are involved in mitragynine action in its smooth muscle.

Next, we investigated the effects of mitragynine using guinea-pig vas deferens. It is reported that the smooth muscle contraction produced by electrical transmural stimulation in guinea-pig vas deferens results from norepinephrine and ATP released from nerve endings by excitation of the sympathetic neurones (Sneddon et al., 1982). The present study supported these findings: The twitch contraction of vas deferens was abolished by tetrodotoxin, but was not affected by hexamethonium. An α_1-adrenoceptor antagonist, prazosin, or desensitization of the ATP receptor by α,β-methylene ATP partly reduced the contractile response. The combined treatment resulted in the complete inhibition of electrically induced contraction. Thus, the electrically-induced contraction is due to the excitation of postganglionic sympathetic neurones, leading to co-release of norepinephrine and ATP from the nerve terminal. Opioid receptors are known to be located in the vas deferens (Traynor, 1994), but morphine failed to inhibit the electrically induced contraction in the guinea-pig vas deference. In addition, the inhibitory effect of mitragynine was not reversed by naloxone. These results suggest that opioid receptors are not involved in its inhibitory effect of this tissue.

In this tissue, mitragynine almost abolished the electrically induced contraction of the vas deferens but failed to affect the responses to norepinephrine or to ATP. Additionally, it did not affect KCl-induced contraction in the presence of tetrodotoxin, prazosin and α,β-methylene ATP. This KCl-induced contraction results from the excitation of smooth muscle because the neurogenic factors elicited by KCl were eliminated under the present condition. Consequently, the effects of mitragynine on the receptors and the contractile mechanism of the vas deferens smooth muscle can be negligible at a concentration less than 30 μM. Taken together, mitragynine acts not on the smooth muscle, but

mainly on the sympathetic nerve, leading to inhibition of the neurogenic contraction of the vas deferens. Thus, mitragynine is thought to inhibit neurotransmitter release elicited by nerve stimulation.

Neuronal Ca^{2+} channels play an essential role in neurogenic contraction of the vas deferens. We noted the effect of mitragynine on neuronal Ca^{2+} channels in N1E-115 neuroblastoma cells. By using the patch clamp technique, mitragynine was found to block T- and L-type Ca^{2+} channel currents in neuroblastoma cells. Mitragynine reduced the amplitude of both T- and L-type Ca^{2+} channel currents without altering the channel kinetics. The inhibitory effect was reversible by washout. This is direct evidence that mitragynine blocks Ca^{2+} channels in neuronal cells. Additional evidence for the effect of mitragynine on Ca^{2+} channels is provided by the experiments where $[Ca^{2+}]i$ was measured with the fluorescent dye fura-2 in neuroblastoma cells. The cells were stimulated by depolarization with KCl, resulting in an increase in intracellular Ca^{2+}. Mitragynine inhibited the increase in $[Ca^{2+}]i$ in response to KCl stimulus in neuroblastoma cells. Mitragynine was found to inhibit the electrically stimulated contraction of guinea-pig vas deferens, and to block Ca^{2+} channels in N1E-115 neuroblastoma cells. It is speculated that mitragynine inhibits the neurogenic contraction of the vas deferens through the blockade of neuronal Ca^{2+} channels. Some Ca^{2+} channel blockers have been reported to exhibit analgesic properties in some pain tests (Miranda et al., 1993; Chaplan, 2000). It is thought that the decrease of neurotransmitters through the blockade of neuronal Ca^{2+} channels may lead to the inhibition of pain transduction. Taken together, the neuronal Ca^{2+} channel-blocking effect of mitragynine may contribute to its analgesic effects.

Summary

In the present chapter, mitragynine was found to inhibit the electrically stimulated contraction of guinea-pig ileum and vas deferens, and to block Ca^{2+} channels in N1E-115 neuroblastoma cells. It is suggested that mitragynine inhibits the contraction of the guinea-pig ileum and vas deferens through the opioid receptors and blockade of neuronal Ca^{2+} channels, respectively.

Concluding Remarks

Mitragyna speciosa has long been used in Thailand, Malaysia, Indonesia, and Papua New Guinea for its opium- and coca-like effects. The use of this herb has now been banned in Thailand and Malaysia because of its narcotic effect. However, the herb is not under any control in many other countries. In this study, I found a novel and potent opioid agonist, 7-hydroxymitragynine, that is a minor constituent of *Mitragyna speciosa*.

Discovery of 7-hydroxymitragynine from Mitragyna speciosa as an opioid agonist

We previously found that the antinociceptive effect of the main constituent, mitragynine, is less potent than that of the crude extract of *Mitragyna speciosa*. This finding suggests that one nor more minor constituents of *Mitragyna speciosa* have a very potent antinociceptive effect. In the present study, I studied the opioid agonistic effect of other constituents of *Mitragyna speciosa* using *in vitro* assays. Among them, 7-hydroxymitragynine showed the most potent opioid effect, which suggested that the opioid effect of *Mitragyna speciosa* is mostly based on the activity of 7-hydroxymitragynine.

Opioid agonistic effects and involvement of μ-opioid receptors on the effect of 7-hydroxymitragynine

7-Hydroxymitragynine showed selectivity for μ-opioid receptors in isolated guinea-pig ileum, mouse vas deferens contraction, and receptor-binding assays. 7-Hydroxymitragynine has full agonist properties on μ-opioid receptors *in vitro*. In *in vivo* assays, 7-hydroxymitragynine was found to be a potent μ-opioid antinociceptive compound. 7-Hydroxymitragynine showed potent antinociceptive activities when administered subcutaneously. It was about 4–6 fold more potent than that of morphine. Interestingly, this alkaloid is effective when administered orally. The effect was 14–22 fold more potent than that of morphine when orally administered, and had a favorable bioavailability (oral/subcutaneous dose ratio). In addition, it induced a more rapid effect than morphine. These results obtained in this study, strongly support the traditional oral administration of *Mitragyna speciosa*.

Side effects of 7-hydroxymitragynine

Morphine plays an important role as pain relieving agent, but it has a number of side effects, e.g., constipation, tolerance, and dependence. I evaluated the side effects of 7-hydoxymitragynine in comparison with morphine. Repeated subcutaneous administration of 7-hydroxymitragynine resulted in the development of tolerance and cross-tolerance to morphine. Naloxone-induced withdrawal signs were elicited equally in mice chronically treated with 7-hydroxymitragynine or morphine. On the gastrointestinal transit study, 7-hydroxymitragynine was less constipating than morphine at the equi-antinociceptive doses.

Potential utility of 7-hydroxymitragynine as an opioid analgesic

Clinical studies have demonstrated that when opioids are used to control cancer pain, physical dependence and analgesic tolerance are not a major concern. Constipation is a major problem during morphine administration, however. Therefore, 7-hydroxymitragynine is superior to morphine as an analgesic because 7-hydroxymitragynine was less constipating than morphine. 7-Hydroxymitragynine is structurally different from clinically used opioid agonists, such as morphine, fentanyl, and buprenorphine. It is speculated that the pharmacophore binding of 7-hydroxymitragynine to opioid receptors is difference from that of morphine. This may lead to a potential difference between the opioid effects of 7-hydroxymitragynine and morphine. Furthermore, the antinociceptive effect of 7-hydroxymitragynine is more potent than that of morphine, especially when administered orally. Therefore, the study of the pharmacological effects of alkaloids derived from 7-hydroxymitragynine is useful for the development of novel opioid agonists. Further studies of 7-hydroxymitrgynine and its related compounds will address development of novel analgesics for clinical management of pain, like the development of analgesics that have morphinan structures.

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List of publications

(Main Thesis Publications)

1. **Matsumoto, K.**; Takayama, H.; Ishikawa, H.; Aimi, N.; Ponglux, D.; Watanabe, K.; Horie, S.: Partial agonistic effects of 9-hydroxycorynantheidine on μ-opioid receptor in the guinea-pig ileum. Life Sci. 78, 2265-2271 (2006)

2. **Matsumoto, K.**; Yamamoto, L.T.; Watanabe, K.; Yano, S.; Shan, J.; Pang, P.K.; Ponglux, D.; Takayama, H.; Horie, S.: Inhibitory effect of mitragynine, an analgesic alkaloid from Thai herbal medicine, on neurogenic contraction of the vas deferens. Life Sci. 78, 187-194 (2005)

3. **Matsumoto, K.**; Horie, S.; Takayama, H.; Ishikawa, H.; Aimi, N.; Ponglux, D.; Murayama, T.; Watanabe, K.: Antinociception, tolerance and withdrawal symptoms induced by 7-hydroxymitragynine, an alkaloid from the Thai medicinal herb *Mitragyna speciosa*. Life Sci. 78, 2-7 (2005)

4. **Matsumoto, K.**; Horie, S.; Ishikawa, H.; Takayama, H.; Aimi, N.; Ponglux, D.; Watanabe, K.: Antinociceptive effect of 7-hydroxymitragynine in mice: Discovery of an orally active opioid analgesic from Thai medicinal herb *Mitragyna speciosa*. Life Sci. 74, 2143-2155 (2004)

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1. Horie, S.; Koyama, F.; Takayama, H.; Ishikawa, H.; Aimi, N.; Ponglux, P.; **Matsumoto, K.**; Murayama, T.: Indole alkaloids of a Thai medicinal herb, *Mitragyna speciosa*, that has opioid agonistic effect in guinea-pig ileum. Planta Med. 71, 231-236 (2005)

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3. **Matsumoto, K.**; Hatori, Y.; Murayama, T.; Tashima, K.; Wongseripipatana, S.; Misawa, K.; Kitajima, M.; Takayama, H.; Horie, S.: Antinociception and inhibition of gastrointestinal transit by 7-hydroxycorynantheidine isolated from Thai herbal medicine *Mitragyna speciosa* through μ-opioid receptors. (*to be submitted*)

(Other Publications)

1. **Matsumoto, K.**; Sakai, H.; Takeuchi, R.; Tsuchiya, K.; Ohta, K.; Sugawara, F.; Abe, M.; Sakaguchi, K.: Effective form of sulfoquinovosyldiacyglycerol (SQDG) vesicles for DNA polymerase inhibition. Colloids Surf. B: Biointerfaces 46, 175-81 (2005)

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3. **Matsumoto, K.**; Sakai, H.; Ohta, K.; Kameda, H.; Sugawara, F.; Abe, M.; Sakaguchi, K.: Monolayer membranes and bilayer vesicles characterized by alpha- and beta-anomer of sulfoquinovosyldiacyglycerol (SQDG). Chem. Phys. Lipids 133, 203-214 (2005)

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5. Yamamoto, Y.; Sahara, H.; Takenouchi, M.; Matsumoto, Y.; Imai, A.; Fujita, T.; Tamura, Y.; Takahashi, N.; Gasa, S.; **Matsumoto, K.**; Ohta, K.; Sugawara, F.; Sakaguchi, K.; Jimbow, K.; Sato, N.: Inhibition of $CD62L^{+}$ T-cell response in vitro via a novel sulfo-glycolipid, beta-SQAG9 liposome that binds to CD62L molecule on the cell surface. Cell. Immunol. 232, 105-115 (2004)

Acknowledgements

I would like to express my gratitude to Professor Shingo Yano of Department of Molecular Pharmacology and Pharmacotherapeutics, Graduate School of Pharmaceutical Sciences, Chiba University for his kindness, supervision and continuous encouragement.

I am sincerely grateful to Professor Syunji Horie of Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University for his helpful and constructive advice, kindness and continuous encouragement during the execution of this research work. I also express my grateful thanks to Emeritus Professor Kazuo Watanabe of Chiba University for his invaluable guidance, supervision, kindness and continuous encouragement.

I am deeply indebted to Professor Hiromitsu Takayama and Dr. Hayato Ishikawa of Department of Molecular Structure and Biological Function, Graduate School of Pharmaceutical Sciences, Chiba University for the generous gift of mitragynine-related compounds. I also express my sincere thanks to Dr. Norio Aimi while he was a professor at Graduate School of Pharmaceutical Sciences in Chiba University. Thanks are also extended to Dr. Dhavadee Ponglux while she was a professor at Faculty of Pharmaceutical Sciences in Chulalongkorn University for the generous gift of the crude extract of *Mitragyna speciosa*.

I also express my sincere thanks to Research Associate Shizuko Tsuchiya of Department of Molecular Pharmacology and Pharmacotherapeutics, Graduate School of Pharmaceutical Sciences, Chiba University for her helpful suggestions and kindness.

I thank to all the people for their kindness and assistance rendered to me during the execution of this research work.

Finally, I thank to my wife Ayumi and parents for their continual encouragement and sustained support throughout the accomplishment of this thesis.

This thesis for the doctorate in pharmaceutical sciences was examined by the following referees authorized by the Graduate School of Pharmaceutical Sciences, Chiba University.

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