(0 min)-55% B (10 min)-85% B (15 min) のグラジエント条件で用い，流速を 0.4 ml/min とした．カラム温度は，40℃ とした．MS 検出器におけるイオン化は，electron spray ionization (ESI) のポジティブモードで行い，キャピラリー電圧，3000 V，コーン電圧，30 V，脱溶媒ガス流量は，750 l/min，コーンガス流量，50 l/min，脱溶媒温度は，350° C，イオン源温度は，120℃ とした．またスキャン範囲は，100–1000 amu とした．

また，定量は，各濃度 (3, 10, 30, 100 μg/ml) の標準溶液を調製し，疑似分子イオンピーク値 (*m/z* 433 $[M+H]^+$) におけるマスクロマトグラムのピーク面積を用いた絶対検量線法により行った．分析法のバリデーションは，各濃度について分析法の全操作を 3 回繰り返し行った結果から評価した．

3. ARMS 分析　乾燥試料 30 mg より，DNeasy Plant Mini Kit (Qiagen) を用いて，genomic DNA を抽出，精製した．Genomic DNA 溶液 0.5 μl を鋳型として，polymerase chain reaction (PCR) を行い，5S rRNA NTS 領域を増幅した．PCR 産物，1 μl をアガロースゲル電気泳動 (2%, 100 V, 30 min) により分析した．PCR 酵素には，Nova Taq Hot Start DNA polymerase (Merck) を用い，PCR 試薬には，Ampdirect plus (Shimadzu) を用いた．プライマーは，文献[1]記載の 5S-P1, 5S-P2, SD-F, SD3 を使用した．この内，5S-P1 及び 5S-P2 は，植物の 5S rRNA の配列に保存性の高い領域に設計されたプライマーであり，各試料から調製した DNA が，PCR の使用に適した品質を保持しているかを確認するための positive primer である．一方，SD-F 及び SD3 は，*S. divinorum* に特異的な配列に設計されたプライマーであり，*S. divinorum* 由来の genomic DNA を鋳型に用いた際にのみ，PCR 産物の増幅が起こると期待される specific primer である．PCR のタイムプログラムは，ホットスタート，95℃ 10 min，続いて，95℃ 15 sec, 56℃ 15 sec, 72℃ 25 sec を 40 サイクルののち，終了反応，72℃ 7 min の条件で行った．

RESULTS AND DISCUSSION

分析法のバリデーションの評価結果を Table 2 に示した．1 の検量線は，10–100 μg/ml の範囲で，良好な直線性を示した．本条件において，Sa-0 の抽出操作を 6 回繰り返し行った結果，定量値のバラツキが小さかった (CV 2.7%) ため，各検体の分析は，1 回とした．

Table 2. Calibration Curve, Accuracy and Precision in Quantitative Determination

Conc. (μg/ml)	RSD (%, n=3)	Calibration curve	Lineality (R^2)	Range (μg/ml)
3	14.8	y=31.9x+311.7	0.9954	10–100
10	9.6			
30	6.7			
100	7.8			

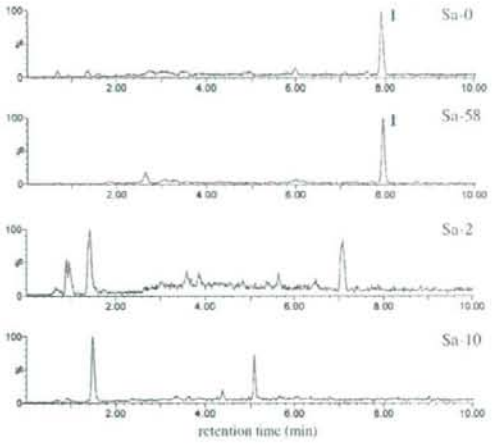

Fig. 2. Mass Chromatograms (*m/z* 433) on UPLC/MS Analysis
1: Salvinorin A

UPLC/MS 分析の結果の一部を Fig. 2 に示した．1 の標品は，上記条件で，保持時間，約 7.9 分で溶出した．違法ドラッグ市場に流通する *S. divinorum* 製品 (Sa-0, -58, -59, -64) からは，1 と同一の保持時間にピークが認められ，そのマススペクトルも標品のものと一致した．一方，園芸植物試料においても，1 と同一の保持時間にピークを検出する検体が認められたが，疑似分子イオンピーク 433 $[M+H]^+$ におけるマスクロマトグラム (*m/z* 433.1–433.2) において，該当するピークは，検出されなかった．また，それぞれのマススペクトルは，いずれも 1 の標品のものとは一致しなかった．したがって，本分析条件において，今回，試験を行った園芸植物試料 16 種から，1 は検出されないことを確認

した．

違法ドラッグ市場品，Sa-0, -58, -59, -64 における **1** の含量は，それぞれ，0.23, 0.37, 0.58, 0.19%であり，この結果は，先に東京都健康安全センターが行った調査結果[3]と同様の傾向を示した．なお，本方法における **1** の検出限界及び定量限界は，それぞれ，1 ng (S/N=3) 及び 10 ng (S/N=10) であった．

ARMS 分析の結果を Figs. 3 and 4 に示した．Positive primer (5S-P1 及び 5S-P2) を用いた PCR では，いずれの試料も約 500 bp に PCR 産物を生成しており (Fig. 3, Fig. 4(A),(B))，各試料より，PCR 法を適用可能な品質の DNA が調製されていることが確認できた．一方，*S. divinorum* specific primer (SD-F 及び SD3) を用いた PCR では，*S. divinorum* 試料のみに，約 320 bp の PCR 産物を認めた (Fig. 3, Fig. 4(C),(D))．したがって，本方法による *S. divinorum* の基原種鑑別は，非常に精度の高い方法であることが確認された．また，文献[11]の方法では，PCR に，50 サイクルを要しているが，本研究では，試料由来の PCR 酵素阻害物質の作用を抑える働きを持つ Ampdirect plus を利用することにより，PCR におけるサイクル数を 40 に抑えるとともに，伸長時間も 25 秒に短縮しており，PCR に要する時間を大幅に短縮することに成功した．なお，本条件において，specific primer による増幅の際に，目的の約 320 bp の PCR 産物のほかに，約 900 bp の PCR 産物が確認された (lane 9 in Fig. 4(C) (D))．これは，文献[11]とは異なる酵素系を使用したことによる非特異的増幅に由来するもの

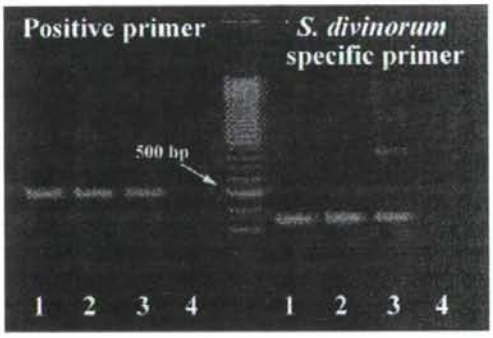

Fig. 3. ARMS Assay for the Commercial *S. divinorum* Products

Lanes 1 to 3: Sa-58, 59 and 64, lane 4: no template control, Positive primer: 5S-P1 and 5S-P2, *S. divinorum* specific primer: SD-F and SD3.

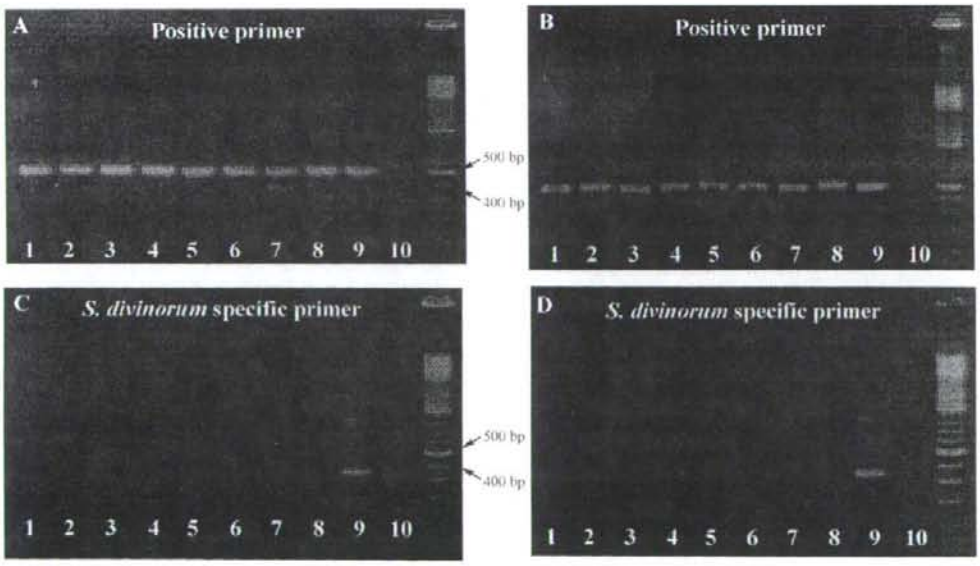

Fig. 4. ARMS Assay for the Commercial *Salvia* Cultivars

A and B show the PCR products amplified using positive primer (5S-P1 and 5S-P2). C and D show the PCR products amplified using *S. divinorum* specific primer (SD-F and SD3). Lanes 1 to 4 in A and C: Sa-1 to -4, lanes 5 to 8 in A and C: Sa-6 to -9, Lanes 1 to 8 in B and D: Sa-10 to -17, Lanes 9 and 10 in all panels mean positive control (Sa-58) and no template control, respectively.

であると思われる．PCR における温度プログラムを検討することにより，この PCR 産物の産生を抑制できるものと思われるが，目的の約 320 bp の PCR 産物が明瞭に検出できるため，本研究では，検討を行わなかった．

以上の結果から，園芸市場に流通する *Salvia* 属植物は，**1** を含有せず，*S. divinorum* の指定薬物への移行に際し，特に問題にならないことが明らかになった．また，文献既報の ARMS 法による基原種鑑別法は，非常に精度が高く，アメリカ大陸原産の *Salvia* 属植物との間でも良好な鑑別が可能であることが示された．本鑑別法は，*S. divinorum* が指定薬物となった現在，塩基配列解析を必要としない簡便な方法として，非常に有用であると考えられる．

Acknowledgements 本研究を行うに当たり，**1** の標品を御恵与頂きました徳島文理大学香川薬学部の代田修先生に御礼申し上げます．また，本研究は，厚生労働科学研究費補助金により行われた．

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work Programme for Research, which have added the obligation to address alternative methods in the field of pharmaceuticals, in September 2006 ECVAM has created a new Action on Validation for Pharmaceuticals. The main objective of the action is to validate new methodologies for a better prediction of safety profiles of pharmaceuticals. Non animal-based tests, relevant for target organ (e.g. liver, kidney, brain) and target system-specific toxicities (e.g. the haemopoietic system and the immune system) will be developed and validated to be incorporated into testing strategies for the estimation of human systemic toxicity. As an example, the optimisation of a set of in vitro assays to detect immunotoxicity is ongoing. Different endpoints have been assessed and an appropriate number of chemical substances have been be chosen, representatives of the full range of in vivo responses and for which good human and/or animal bioavailability data are available either from databases or literature. The reproducibility of the assays and the standardised protocols will be documented.

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P27

A preliminary study for assessing the feasibility of sebum sampling for monitoring human exposure to environmental chemicals following inadvertent or malicious release

James C. Wakefield [1], Kalwinder Kaur [2], Robert P. Chilcott [1,2]

[1] *CBRN and Chemical Toxicology Group, Chemical Hazards and Poisons Division HQ, Centre for Radiation, Chemical and Environmental Hazards, Health Protection Agency, Chilton, Didcot, Oxon, OX11 0RQ, United Kingdom*

[2] *School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom*

E-mail address: james.wakefield@hpa.org.uk (J.C. Wakefield).

Sebum is an oily substance secreted onto the skin surface by the sebaceous glands, and comprises a mixture of wax esters, triglycerides, fatty acids and squalene. It forms a thin layer over the surface of the skin, particularly in regions which are normally subject to constant environmental exposure such as the face (Kligman, 1963). As such, environmental chemicals which are lipophilic in nature may preferentially partition into sebum and so appropriate acquisition of sebum samples may potentially provide a convenient, non-invasive matrix for monitoring exposure of the general public to chemicals in the environment. Sebum samples from the forehead, for example, could be collected in order to confirm, identify or quantify putative exposure following an incident involving either inadvertent or malicious release of toxic chemicals into the environment.

The purpose of this study was to develop an *in vitro* model to assess the feasibility of using sebum as a biological substrate for monitoring exposure to environmental chemicals. The model comprised a layer of artificial sebum (Motwani et al., 2002) of 2 mg cm^{-2} (corresponding to a nominal thickness of 1 μm) deposited on the interior face of a vial cap. The sebum film was exposed to saturated vapours of radiolabelled solvents (^{14}C-benzene and ^{14}C-methanol) to model exposure to environmental chemicals. The solvents were selected due to their contrasting log P values and their relative volatility. The two experimental parameters investigated were (1) effect of exposure duration (0–60 min) on accumulation of material in sebum (absorption kinetics) and (2) effect of time-delay between exposure and sampling (0–60 min) on recovery of material from sebum (i.e. 'signal decay').

A linear, time-dependent increase in the uptake into the sebum film was observed for both benzene and methanol, with no indication that the sebum had reached saturation with either chemical after the maximum 60 min exposure. The maximum rate of uptake for benzene into the sebum film was 0.45 mg/mg sebum/hour. The maximum rate of uptake for methanol into the sebum film was 0.25 mg/mg sebum/hour. The increased uptake of benzene into sebum compared to methanol is considered to be due to the lipophilic nature of benzene having a much higher affinity for the lipid-rich sebum than methanol.

Following removal from the exposure the amount of both benzene and methanol entrapped within the sebum film reduced exponentially. Approximately 90% of the weight of both benzene and methanol taken up into the sebum was lost through desorption to the atmosphere after the first 5 min, followed by a period of slower desorption. A residual, quantifiable amount of both benzene and methanol did, however, remain present within the sebum 60 min post exposure (0.94 μg/mg sebum and 5.12 μg/mg sebum for benzene and methanol, respectively) (Table 1).

This *in vitro* study has confirmed that environmental chemicals may potentially be trapped in the sebum of exposed individuals *in vivo* and as such, sebum samples may be used to monitor exposure to chemicals in the environment. Further work is required to ascertain the effect of more realistic vapour concentrations and the interactions of a wider range of airborne chemicals with sebum.

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P29

Malaysian *Kratom*, a phyto-pharmaceutical of abuse: Studies on the mechanism of its cytotoxicity

Nor Aini Saidin [1], Thomas Randall [1], Hiromitsu Takayama [2], Elaine Holmes [1], Nigel J. Gooderham [1]

[1] *Department of Biomolecular Medicine, Faculty of Medicine, Imperial College, London, SW7 2AZ, UK*

[2] *Graduate School of Pharmaceutical Sciences, University of Chiba, Japan*

E-mail address: n.saidin05@imperial.ac.uk (N.A. Saidin).

We have reported recently that *Mitragyna speciosa* Korth extract (MSE) exerted dose-dependent cytotoxicity in several human can-

Table 1
Uptake and desorption of benzene and methanol into artificial sebum

Off-gassing time (min)	Benzene (μg/mg of sebum)					Methanol (μg/mg of sebum)				
	Exposure time (min)					Exposure time (min)				
	5	10	20	40	60	5	10	20	40	60
0	73.72	118.78	249.23	310.31	410.48	79.43	122.38	149.02	200.65	248.61
60	1.77	4.33	0.53	0.91	0.94	2.82	3.18	4.11	5.76	5.12

Values are expressed as mean ($n = 5$ for each exposure and off-gassing time) in μg/mg of sebum.

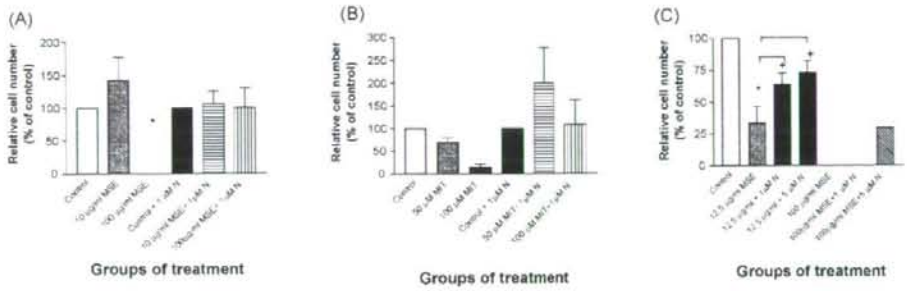

Fig. 1. Trypan blue exclusion assay of SH-SY5Y cells after 24 h treatment with (A) MSE ± naloxone (N) and (B) MIT ± naloxone. Clonogenicity of SH-SY5Y cells (C) after 24 h treatment with MSE ± naloxone. $N = 3 \pm$ S.E.M., $^{*}P < 0.05$ vs. Control and $^{+}P < 0.05$ vs. MSE alone, ANOVA with Tukey post test.

cer cell lines; SH-SY5Y cells (neuronal cells) was the most sensitive cell line examined (Saidin and Gooderham, 2007). To determine whether the cytotoxicity of MSE was accompanied by DNA damage, the Mouse Lymphoma tk gene mutation assay (MLA) was performed and the possible role of metabolic activation in the toxicity of MSE was investigated using lymphoblastoid cells, MCL-5 (metabolically competent) and cHol cells (non-metabolic competent). In parallel experiments, we examined the toxicity of mitragynine (MIT), a major alkaloid present in the extract of *Mitragyna speciosa* Korth (*Kratom*).

Both MSE and MIT are cytotoxic to human neuronal cells (SH-SY5Y), but neither were genotoxic in the MLA in the presence or absence of rat liver S9. In incubations with human lymphoblastoid cells, MSE was toxic, with toxicity being greater in metabolically competent MCL-5 cells compared to the cHol cells (lack of drug metabolism activity). Studies with metabolic inhibitors, ketoconazole, diethyldithiocarbamate, α-naphtoflavone and 3-amino-1,2,4-triazole (ATZ) indicated that CYP2E1 (inhibitable by ATZ) enhanced MSE and MIT toxicity. MIT is reported to exert opiate-like properties via opioid receptors (Watanabe et al., 1997). We therefore examined the effect of naloxone, an opioid antagonist on MSE and MIT cytotoxicity. Naloxone begins to inhibit the MSE toxicity at 100 μg/ml and mitragynine at 50 μM concentrations (Fig. 1). These finding were further confirmed by clonogenicity assay, which assesses longer-term effects.

These data indicate that the cytotoxicity of MSE and its major alkaloid mitragynine, is enhanced in the presence of CYP2E1 and is reversed by opioid antagonism.

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P30

Uptake of Cr (VI) and Cr (III)–GSH complex across rat liver cell nuclei

Vijay Krishna Raghunathan [1], Justice N.A. Tettey [2], M. Helen Grant [1]

[1] *Bioengineering Unit, University of Strathclyde, Glasgow, G4 0NW, UK*
[2] *Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, G4 0NW, UK*

E-mail address: m.h.grant@strath.ac.uk (V.K. Raghunathan).

Cr (VI) is a known carcinogen that readily crosses the cellular membrane and is then reduced by intracellular reductants such as reduced glutathione (GSH) to Cr (III), forming various reactive intermediate compounds (Ding and Shi, 2002). The interaction of Cr (VI) and GSH is known to be one of the causes of DNA damage *in vitro* (Mazzer et al., 2007). This study investigates the occurrence of Cr (III)–GSH in freshly isolated rat hepatocytes incubated with Cr (VI), and the uptake of Cr (VI) and Cr (III)–GSH complex across rat liver cell nuclei.

Cr (III)–GSH complex was synthesised by the method of Levina et al. (2003), and its formation confirmed by liquid chromatography–mass spectrometry (LC–MS). Freshly isolated male Sprague-Dawley rat hepatocytes (2×10^6 cells per ml) were incubated with 0, 50 and 100 μM Cr (VI) in Krebs–Henseleit (KH) buffer, pH 7.4 for 2 h, and GSH depletion and the occurrence of Cr (III)–GSH complex were investigated.

Nuclei (2×10^6 per ml), isolated from male Sprague-Dawley rat liver using Nuclei PURE prep (Sigma Aldrich, UK), were exposed to increasing concentrations of Cr (VI) (0, 0.05, 0.1, 0.5, 5 and 10 μM) plus ^{51}Cr (VI) (5 μCi) for 90 min in KH buffer. The Cr (III)-GSH complex was also incubated with nuclei after formation for 2 h as described above with increasing concentrations of Cr (VI) (0, 0.05, 0.1, 0.5, 5 and 10 μM), ^{51}Cr (VI) (5 μCi) and 70 μM GSH. Uptake of Cr was determined by liquid scintillation counting. Contamination of the nuclear fraction by cytoplasmic protein, microsomes and mitochondria was assessed by measuring lactate dehydrogenase, glucose-6-phosphatase and succinate cytochrome *c* reductase activities respectively. Integrity of the nuclear membrane was determined microscopically by the Trypan blue exclusion test.

Although the incubation of hepatocytes with Cr (VI) (100 μM) resulted in the depletion of intracellular GSH, the Cr (III)–GSH complex was not detected by LC–MS. The uptake of Cr (VI) and Cr (III)–GSH complex across the nuclei increased with increasing concentrations of Cr, and occurred to a similar extent (Table 1). Hneihen et al. (1992) found that Cr (VI), Cr (III) and their GSH complexes bind to salmon sperm nuclear DNA. It is feasible that the Cr (III)–GSH complex generated in situ in hepatocytes binds to nuclear DNA.

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MGM-9 [(*E*)-methyl 2-(3-ethyl-7a,12a-(epoxyethanoxy)-9-fluoro-1,2,3,4,6,7,12,12b-octahydro-8-methoxyindolo[2,3-*a*]quinolizin-2-yl)-3-methoxyacrylate], a derivative of the indole alkaloid mitragynine: A novel dual-acting μ- and κ-opioid agonist with potent antinociceptive and weak rewarding effects in mice

Kenjiro Matsumoto [a,*], Hiromitsu Takayama [b], Minoru Narita [c], Atsushi Nakamura [c], Masami Suzuki [c], Tsutomu Suzuki [c], Toshihiko Murayama [d], Sumphan Wongseripipatana [e], Kaori Misawa [b], Mariko Kitajima [b], Kimihito Tashima [a], Syunji Horie [a]

[a] Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University, 1 Gumyo, Togane, Chiba 283-8555, Japan
[b] Department of Molecular Structure and Biological Function, Graduate School of Pharmaceutical Sciences, Chiba University, 1–33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
[c] Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan
[d] Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Chiba University, 1–33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
[e] Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

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ABSTRACT

Mitragynine is a major indole alkaloid isolated from the Thai medicinal plant *Mitragyna speciosa* that has opium-like properties, although its chemical structure is quite different from that of morphine. We attempted to develop novel analgesics derived from mitragynine, and thus synthesized the ethylene glycol-bridged and C10-fluorinated derivative of mitragynine, MGM-9 [(*E*)-methyl 2-(3-ethyl-7a,12a-(epoxyethanoxy)-9-fluoro-1,2,3,4,6,7,12,12b-octahydro-8-methoxyindolo[2,3-*a*]quinolizin-2-yl)-3-methoxyacrylate]. We hypothesized that a dual-acting μ- and κ-opioid agonist could produce potent antinociceptive effects with fewer rewarding effects compared with μ agonists. In this study, MGM-9 exhibited high affinity for μ- and κ-opioid receptors with Ki values of 7.3 and 18 nM, respectively. MGM-9 showed a potent opioid agonistic effect, and its effects were meditated by μ- and κ-opioid receptor mechanisms in in vitro assays. Subcutaneous and oral administration of MGM-9 produced potent antinociceptive effects in mouse tail-flick, hot-plate, and writhing tests. When administered orally, the antinociceptive effect of MGM-9 was seven to 22 times more potent than that of morphine. The antinociceptive effects of MGM-9 were mediated by both μ- and κ-opioid receptors. Subcutaneous administration of MGM-9 twice daily for 5 days led to antinociceptive tolerance. In the gastrointestinal transit study, MGM-9 inhibited gastrointestinal transit, but its effect was weaker than that of morphine at equi-antinociceptive doses. Furthermore, MGM-9 induced less hyperlocomotion and fewer rewarding effects than morphine. The rewarding effect of MGM-9 was blocked by a μ antagonist and enhanced by a κ antagonist. Taken together, the results suggest that MGM-9 is a promising novel analgesic that has a stronger antinociceptive effect and weaker adverse effects than morphine.

1. Introduction

For the clinical treatment of acute and chronic severe pain, morphine is the standard analgesic. Morphine-related derivatives have been synthesized by simplification and introduction of substituents into the morphine structure in order to develop powerful analgesics without side effects (Corbett et al., 2006). Analgesics such as fentanyl and buprenorphine have been consequently derived from morphine. Most of those used clinically have μ-receptor agonist profiles. Despite their profound utility in the management of pain, they have undesirable side effects such as constipation, development of dependence, and tolerance.

The traditional Thai herbal medicine *Mitragyna speciosa* has long been used in Thailand for its opioid-like effects (Burkill, 1935) and as a replacement for opium (Suwanlert, 1975). This medicinal herb contains many indole alkaloids (Takayama, 2004). Mitragynine,

* Corresponding author. Tel.: +81 475 534 585; fax: +81 475 534 590.
E-mail address: kenjiro@jiu.ac.jp (K. Matsumoto).

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a main constituent of this plant, is an indole alkaloid that is structurally different from morphine (Fig. 1). We have studied the pharmacological activities of mitragynine (Watanabe et al., 1997; Matsumoto et al., 2005b) and found that mitragynine has agonistic effects on opioid receptors, but its antinociceptive effect was less potent than that of the crude extract of *Mitragyna speciosa* (Watanabe et al., 1999) and morphine (Takayama, 2004).

Recently, we studied the opioid agonistic effects of the constituents of *Mitragyna speciosa* using in vitro assays. Among them, 7-hydroxymitragynine (Fig. 1), which has a hydroxyl group at the C7 position of mitragynine, produced the most potent effect, suggesting that the opioid effect of *Mitragyna speciosa* is mostly based on the activity of 7-hydroxymitragynine (Horie et al., 2005). 7-Hydroxymitragynine induced a potent antinociceptive effect in mice, and its effect was more potent than that of morphine when subcutaneously or orally administered and mediated by the μ-opioid receptor mechanism (Matsumoto et al., 2004, 2006). Furthermore, 7-hydroxymitragynine inhibited gastrointestinal transit less potently than morphine at each equi-antinociceptive dose (Matsumoto et al., 2006). We investigated the structural similarities between morphine and 7-hydroxymitragynine using molecular modeling techniques (Matsumoto et al., 2005a), but we could not superimpose all three functional groups, i.e., a nitrogen atom, a benzene residue, and an oxygen atom, on the benzene ring in the structures of morphine and 7-hydroxymitragynine. These functional groups play an important role in producing analgesic activity (Dhawan et al., 1996). Therefore, it is speculated that 7-hydroxymitragynine binds opioid receptor sites other than those that morphine binds.

These pharmacologically and chemically interesting properties of 7-hydroxymitragynine encouraged us to pursue further investigation for the development of novel analgesics, and we have synthesized a large number of mitragynine derivatives. Among them, an ethylene glycol-bridged and C10-fluorinated derivative of mitragynine, MGM-9 (Fig. 1), induced more potent opioid agonistic effects than morphine and 7-hydroxymitragynine in the electrical stimulation assay using a guinea-pig isolated ileum preparation (Takayama et al., 2006).

It is well known that μ-opioids induce potent antinociception, but they also induce psychological dependence during chronic administration. Activation of dopaminergic systems after administration of the μ-opioid agonist induces the development of rewarding effects. In contrast, κ-opioid receptors negatively modulate the activity of dopaminergic neurons and inhibit the rewarding effects mediated by μ-opioid receptors (Narita et al., 2001a,b). Therefore, we hypothesized that a dual-acting μ- and κ-opioid agonist would induce potent antinociceptive effects and fewer rewarding effects than μ agonists such as morphine.

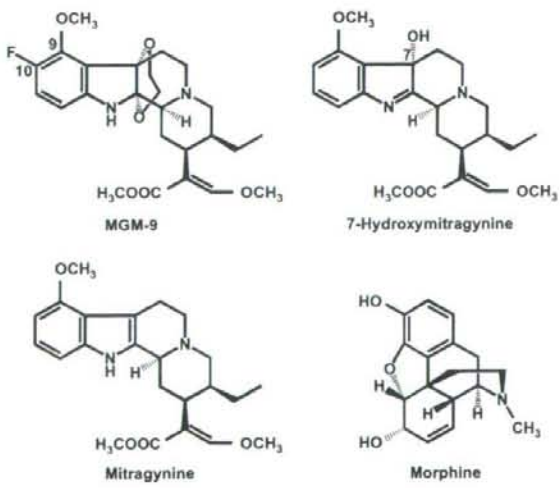

Fig. 1. Chemical structures of MGM-9, 7-hydroxymitragynine, mitragynine, and morphine

In the present study, we clarified the pharmacological profiles of MGM-9 in comparison with morphine and 7-hydroxymitragynine. We investigated the antinociceptive effects of MGM-9 after subcutaneous and oral administration to mice in tail-flick, hot-plate, and writhing tests. To determine the involvement of opioid receptor subtypes in the pharmacological effect of MGM-9, we investigated the opioid receptor selectivity of MGM-9 by a receptor-binding assay, mouse antinociceptive tests, and electrically stimulated guinea-pig ileum and mouse vas deferens assays using selective opioid antagonists. In addition, we investigated whether MGM-9 produces rewarding effects, hyperlocomotion, antinociceptive tolerance, and inhibits gastrointestinal transit to evaluate the morphine-like side effects of MGM-9. As a result, we found that μ- and κ-opioid receptor mechanisms are involved in the opioid agonistic effects of MGM-9. We found that MGM-9, a novel dual acting μ- and κ-opioid agonist, produces stronger antinociceptive effects and weaker adverse effects than morphine.

2. Methods

2.1. Animals

Male ddY-strain mice (Japan SLC, Hamamatsu, Japan) weighing 25–33 g and male Hartley strain guinea-pigs (Japan SLC) weighing 320–510 g were used. Animals were housed in a temperature-controlled room at 24 °C with lights on from 07:00 to 19:00 and 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 and the guidelines approved by the Ethical Committee on Animal Care and Animal Experimentation of Josai International University (#12). 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.

2.2. Drugs

The drugs used in this study were morphine hydrochloride (Takeda Chemical Ind., Osaka, Japan), naloxone hydrochloride (MP Biomedicals, Irvine, CA), naltrindole hydrochloride, nor-binaltorphimine dihydrochloride (norBNI), [D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin (DAMGO), *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl-cyclohexyl] benzeneacetamide methanesulfonate salt (U50,488), (5α,7α,8β)-(+)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69593), (Sigma Chemical Co., St. Louis, MO, USA), 5′-guanidyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2′,3′-indolomorphinan dihydrochloride (GNTI; Tocris-Cookson, Bristol, UK), and β-funaltrexamine hydrochloride (β-FNA; Tocris-Cookson, Bristol, UK). MGM-9 and 7-hydroxymitragynine were synthesized from mitragynine as described previously (Takayama et al., 2002, 2006). The purity (>99%) of these compounds was checked by high-performance liquid chromatography and ^{1}H-nuclear magnetic resonance (500 MHz) analysis.

For electrical stimulation of guinea-pig ileum and vas deferens, MGM-9 and cyprodime hydrobromide were first dissolved in 100% dimethylsulfoxide to yield a 5 mM solution, and then subsequently diluted with distilled water. The other drugs were dissolved in distilled water. For subcutaneous administration, MGM-9 and 7-hydroxymitragynine were dissolved in phosphate-buffered saline (pH 5.3–5.5). The other drugs were dissolved in saline. For oral administration and receptor-binding assays, MGM-9 and 7-hydroxymitragynine were dissolved in 25 mM phosphate buffer (pH 5.3–5.5). The other drugs were dissolved in distilled water.

For the antinociceptive test, naloxone (1 mg/kg), naltrindole (3 mg/kg), GNTI (3 mg/kg), norBNI (20 mg/kg), or β-FNA (40 mg/kg), was administered subcutaneously 15 min, 30 min, 30 min, 24 h, or 24 h, respectively, before drug injection. These protocols were described by Nozaki et al. (2005), Qi et al. (2006), Goncales et al. (2005), and Jinsmaa et al. (2004). For the place conditioning test, β-FNA (10 mg/kg) or norBNI (3 mg/kg) was administered subcutaneously 24 h or 4 h, respectively, before MGM-9 injection. These protocols were described by Narita et al. (2001a,b).

2.3. Receptor binding

The binding assays used to screen compounds are similar to those previously reported (Neumeyer et al., 2000). Guinea-pig brain membrane protein (without

cerebellum, 500 μg) fractions were incubated with 1 nM [^{3}H]DAMGO (50.0 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO), 0.25 nM [^{3}H]naltrindole (50.0 Ci/mmol; American Radiolabeled Chemicals), or 1 nM [^{3}H]U69593 (55.0 Ci/mmol; Amersham Bioscience, Bucks, UK) in a final volume of 1 ml of 50 mM Tris–HCl buffer. Non-specific binding was measured by inclusion of 10 μM naloxone. The incubation periods were 1, 3, and 1 h for [^{3}H]DAMGO, [^{3}H]naltrindole, and [^{3}H]U69593, respectively, at 25 °C. The reaction was terminated by rapid filtration under reduced pressure through glass fiber filters (Whatman GF/C, presoaked in 0.25% polyethyleneimine). After filtration, filters were washed three times with 3 ml of cold 50 mM Tris–HCl and counted in 4 ml of Ultima Gold scintillation cocktail (Perkin-Elmer, Boston, MA, USA). The ability of unlabeled drugs to inhibit specific radioligand binding was expressed as the IC_{50} value, which is 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).

2.4. Electrical stimulation of guinea-pig ileum

The guinea-pig ileum was dissected and placed in Krebs–Henseleit solution (in 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 37 °C 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 5 mm long needle) electrode. After equilibration, the ileum was transmurally stimulated 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. The height of the twitch response to transmural stimulation was measured before and after the drug challenge. The responses were expressed as contraction % of the twitch response to the transmural stimulation before the drug challenge as 100%.

2.5. Electrical stimulation of mouse vas deferens

Mouse vas deferens was dissected and placed in Krebs–Henseleit solution without $MgCl_2$. The tissues were placed under 0.2 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37 °C 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 5 mm long needle) electrode. After equilibration, the tissues were transmurally stimulated with a train of 10 pulses of 0.5 ms duration with 2 ms intervals every 1 min by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Contractions were isometrically 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. The responses were expressed as contraction % of the twitch response to the transmural stimulation before the drug challenge as 100%.

2.6. 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, vehicle, or saline, 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 s was used to prevent tissue damage.

Antinociception in the tail-flick test was quantified using the percentage of maximum possible effect (% MPE) and calculated as: % MPE = [(test latency − pre-drug latency)/(cut-off time − pre-drug latency)] × 100.

2.7. Hot-plate test

Animals were placed on an electrically heated plate at 55 ± 0.2 °C, and the latency period until the occurrence of nociceptive responses such as licking, shaking the legs, or jumping was measured. Prior to treatment with drugs, vehicle, or saline, 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 s was used to prevent tissue damage.

Antinociception in the hot-plate test was quantified using the percentage of maximum possible effect (% MPE) and calculated as: % MPE = [(test latency − pre-drug latency)/(cut-off time − pre-drug latency)] × 100.

2.8. Writhing test

Intraperitoneal injection of 0.6% (v/v) acetic acid at a volume of 0.1 ml/10 g body weight elicits writhing responses about 3–5 min after injection. The degree of nociception was assessed by counting the number of writhing responses (abdominal constrictions accompanied by stretching of the hind limbs) during a 15 min period in freely moving mice. MGM-9, 7-hydroxymitragynine, morphine, saline, or the vehicle was administered subcutaneously 10 min before the injection of acetic acid. MGM-9, 7-hydroxymitragynine, or the vehicle was administered orally 10 min before the injection of acetic acid. Morphine or distilled water was administered orally 30 min before the injection of acetic acid. These time points were established by the time of peak effect for each individual drug and by the protocol previously reported (Wells et al., 2001).

2.9. Development of tolerance

A 5-day consecutive administration regimen was used for the investigation of development of antinociceptive tolerance of drugs, as reported previously (Matsumoto et al., 2005a). Morphine (8 mg/kg), saline, 7-hydroxymitragynine (2 mg/kg), MGM-9 (1 mg/kg), or the vehicle was administrated subcutaneously twice daily (09:00 and 18:30) for 5 consecutive days. Antinociceptive effects were investigated using tail-flick test (described above). Measurements were performed at the time of peak effect for each individual drug, 30, 30, 15, 15, or 15 min after subcutaneous administration of morphine, saline, 7-hydroxymitragynine, MGM-9 or vehicle, respectively, on the first administration of day 1, 3, and 5. The development of tolerance was defined as the reduction of the antinociceptive effect of the each drugs compared with the effect produced by the first administration on day 1.

We investigated whether MGM-9 has the adverse effects induced by κ-opioid selective agonists, such as sedation and salivation. MGM-9 did not induce sedation and salivation in both acute administration and chronic administration at the doses used in the present study.

2.10. Gastrointestinal transit

Mice were fasted, with water available ad libitum, for 18 h before the experiments. Fifteen minutes after subcutaneous administration of MGM-9, 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. Fifteen minutes after oral administration of MGM-9, 7-hydroxymitragynine, or vehicle, and 30 min after oral administration of morphine or distilled water, a charcoal meal was orally administered. These time points were established by the time of peak effect for each individual drug and by the protocol as previously reported (Matsumoto et. al., 2006). Thirty minutes after administration of the charcoal meal, the animal was sacrificed by cervical dislocation, and the small intestine from the pylorus to the ileocecal junction was carefully removed. Both the length of the small intestine from the pylorus to the ileocecal junction and the farthest distance to which the charcoal meal had traveled were measured. For each animal, the 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.

2.11. Locomotor assay

The locomotor activity of mice was measured by an ambulometer as described previously (Narita et al., 1993). Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Counts of hyperlocomotor activity were collected in 10 min intervals for 180 min after drug administration.

2.12. Place conditioning

The place-conditioning procedure is used to evaluate the motivation properties, such as rewarding or aversive effects, of drugs in animals (Suzuki et al., 1991). Place conditioning studies were conducted using a shuttle box (mouse size, 15 cm wide × 30 cm long × 15 cm high) that was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor, and the other was black with a smooth floor to create equally preferable compartments. The place conditioning schedule consisted of three phases (preconditioning test, conditioning, and postconditioning test). The preconditioning test was performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, a neutral platform was inserted along the seam separating the compartments, and an animal that had not been treated with either drugs or each control was then placed on the platform. The time spent in each compartment during a 900 s session was recorded automatically with an infrared beam sensor (KN-80; Natsume Seisakusyo, Tokyo, Japan). We previously reported that chronic treatment with morphine (5 mg/kg, s.c.) (Narita et al., 2005) produced a significant preference for the drug-associated place in the conditioned place preference test. Conditioning sessions (3 days for drugs, 3 days for each control)

were started the day after the preconditioning test and conducted once daily for 6 days. Immediately after subcutaneous administration of morphine (5 mg/kg), 7-hydroxymitragynine (0.5, 1 or 2 mg/kg), MGM-9 (0.5, 1 or 2 mg/kg), vehicle, or saline, the animal was placed for 1 h in the compartment opposite to that in which it had spent the most time in the preconditioning test. On alternate days, the animals and each control were placed in the other compartment for 1 h. On the day after the final conditioning session, these animals were placed in the test apparatus without any restrictions, and then the relative amount of time spent in each compartment was measured (postconditioning). The preference for the drug-paired place was shown as the mean difference between time spent during the postconditioning and preconditioning tests.

2.13. Statistical analysis

The data are expressed as the mean ± SEM. 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

3.1. Receptor binding

The affinities of MGM-9 for the three opioid receptor types were determined by evaluating the inhibition of binding of ligands to μ-, δ-, and κ-opioid receptors (Table 1). MGM-9 had a relatively high affinity for μ- and κ-opioid sites. The Ki value for MGM-9 displacement of [^{3}H]DAMGO and [^{3}H]U69593 binding to μ- and κ-opioid sites were 7.3 ± 0.24 and 18 ± 2.5 nM, respectively. MGM-9 weakly displaced [^{3}H]naltrindole binding to δ-opioid sites (Ki value of 350 ± 28 nM). 7-Hydroxymitragynine interacted with all three opioid sites but bound preferentially to μ-opioid receptors with Ki values of 11 ± 1.6 nM.

3.2. Electrically induced contraction in guinea-pig ileum and mouse vas deferens

In vitro biological activities of MGM-9 were investigated in electrically stimulated guinea-pig ileum and mouse vas deferens assays using μ-, δ-, and κ-opioid selective antagonists (Fig. 2). MGM-9 and morphine inhibited the twitch contraction in a concentration-dependent manner ($n = 5$ or 6). The pD_2 values of MGM-9 and morphine were 8.40 ± 0.02 and 7.15 ± 0.05, respectively. The μ-opioid receptor antagonist cyprodime (1 μM) and the κ-opioid receptor antagonist norBNI (30 nM) partially reversed the twitch contraction inhibited by MGM-9 (30 nM) in guinea-pig ileum (Fig. 2A). Coadministration of cyprodime and norBNI completely reversed the effect of MGM-9 (Fig. 2A). In mouse vas deferens, MGM-9 showed weak activity in the twitch contraction (3 μM, 52.4 ± 3.7%). Naltrindole (30 nM), a δ-opioid receptor antagonist, did not reverse the twitch contraction inhibited by MGM-9 (3 μM) in the mouse vas deferens (Fig. 2B). The dose of selective antagonists used in these experiments completely and selectively reversed the effect of each selective agonist (Matsumoto et al., 2006).

Table 1
Inhibition constants of μ-, δ-, and κ-opioid binding to guinea-pig brain membrane by test compounds

Compound	Ki (nM ± SEM)		
	[^{3}H]DAMGO (μ)	[^{3}H]Naltrindole (δ)	[^{3}H]U69593 (κ)
Morphine	2.7 ± 0.24	310 ± 9.3	55 ± 4.1
MGM-9	7.3 ± 0.24	350 ± 28	18 ± 2.5
7-Hydroxymitragynine	11 ± 1.6	96 ± 13	85 ± 4.8
DAMGO	1.2 ± 0.092	330 ± 46	680 ± 54
Naltrindole	30 ± 1.5	0.11 ± 0.012	8.3 ± 0.47
U69593	2300 ± 140	10000 ± 360	0.66 ± 0.073

The μ-binding sites were labeled with [^{3}H]DAMGO (1 nM), δ-binding sites with [^{3}H]naltrindole (0.25 nM) and κ-binding sites with [^{3}H]U69593 (1 nM). Data are expressed as the mean Ki value ± SEM for three determinations performed in duplicate.

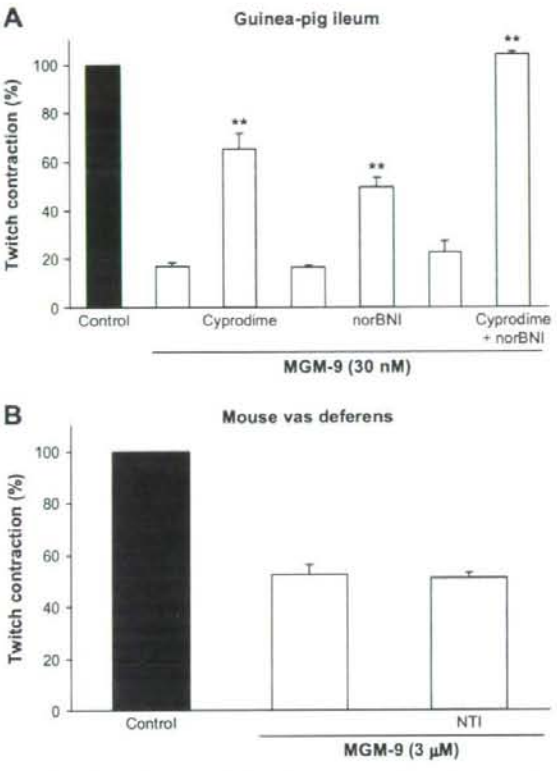

Fig. 2. Effect of cyprodime (1 μM) and nor-binaltorphimine (norBNI, 30 nM) on the twitch contraction inhibited by MGM-9 (30 nM) in the guinea-pig ileum preparation (A). Effect of naltrindole (NTI, 30 nM) on the twitch contraction inhibited by MGM-9 (3 μM) in the mouse vas deferens preparation (B). Control represents the twitch contraction before adding MGM-9 as 100%. Each value represents the mean ± SEM of five animals. **, $P < 0.01$ significantly different from the values before the addition of antagonist (Student's *t*-test).

3.3. Antinociception of MGM-9 in mice tail-flick, hot-plate, and writhing tests by subcutaneous and oral administration

Antinociceptive effects of MGM-9, 7-hydroxymitragynine, and morphine were investigated in acute thermal pain tests in mice. MGM-9 (0.25–2 mg/kg) induced dose-related antinociceptive responses in the tail-flick and hot-plate tests after subcutaneous administration (Fig. 3A,B). 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 MGM-9 were 0.57 mg/kg (0.36–0.90) and 0.70 mg/kg (0.42–1.17) in the tail-flick and the hot-plate tests, respectively. Morphine (1.25–8 mg/kg, s.c.) induced dose-related antinociceptive responses with a peak effect at 30 min in both tests (Fig. 4A,B). Compared to morphine, MGM-9 was eight and six times more potent in the tail-flick and hot-plate tests, respectively (Fig. 5A,C, Table 2). Potent and dose-related antinociceptive responses were exhibited in both tests in response to oral administration of MGM-9 (1–8 mg/kg) (Fig. 3C,D). The effect peaked at 15 and 7.5 min after injection in the tail-flick and hot-plate tests, respectively. The ED_{50} values for MGM-9 were 2.84 mg/kg (1.60–5.05) and 2.98 mg/kg (1.79–4.92) in the tail-flick and

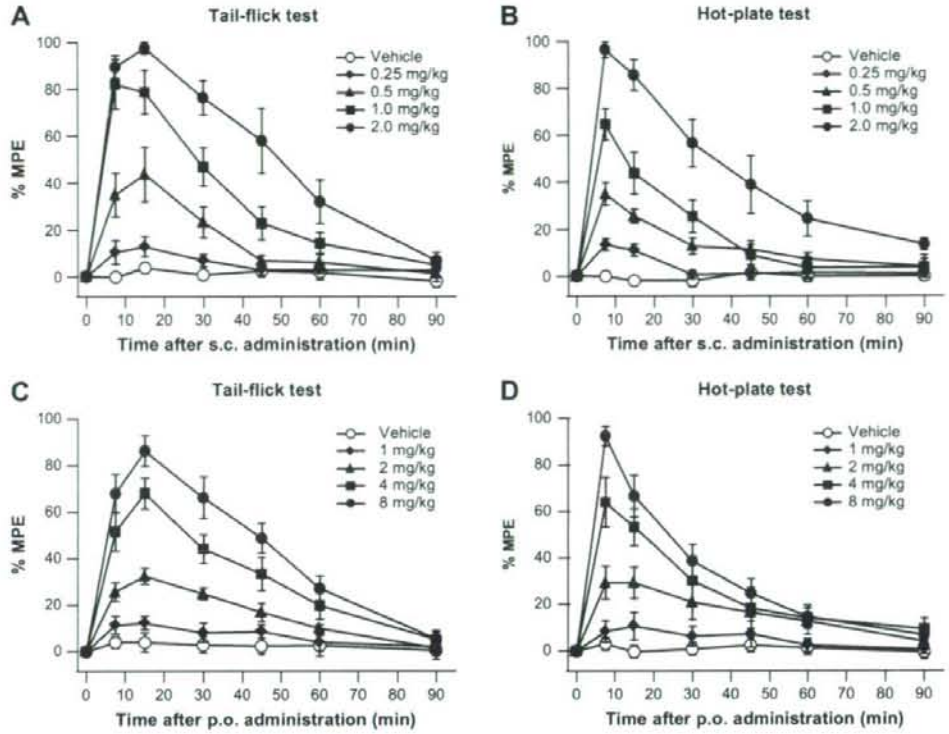

Fig. 3. Time course of the antinociceptive effects produced by subcutaneous (A, B) or oral (C, D) administration of MGM-9 in the tail-flick test (A, C) and hot-plate test (B, D) in mice. Each value represents mean ± SEM of data obtained from eight mice.

hot-plate tests, respectively. Morphine (25–100 mg/kg, p.o.) induced dose-related antinociceptive responses with a peak effect at 60 and 30 min in tail-flick and hot-plate tests, respectively (Fig. 4C,D). Compared to morphine, MGM-9 was 22 and 16 times more potent in the tail-flick and hot-plate tests, respectively (Fig. 5B,D, Table 3).

In the acetic acid writhing test, MGM-9 induced potent and dose-related antinociceptive responses in mice after subcutaneous and oral administration. The ED_{50} values (95% confidence limits) for MGM-9 were 0.06 mg/kg (0.03–0.09) and 0.63 mg/kg (0.40–0.99) after subcutaneous and oral administration, respectively. MGM-9 was about two times more potent than 7-hydroxymitragynine (Fig. 5E,F, Tables 2 and 3). Compared to morphine, MGM-9 was 8 and 7 times more potent after subcutaneous and oral administrations, respectively (Tables 2 and 3).

3.4. Effects of selective antagonists on MGM-9 antinociception in mouse pain models

In order to determine the opioid receptor type selectivity of MGM-9 antinociception, mice were pretreated with selective opioid receptor antagonists in the tail-flick, hot-plate, and writhing tests (Fig. 6). We chose a dose of MGM-9 that produces a response of 80–90% (Fig. 5) to detect the effects of the antagonists easily. In the tail-flick test, the antinociceptive effect of MGM-9 was completely blocked by the non-selective opioid antagonist naloxone, and it was significantly blocked by the irreversible μ-opioid receptor selective antagonist β-FNA and the κ-opioid receptor selective antagonist norBNI in the tail-flick, hot-plate, and writhing tests. The antinociceptive effect of MGM-9 was completely blocked by coadministration of β-FNA and norBNI in the three tests. To confirm the involvement of κ-opioid receptor in the antinociceptive effect of MGM-9, the effect of GNTI, a selective κ-opioid antagonist, was investigated in the tail-flick assay. GNTI (3 mg/kg, s.c.) significantly blocked the antinociceptive effects of MGM-9 (27.4 ± 4.9% MPE, $P < 0.01$, $n = 7$). The antinociceptive effect of U50,488 (10 mg/kg, s.c., 86.6 ± 6.2% MPE, $n = 6$) was completely blocked by the same dose of GNTI (6.1 ± 3.4% MPE, $P < 0.01$, $n = 6$). The selective δ-antagonist naltrindole was ineffective against MGM-9-mediated antinociception in the three tests. When these opioid antagonists were administered subcutaneously alone at the doses used in the present study, they did not produce any change in the three test results (data not shown).

3.5. Development of tolerance due to repeated subcutaneous administration of morphine, 7-hydroxymitragynine, or MGM-9

To investigate the development of antinociceptive tolerance, mice were subcutaneously administered morphine (8 mg/kg), 7-hydroxymitragynine (2 mg/kg), or MGM-9 (1 mg/kg) twice daily for 5 days, and antinociceptive effects were measured on day 1, 3, and 5 using the tail-flick test (Fig. 7). On day 1, each compound showed a similar degree of antinociception in the tail-flick test. To examine tolerance development due to repeated administration, the %MPE on test days 3 and 5 were compared with those on the first test day. Compared with day 1, morphine produced a significant reduction in %MPE on days 3 and 5. MGM-9 and 7-hydroxymitragynine also produced a decrease in %MPE across days, and there was a significant difference on day 5 compared with day 1.

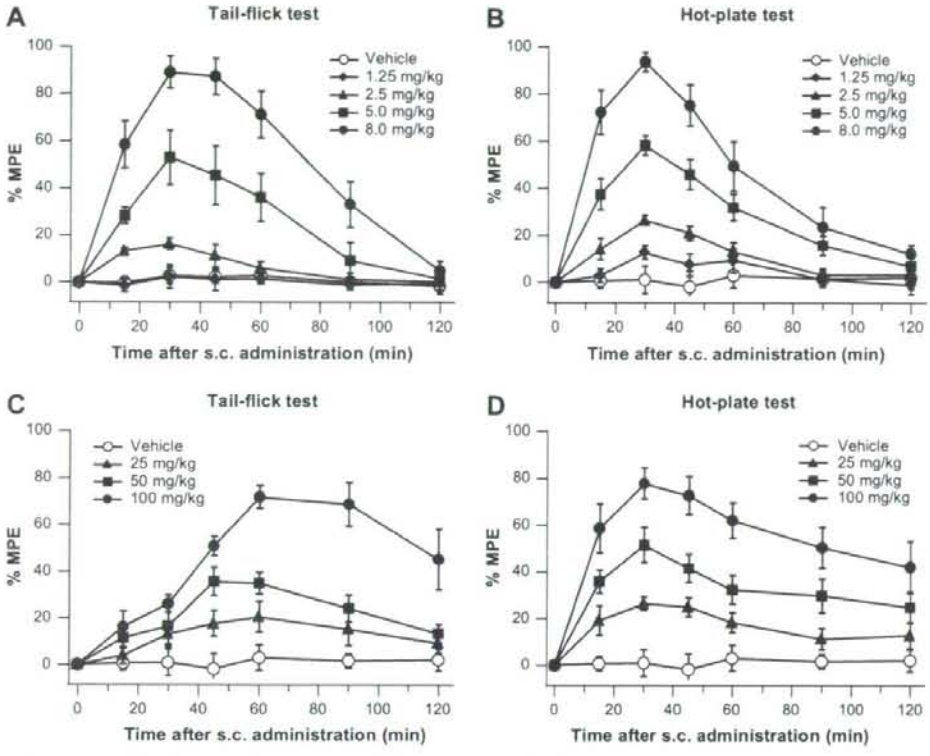

Fig. 4. Time course of the antinociceptive effects produced by subcutaneous (A, B) or oral (C, D) administration of morphine in the tail-flick test (A, C) and hot-plate test (B, D) in mice. Each value represents mean + SEM of data obtained from seven to eight mice.

3.6. Effect of MGM-9 on gastrointestinal transit

The effect of MGM-9 on the passage of a charcoal meal was examined after subcutaneous and oral administration (Fig. 8). MGM-9 (0.25–4 mg/kg), 7-hydroxymintragynine, and morphine dose-dependently inhibited gastrointestinal transit after subcutaneous administration (Fig. 8A). The ED_{50} values (95% confidence limits) for MGM-9, 7-hydroxymitragynine, and morphine were 1.30 mg/kg (0.73–2.31), 1.19 mg/kg (0.54–2.63), and 1.07 mg/kg (0.40–2.86), respectively (Table 2). The oral administration of MGM-9 (2–16 mg/kg) also inhibited gastrointestinal transit dose-dependently (Fig. 8B). The ED_{50} values (95% confidence limits) for MGM-9, 7-hydroxymitragynine, and morphine were 11.1 mg/kg (6.0–20.7), 7.50 mg/kg (3.95–14.19), and 11.7 mg/kg (5.6–24.6), respectively (Table 3).

3.7. Effect of MGM-9 and 7-hydroxymitragynine on spontaneous locomotor activity

To determine the effects of MGM-9 and 7-hydroxymitragynine on the central nervous system, locomotor activity was investigated in mice after subcutaneous administration (Fig. 9). MGM-9 (2, 4 mg/kg) and 7-hydroxymitragynine (1–4 mg/kg) increased locomotive activity in a dose-dependent manner. The effect peaked at 10–20 min after injection (Fig. 9A). As shown in Fig. 9B, 7-hydroxymitragynine (2, 4 mg/kg) significantly increased locomotor activity compared to the vehicle. Morphine (10 mg/kg) induced hyperlocomotion, and its effect peaked at 50–70 min after injection (Fig. 9A,B). MGM-9 (4 mg/kg) significantly increased locomotor activity, but its effect was weaker than that of morphine and 7-hydroxymitragynine.

3.8. Effect of MGM-9 and 7-hydroxymitragynine on place conditioning

We next investigated the rewarding effects of MGM-9 and 7-hydroxymitragynine using the place conditioning test in mice. As shown in Fig. 10A, the 2 mg/kg dose of 7-hydroxymitragynine induced a significant preference for the drug-associated place compared to the vehicle group. Morphine at 5 mg/kg also induced a significant preference compared to the saline group, as reported previously (Narita et al., 2003). In contrast, MGM-9 (0.5–2 mg/kg) did not induce a significant preference for the drug-associated place compared to the vehicle group. The effect of β-FNA and norBNI on the MGM-9 (2 mg/kg, s.c.)-induced place preference is shown in Fig. 10B. Pretreatment with β-FNA (10 mg/kg, s.c.) significantly blocked the MGM-9-induced place preference. On the other hand, pretreatment with norBNI (3 mg/kg, s.c.) significantly enhanced MGM-9-induced place preference. These opioid receptor antagonists alone did not produce either preference or aversion for the drug-associated place (data not shown).

4. Discussion

Our research group has isolated uniquely structured alkaloids from the traditional Thai herb *Mitragyna speciosa*. We previously reported that 7-hydroxymitragynine, a minor constituent of *Mitragyna speciosa*, produced a potent antinociceptive effect in

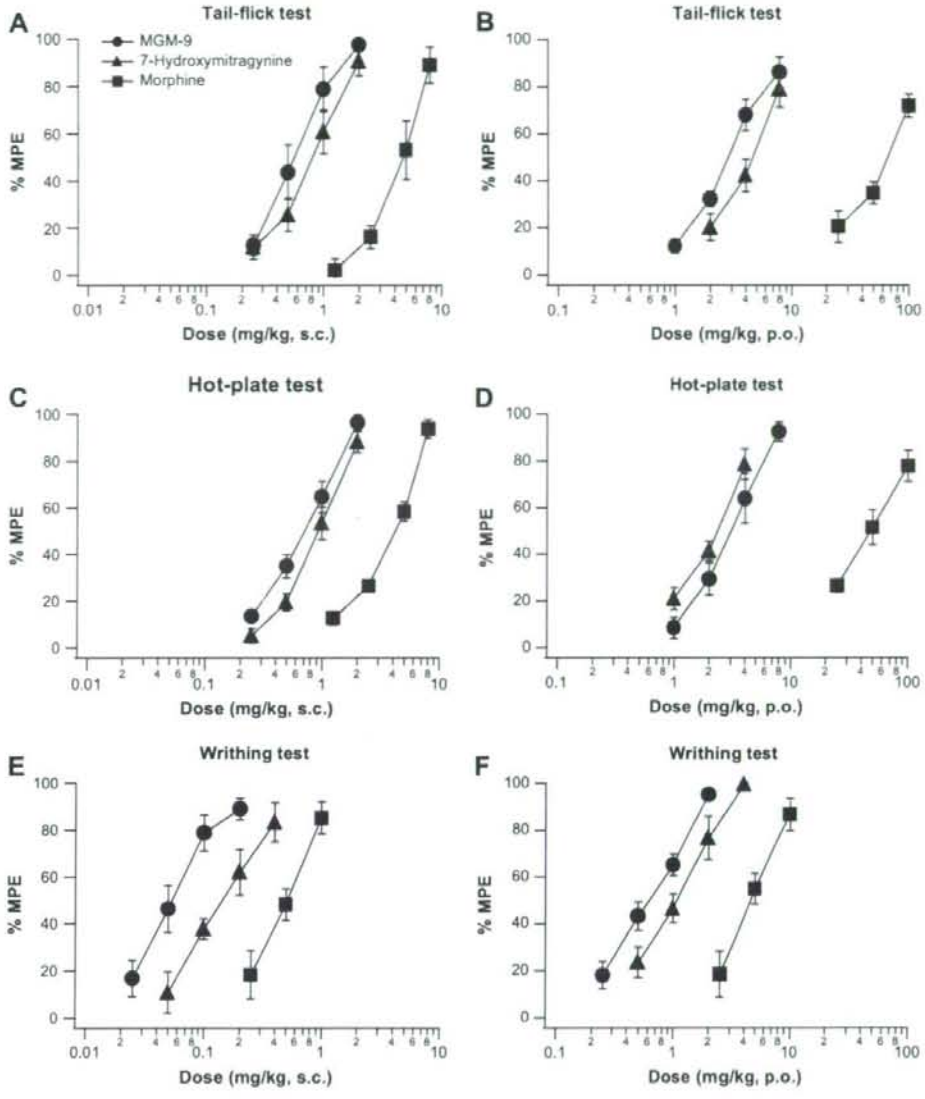

Fig. 5. Dose-response curves for antinociceptive effect of subcutaneous (A, C, E) or oral (B, D, F) administration of MGM-9, 7-hydroxymitragynine, and morphine in tail-flick (A, B), hot-plate (C, D), and writhing (E, F) tests in mice. Each value represents mean ± SEM of data obtained from seven to nine mice.

mice after systemic administration, that its effects were mediated by μ-opioid receptors (Matsumoto et al., 2004), and that the opioid effect of *Mitragyna speciosa* is mostly based on the activity of 7-hydroxymitragynine (Horie et al., 2005). In the course of studying the structure-activity relationship of mitragynine derivatives, we found the ethylene glycol-bridged and C10-fluorinated derivative of mitragynine MGM-9. In the present study, we investigated the pharmacological profiles of MGM-9 in comparison with morphine and 7-hydroxymitragynine in in vitro and in vivo experiments.

Table 2
Antinociceptive effects and inhibition of gastrointestinal transit (IGIT) produced by subcutaneous administration of morphine, 7-hydroxymitragynine and MGM-9 in mice

Compound	Morphine	MGM-9	7-Hydroxymitragynine
Tail-flick	4.57 (3.12–6.69)	0.57 (0.36–0.90)	0.80 (0.48–1.33)
Hot-plate	4.08 (2.75–6.06)	0.70 (0.42–1.17)	0.93 (0.59–1.45)
Writhing	0.50 (0.31–0.80)	0.06 (0.03–0.09)	0.15 (0.09–0.24)
IGIT	1.07 (0.40–2.86)	1.30 (0.73–2.31)	1.19 (0.54–2.63)

ED_{50} represents the median effective dose (mg/kg) (95% confidence limits).

Table 3
Antinociceptive effects and inhibition of gastrointestinal transit (IGIT) due to oral administration of morphine, 7-hydroxymitragynine, and MGM-9 in mice

Compound	Morphine	MGM-9	7-Hydroxymitragynine
Tail-flick	63.0 (37.2–106.8)	2.84 (1.60–5.05)	4.43 (1.57–6.93)
Hot-plate	48.2 (27.5–84.5)	2.98 (1.79–4.92)	2.23 (1.38–3.60)
Writhing	4.60 (2.87–7.38)	0.63 (0.40–0.99)	1.05 (0.62–1.78)
IGIT	11.7 (5.6–24.6)	11.1 (6.0–20.7)	7.50 (3.95–14.19)

ED_{50} represents the median effective dose (mg/kg) (95% confidence limits).

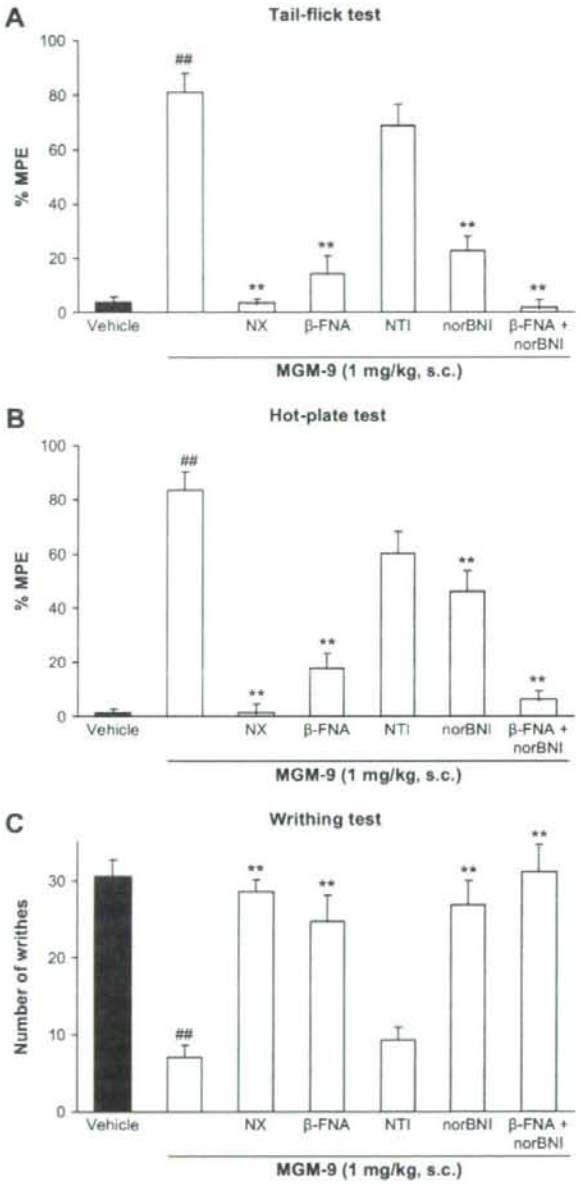

Fig. 6. Effects of opioid receptor antagonists on antinociception by subcutaneous (s.c.) administration of MGM-9. The antinociceptive effect of MGM-9 was determined in mice by the tail-flick (A), hot-plate (B), and writhing (C) tests after s.c. administration of the following antagonists: naloxone (NX, 1 mg/kg), β-funaltrexamine (β-FNA, 40 mg/kg), naltrindole (NTI, 3 mg/kg), and nor-binaltorphimine (norBNI, 20 mg/kg). Measurements were performed 15, 7.5, and 10 min after s.c. administration of MGM-9 in the tail-flick, hot-plate, and writhing tests, respectively. Each value represents mean ± SEM of eight or nine mice. The # denotes values that were significantly different from vehicle-treated mice by Student's *t*-test (##, $P < 0.01$). The asterisk (*) denotes values that were significantly different from mice treated with MGM-9 alone by Bonferroni correction (*, $P < 0.05$, **, $P < 0.01$).

The in vivo studies revealed that MGM-9 produced a dose-dependent and strong antinociceptive effect when subcutaneously and orally administered to mice in tail-flick, hot-plate, and writhing tests. The antinociceptive effect of MGM-9 was about eight and

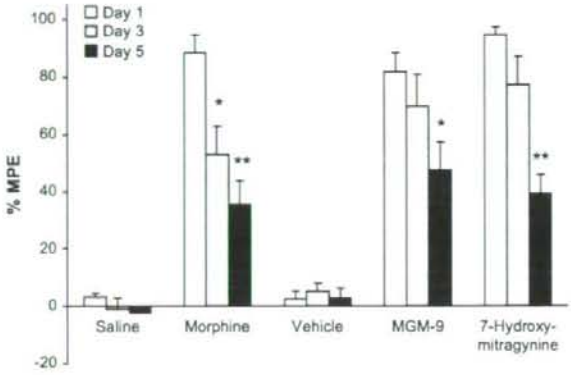

Fig. 7. Development of tolerance to morphine, 7-hydroxymitragynine, or MGM-9-induced antinociception in mice tail-flick test. Morphine (8 mg/kg), 7-hydroxymitragynine (2 mg/kg), or MGM-9 (1 mg/kg) was administrated twice daily for 5 consecutive days. Measurements were performed 30, 15, or 15 min after subcutaneous administration of morphine (8 mg/kg), 7-hydroxymitragynine (2 mg/kg), or MGM-9 (1 mg/kg), respectively, on the first administration of day 1, 3, and 5. Each value represents mean ± SEM of data obtained from eight mice. The asterisk (*) denotes values that were significantly different from those on day 1 for morphine, 7-hydroxymitragynine, or MGM-9 by Bonferroni correction (*, $P < 0.05$, **, $P < 0.01$).

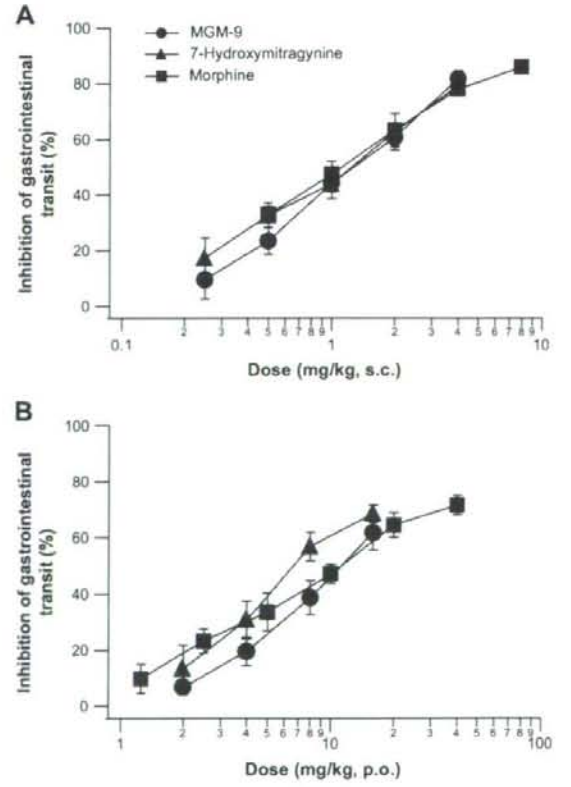

Fig. 8. Dose-response curves of inhibitory effect on gastrointestinal transit of subcutaneous (A) or oral (B) administration of MGM-9, 7-hydroxymitragynine, and morphine in mice. Each value represents mean ± SEM of seven or eight mice.

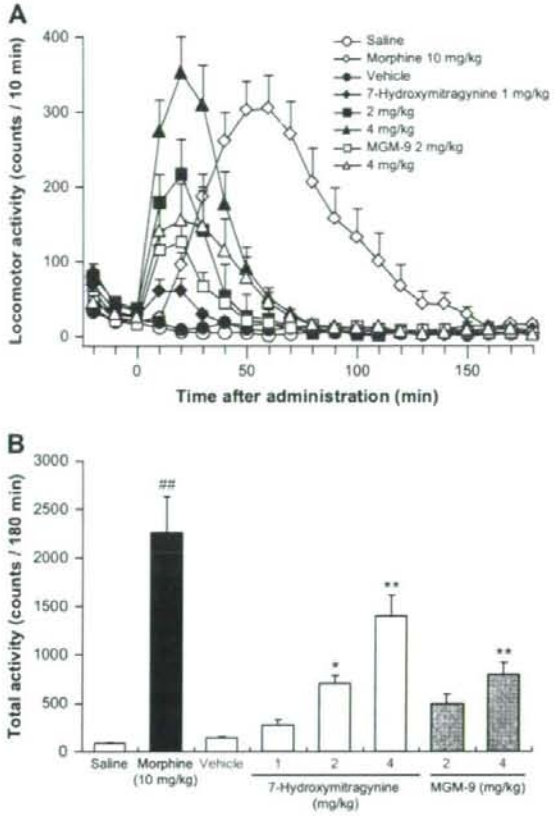

Fig. 9. Time course of the locomotor-enhancing effects of subcutaneous administration of MGM-9, 7-hydroxymitragynine, and morphine in mice (A). Total locomotor activity induced by MGM-9, 7-hydroxymitragynine, and morphine in mice (B). Each value represents mean ± SEM of data obtained from eight to 10 mice. The # denotes values that were significantly different from saline-treated mice by Student's *t*-test (##, $P < 0.01$). The asterisk (*) denotes values that were significantly different from vehicle-treated mice by Bonferroni correction (*, $P < .05$, **, $P < 0.01$).

seven to 22 times more potent than morphine after subcutaneous and oral administration, respectively. Furthermore, MGM-9 showed a more potent antinociceptive effect than 7-hydroxymitragynine. In the receptor binding assay, MGM-9 showed similar binding affinities for μ-, δ- and κ-opioid receptors, as compared with morphine, but the antinociceptive effect of MGM-9 was much stronger than that of morphine. In order to elucidate the reason for this inconsistency, we performed a functional assay using the isolated guinea-pig ileum and compared the potency of MGM-9 with that of morphine. MGM-9 induced opioid agonistic effects about 18 times more potent than morphine in isolated guinea-pig ileum. Then, we found that the strong antinociceptive effect of MGM-9 correlates not with the binding affinity in the receptor binding tests, but with its potency in the experiments with isolated guinea-pig ileum. Possible explanation is the spare receptors in classical receptor theory. Full agonists do not need to bind all the specific receptors in order to induce full intrinsic activity. Therefore, the affinity in the receptor binding assay is somewhat lower than that obtained from the potency in the functional assay. On the other hand, the antinociceptive effect of MGM-9 correlated with the affinity observed in the in vitro functional assay. This may be reason why MGM-9 induces a more potent antinociceptive effect than expected from the receptor-binding assay data.

Natives of Thailand and Malaysia use the leaves of the *Mitragyna speciosa*. When taken orally, the leaves are very effective in increasing work endurance and as a substitute for opium in treating addicts. It is reported that naturally derived indole alkaloids such as 7-hydroxymitragynine from *Mitragyna speciosa* and pseudoakuammigine from *Picralima nitida* exert antinociceptive activity when administered orally (Matsumoto et al., 2004; Duwiejua et al., 2002). Thus, we investigated the antinociceptive effects of MGM-9 via the oral route, based on the traditional usage of *Mitragyna speciosa* and the clinical relevance of this route for administration to human patients. In the present study, MGM-9 induced potent antinociceptive effects especially with oral administration, as 7-hydroxymitragynine did. Interestingly, both MGM-9 and 7-hydroxymitragynine have a favorable bioavailability. The ratios of oral/subcutaneous ED_{50} values of MGM-9 and 7-hydroxymitragynine were much smaller than those of morphine in the tail-flick, hot-plate, and writhing tests. In comparisons of the time courses of the antinociceptive effects of MGM-9 and morphine after subcutaneous or oral administration, the duration of the effect of MGM-9 was shorter than that of morphine. Especially with oral administration, MGM-9 induced a much more potent and rapider effect than morphine. This difference may be due to the difference of the pharmacokinetics of these drugs. It is known that the oral dose of morphine required to elicit an antinociceptive effect is much higher than the parenteral dose because of its high first-pass effect. This pharmacokinetic property of morphine will be involved in its lower potency compared with MGM-9.

We have studied the opioid receptor binding affinities of mitragynine-related compounds. These compounds, e.g., 7-hydroxymitragynine, mitragynine, have a relatively higher affinity for μ-opioid receptors than for δ- and κ-receptors (Takayama et al., 2002). This current study demonstrates that MGM-9 has a high affinity for μ- and κ-opioid receptors. Naloxone completely blocked the antinociceptive effect of MGM-9 in the tail-flick, hot-plate, and writhing tests, confirming the involvement of opioid receptor systems in its effect. The μ-antagonist β-FNA and the κ-antagonist norBNI partially and significantly inhibited MGM-9-induced antinociception in the three tests. Furthermore, a different κ-antagonist GNTI significantly inhibited the effect of MGM-9 as norBNI did. Coadministration of β-FNA and norBNI completely reversed the effect of MGM-9. The selective δ-antagonist naltrindole was ineffective. Isolated guinea-pig ileum was used to examine μ- and κ-opioid receptors, and mouse vas deferens was used to examine δ-opioid receptors. The data obtained from functional bioassays using electrical stimulation in isolated guinea-pig ileum and mouse vas deferens supported the hypothesis that the potent opioid agonistic actions of MGM-9 were mediated by μ- and κ-opioid receptors because MGM-9 showed potent agonistic effects at μ- and κ-opioid receptors in guinea-pig ileum but weak activity in mouse vas deferens. Taken together, the data obtained from in vitro and in vivo assays clarified that the potent antinociceptive effects of MGM-9 resulted from its combined action at both μ- and κ-opioid receptors.

The high antinociceptive potency of the novel dual-acting μ- and κ-opioid agonist MGM-9 allowed us to assess the development of antinociceptive tolerance after chronic administration of MGM-9. Subcutaneous administration of MGM-9 twice daily for 5 consecutive days resulted in a time-dependent decline in the antinociceptive effect in mice, as observed in the cases of morphine and 7-hydroxymitragynine. These results indicate the development of tolerance to MGM-9. We previously reported that the development of tolerance to the antinociceptive effect of 7-hydroxymitragynine and morphine are mediated through μ-opioid receptors (Matsumoto et al., 2005a). Previous studies have shown that repeated subcutaneous administration of κ-opioid agonists such as U50,488 and TRK820 produced a time-dependent decline in the antinociceptive effect in mice (Khotib et al., 2004; Suzuki et al., 2004).

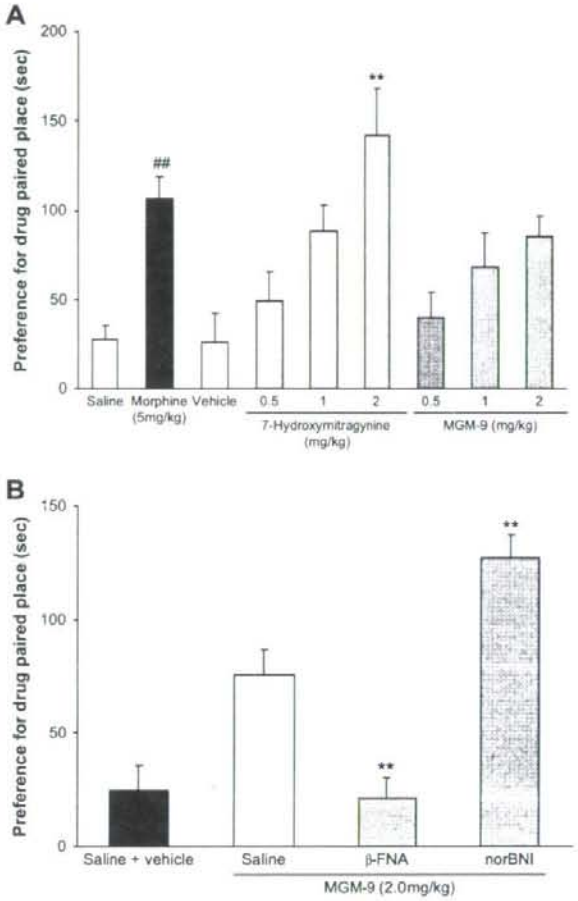

Fig. 10. Conditioned place preference produced by subcutaneous administration of morphine (5 mg/kg), 7-hydroxymitragynine (0.5–2 mg/kg), and MGM-9 (0.5–2 mg/kg) using the conditioned place preference paradigm in mice (A). Each value represents mean ± SEM of time spent in the drug-paired compartment obtained from eight to 12 mice. The # denotes values that were significantly different from saline-treated mice by Student's *t*-test (##, $P < 0.01$). An asterisk (*) denotes values that were significantly different from vehicle-treated mice by Bonferroni correction (**, $P < 0.01$). Effects of pre-treatment with β-funaltrexamine (β-FNA, 10 mg/kg, s.c.) and nor-binaltorphimine (norBNI, 3 mg/kg, s.c.) on the place preference induced by MGM-9 (2 mg/kg, s.c.) (B). Each value represents mean ± SEM of time spent in the drug-paired compartment obtained from six to eight mice. The statistical analysis was done among MGM-9, β-FNA pre-treatment and norBNI pre-treatment groups. The asterisk (*) denotes values that were significantly different from MGM-9-treated mice by Bonferroni correction (**, $P < 0.01$).

Taken together, these results suggest that the antinociceptive tolerance to MGM-9 is mediated by both μ- and κ-opioid receptors in mice.

Constipation is a major problem during administration of a chronic opioid such as morphine. The dose required for its analgesic effect is much higher than that required for its constipating effect; thus, when morphine is used for analgesia, constipation is not a negligible issue (Megens et al., 1998). We investigated the inhibition of gastrointestinal transit to evaluate the constipating effect of MGM-9 and its antinociceptive effect in comparison to morphine and 7-hydroxymitragynine (Tables 2 and 3). The inhibition of gastrointestinal transit (IGIT) ED_{50} value of MGM-9 was much larger than that of its antinociceptive ED_{50}. In the case of morphine, the IGIT ED_{50} value was much smaller than its antinociceptive ED_{50} value. These results suggest that MGM-9 induces constipation less potently than morphine at each equi-antinociceptive dose. In comparison with 7-hydroxymitragynine, the IGIT effects of MGM-9 were weaker at each equi-antinociceptive dose. We previously reported that among the opioid receptors the μ-opioid receptors play a prominent role in 7-hydroxymitragynine- and morphine-induced inhibition of gastrointestinal transit, and that 7-hydroxymitragynine acts more weakly on the peripheral opioid receptors than morphine in the inhibition of gastrointestinal transit (Matsumoto et al., 2006). A previous study using a κ-opioid agonists clearly demonstrated that κ-opioid receptors in mice are also involved in the inhibition of gastrointestinal transit (Bansinath et al., 1991). Therefore, it seems likely that the IGIT of MGM-9 is meditated by both μ- and κ-opioid receptors. The difference between MGM-9 and 7-hydroxymitragynine may be caused not by the subtype selectivity but by the higher lipophilicity of MGM-9 due to the introduction of an ethylene glycol-bridge to mitragynine. Indeed, it has been shown that analgesics with high lipophilicity, such as fentanyl, rapidly penetrate into the brain, leading to the reduction the peripheral side effects (Meert and Vermeirsch, 2005).

It is known that μ-opioids such as morphine induce not only potent antinociception but also rewarding effects following chronic administration in mice. The activation of dopaminergic systems after systemic administration of a μ-opioid agonist induces development

of hyperlocomotion and place preference in mice (Matthes et al., 1999). In contrast, the systemic administration of a κ-opioid agonist negatively modulates the activity of dopaminergic neurons, decreases locomotor activity, and produces place aversion when administered alone (Kuzmin et al., 2000; Narita et al., 2001a,b). In addition, κ-opioid agonists produce a dose-dependent decrease in the hyperlocomotion and conditioned place preference induced by morphine (Narita et al., 1993; Funada et al., 1993). Therefore, we hypothesized that a compound with both μ- and κ-opioid agonistic properties may have a useful pharmacological profile for producing potent antinociceptive effects with fewer rewarding effects compared with μ-agonists. In our experiments, we observed that both morphine and 7-hydroxymitragynine significantly increased locomotor activity and induced a significant place preference after subcutaneous administration. In contrast, MGM-9 did not induce a significant place preference even at doses three to four times higher than its antinociceptive ED_{50} value. Morphine significantly induces a rewarding effect at almost the same doses of the antinociceptive ED_{50} value. These results suggest that MGM-9 induced fewer rewarding effects than morphine and 7-hydroxymitragynine. The place preference induced by the dual-acting μ- and κ-opioid agonist MGM-9 is probably mediated by μ-opioid receptor activation. In contrast, κ-opioid receptor activation negatively modulates the rewarding effect of MGM-9. This is further substantiated by the observation that the μ-receptor antagonist β-FNA blocked MGM-9-induced place preference and that the κ-antagonist norBNI enhanced the place preference. We also confirmed in this study that these opioid receptor antagonists alone did not produce either preference or aversion as reported previously (Narita et al., 2001a,b). Therefore, it seems likely that the μ- and κ-agonistic effects of MGM-9 modulate dopaminergic systems and decrease development of rewarding effects.

In this study, we investigated the pharmacological profile of MGM-9 under normal conditions. Because it is not clear that a dual-acting μ- and κ-opioid agonist is a good candidate for an analgesic for chronic pain with reduced psychological dependence and other side effects, future studies will address the antinociceptive effects and rewarding effects under conditions of neuropathic pain and inflammatory pain in our laboratory. The novel dual-acting μ- and κ-opioid agonist MGM-9 may be a promising new analgesic that has stronger antinociceptive and weaker adverse effects than morphine. Further studies of this uniquely structured alkaloid with an interesting pharmacological profile may lead to development of novel analgesics for clinical management of pain, like the development of analgesics that have morphinan structures.

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—Articles—

指定薬物の分析 Part I：GC-MS 及び LC-MS

花尻(木倉)瑠理,* 河村麻衣子, 内山奈穂子, 緒方 潤,
鎌倉浩之, 最所和宏, 合田幸広

Analytical Data of Designated Substances (Shitei-Yakubutsu) Controlled by the Pharmaceutical Affairs Law in Japan, Part I: GC-MS and LC-MS

Ruri KIKURA-HANAJIRI,* Maiko KAWAMURA, Nahoko UCHIYAMA, Jun OGATA, Hiroyuki KAMAKURA, Kazuhiro SAISHO, and Yukihiro GODA
National Inatitute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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In the last 10 years, many analogs of narcotic substances have been widely distributed in Japan as easily available psychotropic substances and this has become a serious problem. They have been sold as video cleaners, incense and reagents *via* the Internet or in video shops. They are not controlled under the Narcotics and Psychotropics Control Law because their pharmacological effects have not yet been proved scientifically. As a countermeasure to prevrent the abuse fo these substances, the Ministry of Health, Labor and Welfare amended the Pharmaceutical Affairs Law in 2006 so thet 31 non-controlled psychotropic substances (11 tryptamines, 11 phenethylamines, 6 alkyl nitrites, 2 piperazines and salvinorin A) and 1 plant (*Salvia divinorum*) are now controlled as "Designated Substances (Shitei-Yakubutsu)" as of April 2007. Five other compounds (4 phenethylamines and 1 piperazine) were also added to this category in January 2008. In this study, we developed simultaneous analytical methods for these designated substances using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) and present retention times, UV spectra, electron ionization (EI), GC-MS, and electrospray ionization (ESI) LC-MS data.

Key words——psychotropic substances; GC-MS; LC-MS; designated substances; shitei-yakubutsu; drug abuse

緒　　言

近年，麻薬や覚せい剤などの代用として，違法ドラッグ（いわゆる脱法ドラッグ）と呼ばれる様々な化学物質や植物が法律の規制枠を逃れて販売，乱用されており，健康被害や社会的弊害が問題となっている.[1-6] 違法ドラッグとは，一般に，麻薬又は向精神薬には指定されておらず，麻薬又は向精神薬と類似の有害性を有することが疑われる物質（人為的に合成されたもの，天然物及びそれに由来するものを含む）であって，もっぱら人に乱用させることを目的として製造，販売等がされるものを示す．違法ドラッグ市場で取り扱われる化合物は，法的な規制を逃れるため多様化し，一定の基本骨格を持つ様々な新規構造類似化合物が流通している．また，含有成分がある程度判明した違法ドラッグ製品でも，摘発をされると容易に販売名や包装形態等を変えて販売が行われるなど実態把握が困難である製品が多く，さらに，もっぱら乱用に供する目的で流通しているが，目的を偽装（芳香剤，ビデオクリーナー，研究用試薬等）して販売されている製品もあり，従来の薬事法では取締りの実効性に支障が生じていた．これらの問題に対処すべく，平成18年に薬事法が改正され，興奮等の作用を有する蓋然性が高く，保健衛生上の危害が発生する恐れがある薬物や植物を厚生労働大臣が「指定薬物」として指定し，医療等の用途以外の製造，輸入，販売等を禁止することになった．本薬事法改正を受け，平成19年4月より，31化合物1植物（*Salvia divinorum*）が指定薬物として規制されることになった．また，平成20年1月11日には新たに5化合物が指定薬物に加わった．

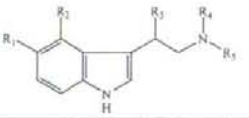
本研究では，現在までに指定薬物に指定された計36化合物について，ガスクロマトグラフィー・質

国立医薬品食品衛生研究所
*e-mail: kikura@nihs.go.jp

量分析（GC-MS）及び液体クロマトグラフィー・質量分析（LC-MS）による一斉分析法を検討したので報告する．

実験方法

1. 分析対象化合物及び試薬　分析対象とした指定薬物36化合物（トリプタミン系化合物11種類，フェネチルアミン系化合物15種類，ピペラジン系化合物3種類，亜硝酸エステル類6種類及びサルビノリンA）の名称，略称及び構造をFig. 1-1から1-3に示した．PMMP塩酸塩及びBDB塩酸塩（1 mg/ml メタノール溶液）はCereliant社製，4MPP二塩酸塩，インダン-2-アミン（2-アミノインダン）塩酸塩，イソプロピルアルコール，*tert*-ブチルアルコール，イソブチルアルコール，ブチルアルコール，イソペンチルアルコール，シクロヘキシルアルコールは和光純薬社製，5-MeO-DMT，亜硝酸ブチル（95%），亜硝酸イソブチル（95%），亜

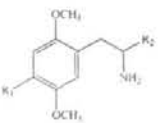


I. Tryptamines

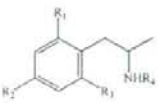
R_1	R_2	R_3	R_4	R_5	Compounds
H	H	H	CH_3	$CH(CH_3)_2$	*N*-Isopropyl-*N*-methyltryptamine (MIPT)
H	H	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	*N,N*-Dipropyltryptamine (DPT)
H	H	H	$CH(CH_3)_2$	$CH(CH_3)_2$	*N,N*-Diisopropyltryptamine (DIPT)
OCH_3	H	CH_3	H	H	1-(5-Methoxy-1*H*-indol-3-yl)propan-2-amine (5-MeO-AMT)
OCH_3	H	H	CH_3	CH_3	5-Methoxy-*N,N*-dimethyltryptamine (5-MeO-DMT)
OCH_3	H	H	CH_2CH_3	CH_2CH_3	*N,N*-Diethyl-5-methoxytryptamine (5-MeO-DET)
OCH_3	H	H	CH_3	$CH(CH_3)_2$	*N*-Isopropyl-5-methoxy-*N*-methyltryptamine (5-MeO-MIPT)
OCH_3	H	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	5-Methoxy-*N,N*-dipropyltryptamine (5-MeO-DPT)
OCH_3	H	H	$CH_2CH=CH_2$	$CH_2CH=CH_2$	*N,N*-Diallyl-5-methoxytryptamine (5-MeO-DALT)
H	OH	H	$CH(CH_3)_2$	$CH(CH_3)_2$	4-Hydroxy-*N,N*-diisopropyltryptamine (4-OH-DIPT)
H	$OCOCH_3$	H	$CH(CH_3)_2$	$CH(CH_3)_2$	4-Acetoxy-*N,N*-diisopropyltryptamine (4-AcO-DIPT)

Fig. 1-1. Structures of "Designated Substances (Shitei-Yakubutsu)" (tryptamines)
No marked compounds have been controlled as designated substances since April in 2007 and asterisk-marked compounds from January 2008.

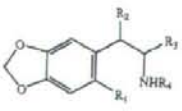
II. Phenethylamines



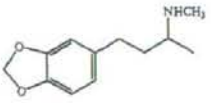
R_1	R_2	Compounds
Cl	H	2-(4-Chloro-2,5-dimethoxyphenyl)ethanamine (2C-C)
I	H	2-(4-Iodo-2,5-dimethoxyphenyl)ethanamine (2C-I)
I	CH_3	1-(4-Iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI)*
CH_2CH_3	H	2-(4-Ethy-2,5-dimethoxyphenyl)ethanamine (2C-E)
SCH_2CH_3	H	2-(4-Ethylsulfanyl-2,5-dimethoxyphenyl)ethanamine (2C-T-2)
$SCH(CH3)_2$	H	2-(2,5-Dimethoxy-4-isopropylsulfanylphenyl)ethanamine (2C-T-4)



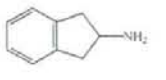
R_1	R_2	R_3	R_4	Compounds
OCH_3	OCH_3	OCH_3	H	1-(2,4,6-Trimethoxyphenyl)propan-2-amine (TMA-6)
H	OCH_3	H	CH_3	1-(4-Methoxyphenyl)-*N*-methylpropan-2-amine (PMMA)
H	F	H	H	1-(4-Fluorophenyl)propan-2-amine (4FMP)



R_1	R_2	R_3	R_4	Compounds
OCH_3	H	CH_3	H	1-(2-Methoxy-4,5-methylenedioxyphenyl)propan-2-amine (MMDA-2)
H	H	CH_2CH_3	H	1-(3,4-Methylenedioxyphenyl)butan-2-amine (BDB)
H	=O	CH_2CH_3	CH_3	2-Methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one (bk-MBDB)*
H	=O	CH_3	CH_2CH_3	2-Ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one (Ethylone, bk-MDEA)*



N-Methyl-4-(3,4-methylenedioxyphenyl)butan-2-amine (HMDMA)



Indan-2-amine (2-aminoindan)*

Fig. 1-2. Structures of "Designated Substances (Shitei-Yakubutsu)" (phenethylamines)

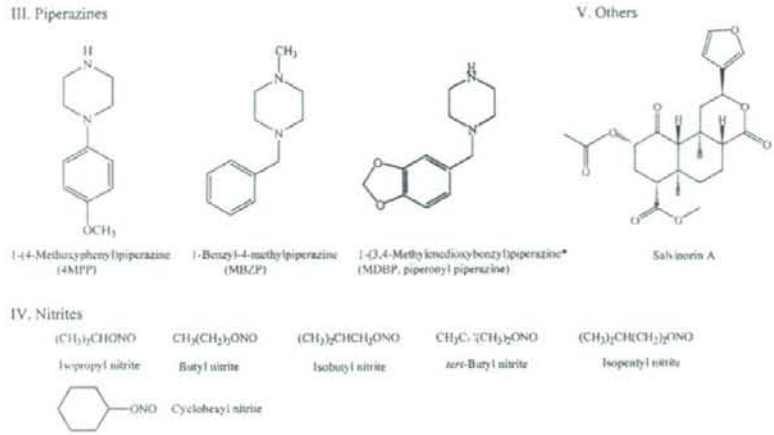


Fig. 1-3. Structures of "Designated Substances (Shitei-Yakubutsu)" (piperazines, alkyl nitrites and salvinorin A)

硝酸 *tert*-ブチル（90%），亜硝酸イソペンチル（96%）は Aldrich 社製，MDBP は東京化成工業製を使用した．また，サルビノリン A は徳島文理大学香川薬学部の代田修先生よりご供与頂いた．その他の化合物は，国立医薬品食品衛生研究所において指定薬物分析標品として調製し，NMR 及び質量分析により構造を確認するとともに，HPLC 及び TLC により純度を確認したものを使用した．[7] LC-MS の移動相に使用したアセトニトリルは HPLC グレードを使用した．その他試薬は市販特級品を使用した．

2. 試料調製法

2.1. 亜硝酸エステル類以外　試料をメタノールに溶解し，0.1 mg/ml の試験溶液を作成した．bk-MDEA 及び bk-MBDB 以外のフェネチルアミン系化合物の塩酸塩を GC-MS で分析する場合は，塩基として測定をした．すなわち，GC-MS 測定用の試験溶液 1 ml を窒素気流下で蒸発乾固させたのち，蒸留水 1 ml で再溶解し，アンモニアアルカリ性として酢酸エチル 1 ml で抽出した溶液を測定溶液とした．

2.2. 亜硝酸エステル類　瀬戸らの方法[8,9]を一部修飾して分析を行った．試料 100 μl にアセトンを加え正確に 10 ml として試験溶液とした．ガラスバイアル瓶に試験溶液 0.05 ml，1M リン酸緩衝液（pH 7）0.5 ml，蒸留水 0.45 ml を加え，タフボンドディスク(シリコンセプタム)で蓋をして密閉し，ヘッドスペース注入測定溶液とした．

3. GC-MS 分析条件

3-1. 亜硝酸エステル類以外　装置：Agilent 社製 6890N GC 及び 5975 MSD，カラム：HP-1MS（30 m × 0.25 mm i.d.，膜厚 0.25 μm, Agilent 社製），キャリアーガス：He, 0.7 ml/min，注入法：スプリットレス，注入量：1 μl，注入口温度：200 ℃，カラム温度：80℃（1 min hold）-5℃/min-190 ℃（15 min hold）-10℃/min-310℃（5 min hold），イオン化法：EI 法，検出器温度：280℃

3-2. 亜硝酸エステル　装置：Agilent 社製 6890N GC，5975 MSD 及びヘッドスペース注入装置 G1888，カラム：AQUATIC-2（60 m×0.25 mm i.d.，膜厚 1.40 μm, GL sciences 社製），キャリアーガス：He，1.0 ml/min，注入口温度：200℃，スプリット比：15：1，検出器温度：220℃，イオン化法：EI 法，カラム温度：40℃（3 min hold）-15℃/min-115℃（7 min）-20℃/min-240℃/min（3 min）

3-3. ヘッドスペース注入条件　平衡化温度：45℃，ループ温度：60℃，トランスファーライン温度：80℃，平衡化時間：10 分，注入：1 ml.

4. LC-MS 分析条件　装置：Agilent 社製 1100 シリーズ LC/MSD，カラム：Atlantis T3（2.1×150 mm, 5 μm, Waters 社製），移動相 A：10 mM ギ酸アンモニウム緩衝液（pH 3），移動相 B：アセトニトリル，グラジエント条件：A/B 90/10（0 min）-80/20(50 min)-30/70(60 min, 10 min hold)，流速：0.3 ml/min，カラム温度：40 ℃，注入量：1 μl，検