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ORIGINAL ARTICLE

Evaluation of carboxamide-type synthetic cannabinoids on the functional activities at cannabinoid receptors and biological effects via inhalation exposure test

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Abstract

Purpose Three synthetic carboxamide-type cannabinoids (5F-MDMB-PICA, 5F-EMB-PINACA, and AMB-CHMICA) were evaluated in terms of their in vitro activities at the cannabinoid receptors CB_1 and CB_2 and in vivo biological effects when smoking the synthetic cannabinoids to assess their biological effects.

Methods [^{35}S]Guanosine-5′-*O*-(3-thio)-triphosphate binding assays were performed to investigate the half maximal effective concentration values of the test compounds at the CB_1 and CB_2 receptors. Additionally, the biological effects were evaluated by observing and scoring the behavior of mice with equipment in which they inhaled smoke from a herbal mixture containing the test compounds.

Results All three synthetic cannabinoids tested in this study activated the CB_1 and CB_2 receptors in vitro. 5F-MDMB-PICA showed less than 1 nM of the half maximal effective concentration value for both receptors. Therefore, it was suggested that 5F-MDMB-PICA was the strongest CB_1 and CB_2 receptor agonist in comparison with synthetic cannabinoids evaluated in the past. The degree of the various biological effects, specifically passivity, spontaneous activity, abnormal gait, abnormal position, and grip strength, when smoking the synthetic cannabinoids corresponded to the functional activity at the CB_1 receptor. However, some biological effects differed between 5F-MDMB-PICA and 5F-MDMB-PINACA, used as a positive control, and AMB-CHMICA induced some biological effects in contrast to the other tested synthetic cannabinoids.

Conclusion This study provides information regarding the biological effects when smoking synthetic cannabinoids from the functional activities at the CB_1 and CB_2 receptors, considering their way of inhalation and thermal degradation.

Keywords NPS · Carboxamide-type synthetic cannabinoid · 5F-MDMB-PICA · 5F-EMB-PINACA · AMB-CHMICA · Activities at CB_1 and CB_2 receptors · Inhalation exposure test

Introduction

Synthetic cannabinoids (SCs) are one of the most prevalent groups of new psychoactive substances (NPS) and have been defined as substances of abuse that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances [1, 2]. Products produced by mixing SCs with plant material or spraying plant material with SC solution have been distributed in drug markets. The governments of various nations have regulated SCs that have been detected in products, but the illicit manufacturers of NPS have synthesized new SCs to circumvent the regulation, and thus, various SCs have globally emerged and

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prevailed. The number of new SCs detected in various countries reached a peak in 2014 and has subsequently decreased. Nevertheless, the prevalence of abuse of SCs has continued, and many fatal cases have been reported [3].

SCs are strong cannabinoid receptor type 1 (CB_1 receptor) and cannabinoid receptor type 2 (CB_2 receptor) agonists and can be classified as naphthoylindoles (e.g., JWH-018), naphthoylindazoles (e.g., THJ-018), indazole carboxamides (e.g., ADB-PINACA), or indole carboxamides (e.g., ADBICA) in terms of their chemical structures. In this work, we classified the above indazole and indole carboxamides as carboxamide-type SCs. This class of SCs is derived by substituting an amino acid ester for a naphthalene ring in the previous generation naphthoylindoles and naphthoylindazoles. The basic chemical structure of the class of SCs was described in a patent by Pfizer in 2009 [4]. Carboxamide-type SCs have been associated with several serious accidents. In 2014, AMB-FUBINACA was found in Sweden [5] and Louisiana (United States) and the compound caused intoxication of 33 people in New York City in 2016. The mass media reported the intoxication as a "zombie" outbreak because of the appearance of the intoxicated people [6]. In 2014, in Japan, 21 reported traffic collisions were related to 5F-AMB, which injured or killed many people [7]. 5F-MDMB-PINACA also caused 24 reported traffic collisions which, fortunately, were not serious accidents [7]. However, in 2014, 5F-MDMB-PINACA caused approximately ten smoking-related deaths in Japan [8]. These SCs have been regulated in each country; however, 5F-MDMB-PINACA and AMB-FUBINACA were the most seized NPS in 2017 and 2018 [3]. Therefore, it has been suggested that these SCs have continued to be prevalent around the world.

5F-MDMB-PICA is classified as a carboxamide-type SC. This SC was found in Germany in 2016 [9, 10]. Risseeuw M. D. P. et al. discovered the SC in a powder form in an unlabeled product and 27 herbal incense products in Belgium [11]. Banister et al. evaluated 5F-MDMB-PICA regarding the functional activity at the CB_1 and CB_2 receptors and found that the half maximal effective concentration (EC_{50}) value for the CB_1 receptor was 0.45 nM [12]. The results showed that 5F-MDMB-PICA was a strong CB_1 receptor agonist and is, therefore, considered a public health threat.

Therefore, we targeted the carboxamide-type SCs that have been distributed in Europe. We investigated the biological effects of 5F-MDMB-PICA, as well as 5F-EMB-PINACA and AMB-CHMICA, which have been detected in Sweden and Slovenia in 2015, respectively [13]. AMB-CHMICA was also found in Turkey between 2016 and 2017 [14].

As SCs are typically smoked like cigarette [15–17], we used previously developed techniques in which mice are exposed to smoke emitted by burning plants sprayed with SCs solution just like cigarette burning to evaluate the biological effects of the SCs [18, 19]. The evaluation of SCs using these techniques is expected to accurately assess the risk of the SCs against human body by considering the way of inhalation and thermal degradation, and to estimate the effects on users by comparing the results of the compounds previously investigated before they were considered as threat to public health.

Materials and methods

Reagents

Methyl 1*H*-indole-3-carboxylate and (bromomethyl) cyclohexane were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Methyl 1*H*-indazole-3-carboxylate and D-*tert*-leucine methyl ester hydrochloride were purchased from Accela ChemBio Co., Ltd. (San Diego, CA, USA), 1-bromo-5-fluoropentane from Fluorochem Ltd. (Hadfield, UK), L-*tert*-leucine methyl ester hydrochloride and D-valine methyl ester hydrochloride from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and L-valine methyl ester hydrochloride was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other reagents used in this study were purchased from FUJIFILM Wako Pure Chemical Corporation. Purified water was obtained from tap water using the Elix Advantage-10 from Merck Millipore (Burlington, MA, USA).

Chemical synthesis

Figure 1 shows the synthetic pathways of the SCs tested in this study. All of the test compounds required methyl 1*H*-indole-3-carboxylate (compound **1**) or methyl 1*H*-indazole-3-carboxylate (compound **2**) as starting material and were synthesized by referring to a previously described method [20, 21]. Compounds **1** or **2** were *N*-alkylated by alkyl halide under basic conditions to obtain compounds **3–5**. Subsequently, compounds **6–8** were yielded via the deprotection of compounds **3–5** by the hydrolysis of the methyl group. The test compounds (compounds **9–11**, including each enantiomer) were synthesized by chlorination of compounds **6–8** and then amidation with the amino acid derivatives. 5F-EMB-PINACA was synthesized as previously described in [22]. The details of the chemical synthesis of 5F-MDMB-PICA and AMB-CHMICA, which were synthesized in this study, are described in the Supplementary Material. The chemical structures of the SCs tested in this study are shown in Fig. 2.

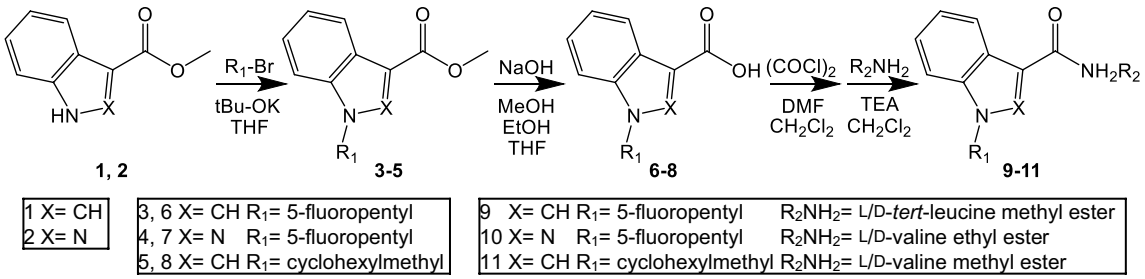
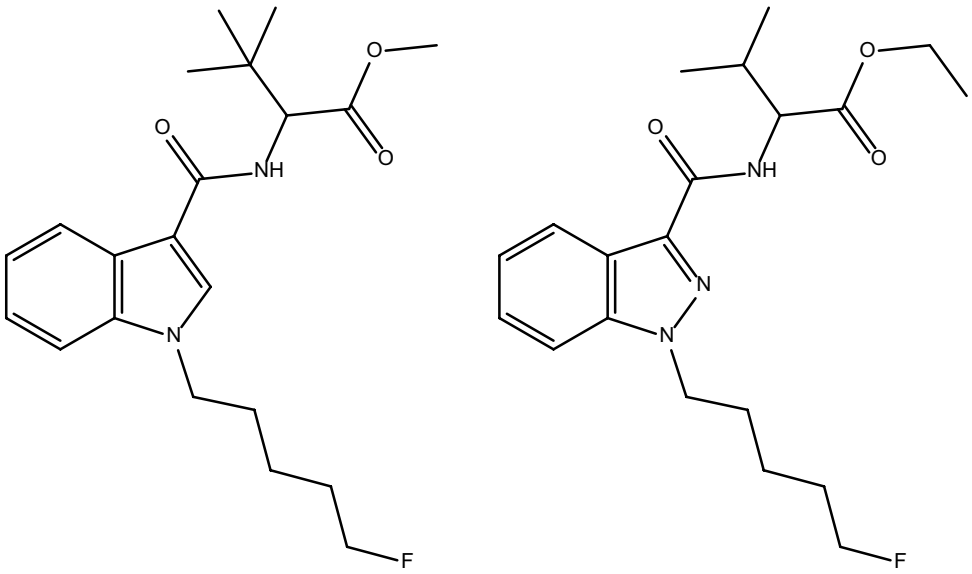


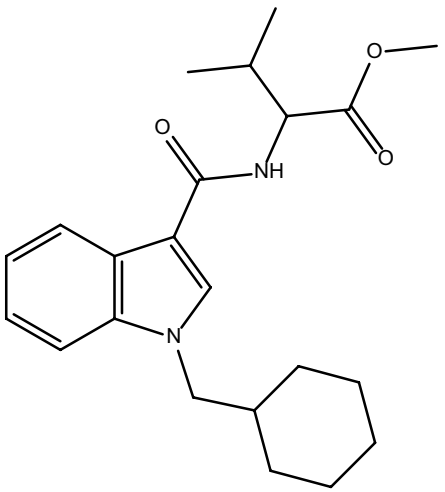
Fig. 1 Synthesis route for the synthetic cannabinoids dealt with in this study. *tBu-OK* potassium *tert*-butoxide, *THF* tetrahydrofuran, *MeOH* methanol, *EtOH* ethanol, *$(COCl)_2$* oxalyl chloride, *DMF N,N*-dimethylformamide, *CH_2Cl_2* dichloromethane, *TEA* triethylamine

Fig. 2 Chemical structures of the synthetic cannabinoids dealt with in this study

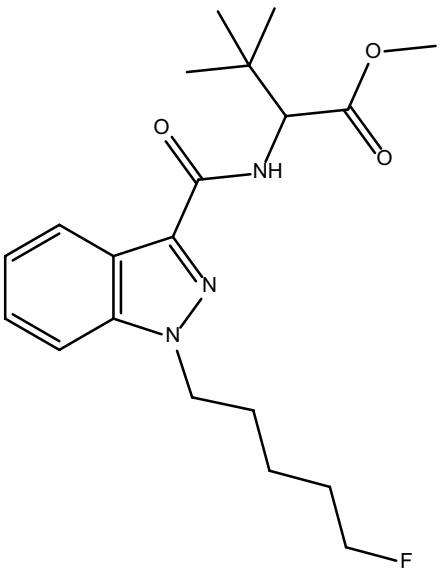


(a) 5F-MDMB-PICA (**9**)

(b) 5F-EMB-PINACA (**10**)



(c) AMB-CHMICA (**11**)



(d) 5F-MDMB-PINACA

In vitro assays to evaluate the CB_1 and CB_2 receptor activities

[^{35}S]Guanosine-5′-*O*-(3-thio)-triphosphate ([^{35}S]GTPγS) binding assays were performed to evaluate the CB_1/CB_2 cannabinoid receptor activity. These assays were performed at ADME and Tox. Research Institute, Sekisui Medical Co., Ltd. (Tokai-mura, Ibaraki, Japan). The assay conditions were as previously described in [23], except for the tested concentration levels of the compounds ranging from 1×10^{-12} to 1×10^{-5} M for 5F-MDMB-PICA and AMB-CHIMICA and from 1×10^{-11} to 1×10^{-4} M for 5F-EMB-PINACA. CP-55,940 was used as a positive control. The agonistic activities (EC_{50} value: concentration showing a 50% response rate) of the test compounds to the cannabinoid receptors CB_1 and CB_2 were measured.

Inhalation exposure test

An inhalation exposure test was performed to examine the biological effects of the test compounds at the Tokyo Metropolitan Institute of Public Health (Tokyo, Japan). The method of the test was as previously described [18, 19]. We referred to Inomata et al.'s study [19] for the results from marshmallow (*Althaea officinalis*) as a negative control and 5F-MDMB-PINACA (Fig. 2) as a positive control. The conditions and evaluation methods for the present test are described below.

Equipment

Figure 3 shows the schema of the equipment for inhalation exposure. The equipment was composed of a respiratory apparatus (g; SN-480-5, Shinano Seisakusho, Tokyo, Japan), a combustion part (A), an exposure box (B; made in vinyl chloride, content 8020 cc, Maeda Seisakusho Co., Ltd, Tokyo, Japan), and two gas recovering bottles (C; SPC midget impinger, SIBATA SCIENTIFIC TECHNOLOGY LTD., Saitama, Japan). The flow of air from the combustion part to an exhaust port (f) was as follows: the smoke from the marshmallow burned in the combustion part (A) was absorbed in the amount of air set by the respiratory apparatus (g) and introduced into the air inlet (b) of the exposure box (B). The smoke flowed into the box was diffused by a fan (c; E122535H, Earth Corporation, Tokyo, Japan) that was installed at the air supply part of the box and then exhausted from the exhaust port (f) of the box in the amount of air set by the respiratory apparatus (g). Refer to the Supplementary Material for information about the two gas recovering bottles (C).

Sample preparation

The carboxamide-type SCs tested in this study were used as a mixture of equal amounts of (*S*)- and (*R*)-enantiomers. For burning the test compounds like cigarettes, 15 mg of the test compound was added to 0.25 g of the lobe part of marshmallow, and the mixture was filled into half a cigarette paper.

Experimental animals and rearing conditions

Male Crl; 5-week old CD1 (ICR) mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The mice were acclimated for 1 week before being used for the test. They were housed individually in plastic cages and had free access to solid feed (CE-2, Clea Japan, Inc., Tokyo, Japan) and tap water. The animal room was maintained at 24 ± 1 °C with a relative humidity of 50 ± 5%, with 10 ventilations per h (through a HEPA filter), and on a 12 h light/dark cycle.

Method of inhalation exposure

The marshmallow filled into half a cigarette paper was set on the combustion part (Fig. 3a) of the equipment. Five mice (Fig. 3d) were used per one compound, and each mouse (n = 5) was put into each part of the exposure box (Fig. 3b) partitioned with nets (Fig. 3e). After the box was closed, the marshmallow was ignited while the air was flowing into the box from the respiratory apparatus (Fig. 3g) at a flow rate of 600 mL/min. After the combustion, the respiratory apparatus was left running.

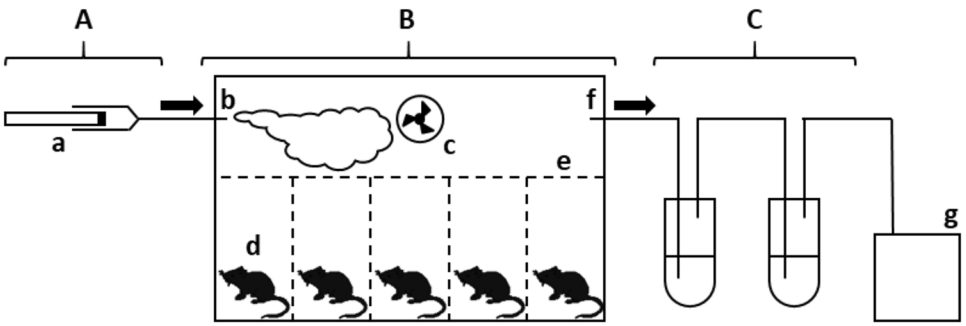


Fig. 3 Schema of the equipment for the inhalation exposure test used in this study **A** a combustion part, **B** an exposure box, **C** two gas recovering bottles; (a) marshmallow filled into a cigarette paper, (b) an air inlet, (c) a fan, (d) mice, (e) nets partitioning the box, (f) an exhaust port, (g) a respiratory apparatus. The black arrows show the air flow direction

Evaluation test for biological effects

The subsequent evaluations were performed 15, 30, and 60 min after the combustion. Incidentally, the measurement of body temperature was also performed before the exposure.

Observation of the general, neurological, and autonomic behaviors We prepared score sheets for the observation of the general, neurological, and autonomic behaviors by improving the observation methods of Irwin [24] (refer to [18] for the score sheets) and calculated the mean of the scores for the five mice.

The normal behavior of a mouse was scored as zero points. Suppressed behavior was given scores on a scale of − 1 to − 3, and excited behavior was given scores on a scale of + 1 to + 3 depending on the strength of behavior (suppressive behavior: − 1, − 2, and − 3 and excitement behavior: + 1, + 2, and + 3). The mean of each observation was calculated to evaluate the strength of the biological effects of each item.

When the absolute value of the mean for five mice (from now on, referred to as "the absolute value") was within the limits of 0–0.4 regarding the strength of the biological effects, it was decided that no biological effect was observed. When the absolute value was within the limits of 0.6–0.8, an effect was suspected. When the absolute value was within the limits of 1.0–3.0, an effect was observed.

Measurement of body temperature The rectal temperatures of mice were measured using a temperature recorder for mice (AD-1687, A&D Company, Limited, Tokyo, Japan). A range of ±0.5 °C before exposure was regarded as normal, and the score of suppressive behavior was counted down (− 1, − 2 and − 3) every time the temperature dropped 1 °C from the lower limit of the normal temperature range. The score was counted up (+ 1, + 2 and + 3) every time the temperature rose 1 °C from the upper limit of the temperature range. Thereafter, the strength of the effect was evaluated from the mean of five mice.

Catalepsy test The forelimb of a mouse was suspended on a crossbar that was made of a galvanized steel wire and positioned at 6.5 cm height. The hindlimb of the mouse was positioned on a stage and it kept standing with its hindlimb. Thereafter, the time the mouse remained in the position was measured. When the mouse remained still for more than 30 s, the time was measured up to 90 s, and the catalepsy was determined as positive. When the mouse remained still for less than 30 s, the time was measured three times. When the mouse moved in less than 30 s all the three times, the catalepsy was determined as negative.

Results

Functional activity at the CB_1 and CB_2 receptors

Table 1 shows the results of the [^{35}S]GTPγS binding assays performed in this study. (*S*)-5F-MDMB-PICA had the most substantial potency at both the receptors in the tested compounds. Both the enantiomers of AMB-CHMICA activated to a CB_1 receptor more selectively than the other tested compounds. Compared with (*R*)-enantiomers, the (*S*)-enantiomers of all tested compounds acted as CB_1 receptor agonists, which was similar to the results described in a previous study [22].

Observation of the biological effects

Table 2 describes the evaluation of the general, neurological, and autonomic behaviors.

General behavior

Aggressiveness, stereotype, and vocalizations were not observed for all the tested compounds. The degree of passivity was strong in the order of 5F-EMB-PINACA < 5F-MDMB-PICA < 5F-MDMB-PINACA. Furthermore, the order corresponded to the functional activity at the CB_1 receptor. Additionally, 5F-MDMB-PINACA strongly induced passivity for 1 h, and the passivity from 5F-MDMB-PICA and 5F-EMB-PINACA decreased over time. AMB-CHMICA did not induce passivity.

Grooming was suppressed by marshmallow, and the suppression decreased over time. The suppression by AMB-CHMICA also decreased over time, but AMB-CHMICA suppressed grooming more strongly than marshmallow. 5F-MDMB-PINACA, 5F-MDMB-PICA, and

Table 1 Activities of test compounds at human CB1 and CB2 receptors

Compound name	EC50 (nM)	
	CB1	CB2
(S)-5F-MDMB-PICA	0.815	0.590
(R)-5F-MDMB-PICA	56.2	76.2
CP-55,940	2.33	0.399
(S)-5F-EMB-PINACA[a]	4.96	6.91
(R)-5F-EMB-PINACA[a]	35.9	1.68
CP-55,940	1.90	0.150
(S)-AMB-CHMICA	14.3	783 × 10
(R)-AMB-CHMICA	62.0	178 × 10
CP-55,940	2.62	1.59

[a]Referred in [22]

Table 2 Mean values of the score in General, Neurogical, and Autonomical behavior ($n = 5$)

Observation time (h)	Marshmallow[a]			5F-MDMB-PINACA[a]			5F-MDMB-PICA			5F-EMB-PINACA			AMB-CHMICA		
	0.25	0.5	1	0.25	0.5	1	0.25	0.5	1	0.25	0.5	1	0.25	0.5	1
Aggressiveness	0	0	0	0.2	0	0	0	0	0	0.6	0	0.6	0.8	0.6	0.6
Passivity	0	− 0.6	− 0.6	− 2.8	− 3	− 3	− 2.8	− 2.6	− 2	− 1.2	− 1.4	− 0.6	− 0.2	0	0
Stereotype	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Grooming	− 2.8	− 1.4	− 0.4	− 3	− 3	− 3	− 3	− 3	− 3	− 3	− 3	− 3	− 3	− 2.6	− 1.4
Vocalization	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0
Sound response	0	0.2	0	− 2.6	− 3	− 3	− 2.6	− 2.4	− 1.6	− 1.2	− 0.2	0	0.2	0.4	0.6
Touch response	0	− 0.2	− 0.4	− 2.6	− 3	− 3	− 2.8	− 3	− 2.2	− 1.8	− 1.4	− 0.2	0.6	0.6	0.8
Pain response	− 0.4	− 0.2	− 0.2	− 2	− 2.4	− 3	− 2.8	− 2.6	− 2.4	− 1	− 1	− 0.2	0.2	1.4	1
Verticalness	− 0.6	− 1.6	− 0.8	− 2.2	− 3	− 3	− 3	− 3	− 3	− 2.6	− 2	− 2	− 2.6	− 2.4	− 1.4
Spontaneous activity	− 0.8	− 0.2	− 0.2	− 2.4	− 3	− 3	− 2.6	− 2.8	− 2.4	− 3	− 1.4	− 1.6	− 2.2	− 1.4	− 0.6
Abnormal gait	0	0	0	− 3	− 3	− 3	− 2.8	− 3	− 2.2	− 3	− 1.8	− 1.2	− 1	− 0.6	− 0.6
Abnormal position	0	0	0	− 3	− 3	− 3	− 3	− 3	− 2.8	− 3	− 1	− 0.6	− 1.2	− 0.6	− 0.4
Muscle tone	0	0	0	2.4	− 2.6	− 2.6	− 2.8	− 3	− 2.8	1.4	− 1.8	0	− 1.4	0	0
Straub tail reaction	0	0	0	0	0.4	0.4	0.4	1.0	1.4	1.4	1.2	0	0	0	0
Righting reflex	0	0	0	− 3	− 3	− 3	− 2.6	− 1.6	− 1.2	− 0.8	0	0	0	0	0
Pinna reflex	0	− 0.2	− 0.4	− 2.8	− 3	− 3	− 2.8	− 2.6	− 2	− 1	− 0.8	0	0.8	1.2	1.2
Corneal reflex	0	0	0	− 2.8	− 3	− 3	− 2.8	− 2.4	− 2	− 0.2	− 0.8	0	0.4	1.2	1.2
Tendon reflex	0	− 0.6	0.2	− 2.8	− 2.8	− 3	− 2.6	− 2.2	− 2	− 0.2	− 0.8	0.4	0.6	1.4	1
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsion	0	0	0	3.0	1.2	0	0.4	1.2	0	2	0	0	0	0	0
Grip strength	0	0.4	0	− 3	− 3	− 3	− 3	− 1.8	− 1.4	− 2.6	− 1	0	− 1	− 0.4	− 0.4
Detached finger	0	0	0	− 1	− 2	− 1.6	0	− 1.2	− 1	− 2	− 0.8	− 0.4	0	0	0
Exophthalmos	0	0	0	0	0	0	0.4	0.4	0.2	0	0	0	0	0	0
Pupil size	0	0	− 0.2	2	1	0.6	1.8	1.6	1.4	0.6	0.6	0.4	0.2	− 0.2	0
Palpebral opening	− 1.4	0	0	− 0.6	− 0.8	− 0.8	− 1.6	− 1.6	− 1.2	− 2	0	0	− 1.2	0	− 0.2
Shed tears	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Respiratory	rate	0	0	0	− 2	− 2	− 2	− 1.8	− 2	− 3	− 1.4	0	0	− 0.4	0
Heart rate	0	0	0	− 2	− 2	− 2	− 1.8	− 2	− 3	− 1.6	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Temperature	− 2	− 2	− 0.6	− 3	− 3	− 3	− 3	− 3	− 3	− 3	− 3	− 2.6	− 2.2	− 2.4	− 2
Skin color	0	0	0	1	− 0.2	0	− 2	− 2	− 3	0	0	0	0	0	0

[a]Referred in [19]

5F-EMB-PINACA drastically suppressed grooming. All mice did not groom for 1 h.

The suppression of the sound, touch, and pain responses were strong in the order of 5F-EMB-PINACA < 5F-MDMB-PICA < 5F-MDMB-PINACA, and the suppression of these responses by 5F-MDMB-PINACA increased over time. The suppression from 5F-MDMB-PICA and 5F-EMB-PINACA decreased over time. Contrary to these SCs, AMB-CHMICA excited these responses, and the pain response was especially observed 30 min and 1 h after combustion.

For verticalness, 5F-MDMB-PINACA increased the degree of the suppression over time, whereas 5F-EMB-PINACA and AMB-CHMICA decreased the degree over time. 5F-MDMB-PICA strongly suppressed the behavior for 1 h.

Neurological behavior

Tremors were not observed for all the test compounds. The degree of the suppression of spontaneous activity, abnormal gait, abnormal position. and grip strength was strong in the order of AMB-CHMICA < 5F-EMB-PINACA < 5F-MDMB-PICA < 5F-MDMB-PINACA. The order corresponded to the functional activity at the CB_1 receptor. These behaviors were drastically suppressed for 1 h by 5F-MDMB-PINACA. The grip strength was strongly suppressed by 5F-MDMB-PICA but decreased over time. The suppression of these behaviors by 5F-EMB-PINACA and AMB-CHMICA also decreased over time.

The results were drastic for muscle tone. An absence of muscle tone was induced by 5F-MDMB-PICA for 1 h, and AMB-CHIMICA suppressed muscle tone 15 min after combustion. In regard to 5F-MDMB-PINACA and 5F-EMB-PINACA, the excitement of the muscle tone was observed 15 min after combustion. The behavior drastically changed into suppression 30 min after combustion, and the suppression continued with 5F-MDMB-PINACA. However, 5F-EMB-PINACA neither excited nor suppressed the behavior 1 h after combustion.

A Straub tail reaction was observed for 5F-MDMB-PICA and 5F-EMB-PINACA. 5F-EMB-PINACA excited the reaction 15 min after combustion, but the excitement decreased over time, and 1 h after combustion, the behavior was no longer observed. The excitement of the behavior by 5F-MDMB-PICA increased over time.

For righting, pinna, corneal, and tendon reflexes, 5F-MDMB-PINACA suppressed the behaviors the strongest. The suppression of the behaviors by 5F-MDMB-PICA decreased over time. 5F-EMB-PINACA hardly suppressed the behaviors. AMB-CHMICA showed the opposite results of the other SCs. The SC did not affect the righting reflex but excited the other reflexes.

5F-MDMB-PINACA and 5F-EMB-PINACA strongly induced convulsions 15 min after combustion. The convulsions decreased over time and were absent 30 min after combustion with 5F-EMB-PINACA and 1 h after combustion with 5F-MDMB-PINACA. 5F-MDMB-PICA induced convulsions 30 min after combustion but was absent 1 h after combustion. AMB-CHIMICA did not induce convulsions.

5F-EMB-PINACA induced detached finger the strongest 15 min after combustion, but the degree decreased over time. 5F-MDMB-PINACA and 5F-MDMB-PICA strongly induced the behavior 30 min after combustion, and the degree did not change at 1 h. The degree of the behavior at 1 h after combustion was strong in the order: 5F-EMB-PINACA < 5F-MDMB-PICA < 5F-MDMB-PINACA. AMB-CHIMICA did not induce the behavior.

Autonomic behavior

Exophthalmos, shed tears, and salivation were not observed for all the test compounds. 5F-MDMB-PINACA and 5F-MDMB-PICA increased the pupil size to the same degree, but the effect of 5F-MDMB-PINACA rapidly decreased.

The behaviors of the palpebral opening of marshmallow, 5F-EMB-PINACA, and AMB-CHMICA were similar. 5F-MDMB-PICA suppressed the palpebral opening for 1 h after combustion; however, the behavior was not observed for 5F-MDMB-PINACA.

The respiratory and heart rates indicated a similar time-course for each tested compound, except for AMB-CHMICA, which did not affect these rates. 5F-EMB-PINACA suppressed the behaviors 15 min after combustion. 5F-MDMB-PINACA and 5F-MDMB-PICA strongly suppressed the behaviors for 1 h. The degree of the suppression from 5F-MDMB-PINACA was constant, but those of 5F-MDMB-PICA increased over time.

The body temperature decreased with marshmallow and returned to normal 1 h after combustion; however, the SCs decreased the temperature for 1 h after combustion. The body temperature of the mice treated with 5F-EMB-PINACA and AMB-CHMICA returned to normal as time passed. 5F-MDMB-PINACA and 5F-MDMB-PICA sharply decreased the temperature for 1 h. By contrast, SCs result in tachycardia and hyperthermia in humans [7]. However, it is unclear why SCs induce the opposite effect in mice.

There was no change in skin color with 5F-EMB-PINACA or AMB-CHMICA. However, 5F-MDMB-PINACA resulted the color to be a little red 15 min after combustion, whereas 5F-MDMB-PICA changed the skin color to white for 1 h.

Catalepsy test

Table 3 shows the results of the catalepsy test. 5F-MDMB-PICA, 5F-EMB-PINACA, and AMB-CHMICA showed that the mice recovered from catalepsy over time, but the time to recover was different between the SCs. The catalepsy caused by AMB-CHMICA lasted the shortest amount of time. For 5F-EMB-PINACA, the catalepsy of all mice recovered 1 h after combustion. The number of mice with catalepsy from 5F-MDMB-PICA decreased over time, but in some mice, it was also observed 1 h after combustion. When exposed to 5F-MDMB-PINACA, the mice were exhausted and could not hold on to the crossbar, thus jumping around or going into convulsion 15 min after combustion. These mice were determined as negative for catalepsy. Catalepsy generally disappears over time; therefore, it was presumed that catalepsy was not observed due to the drastic effect of 5F-MDMB-PINACA.

Table 3 The number of positive mice in catalepsy test ($n = 5$)

Observation time (h)	0.25	0.5	1
Marshmallow[a]	0	0	0
5F-MDMB-PINACA[a]	0	2	4
5F-MDMB-PICA	5	3	2
5F-EMB-PINACA	4	1	0
AMB-CHMICA	2	0	0

[a]Referred in [19]

Discussion

Functional assays for carboxamide-type SCs at the CB_1 and CB_2 receptors have been previously performed. Banister S. D. et al. evaluated 5F-MDMB-PICA, AMB-CHMICA, and 5F-MDMB-PINACA using a fluorometric imaging plate reader membrane potential assay and reported that the EC_{50} values of each SC at the CB_1 receptor were 0.45, 3.50, and 0.59 nM, respectively [12]. Thus, 5F-MDMB-PICA was stronger than AMB-CHMICA and equivalent to 5F-MDMB-PINACA. Noble et al., Antonides et al., and Wouters et al. used β-arrestin2 for functional assays [25–27]. The EC_{50} value of 5F-MDMB-PICA at the CB_1 receptor was evaluated as 3.26 nM by Noble et al. [25], and that of (*S*)-5F-MDMB-PINACA was evaluated as 1.78 nM by Antonides et al. [26]. Wouters et al. reported that EC_{50} values of 5F-EMB-PINACA and 5F-MDMB-PINACA were 8.76 and 0.84 nM, respectively [27]. On treating the EC_{50} values of 5F-MDMB-PINACA evaluated by Antonides et al. and Wouters et al. equally (1.78 and 0.84 nM, respectively) and comparing the SCs tested by them as (*S*)-enantiomers, 5F-MDMB-PICA was found to be stronger than 5F-EMB-PINACA and equivalent to 5F-MDMB-PINACA. Although the functional assay methods differed, the order of strength of the functional activities for carboxamide-type SCs (AMB-CHMICA < 5F-EMB-PINACA < 5F-MDMB-PICA ≈ 5F-MDMB-PINACA) corresponded between all studies.

The activation of CB_1 receptor is involved in severely acute adverse effects when consuming SC products [28]. 5F-MDMB-PICA possesses functional activity at CB_1 receptor similar to that of 5F-MDMB-PINACA, resulting in many traffic collisions and fatal cases in Japan, and is expected to be a threat to public health.

The degrees of various behaviors of mice by the inhalation exposure test with the SCs corresponded to the functional activity at the CB_1 receptor, e.g., passivity and suppression of spontaneous activity, abnormal gait, abnormal position, and grip strength. 5F-MDMB-PINACA was estimated to possess strong functional activity and induced various behaviors. 5F-MDMB-PICA possessed the equivalent functional activity to 5F-MDMB-PINACA and also induced similar behaviors in an equivalent or weaker manner. The use of 5F-MDMB-PINACA resulted in several fatal cases in Japan [8]. The results suggest that 5F-MDMB-PICA also caused health hazard and fatalities as with 5F-MDMB-PINACA when smoked. However, given that the behavior on muscle tone was different between these SCs, 5F-MDMB-PICA was expected to cause different symptoms than those observed for 5F-MDMB-PINACA. Some carboxamide-type SCs induce catalepsy accompanied by muscle rigidity in the extremities for human. When abusers of the SCs drive in cars, they are not able to release the accelerator due to catalepsy and muscle rigidity, causing terrible traffic accidents. Because 5F-MDMB-PINACA has excessive potency, drivers using the SC are not able to drive at all, and fortunately, accidents are not caused [7]. 5F-MDMB-PICA did not induce muscle rigidity for mouse. If drivers took 5F-MDMB-PICA, they would continue to drive in the state of inebriation caused by the SC and cause traffic accidents.

The effects on the behaviors from exposure to 5F-EMB-PINACA and AMB-CHMICA decreased over time. In Japan, traffic accidents were caused by the consumption of herbal products containing 5F-AMB, and the users of the products recovered rapidly within an hour after consumption [7]. Furthermore, the inhalation exposure test was performed with a product containing 5F-AMB, which also contained AB-CHMINACA, another SC, and the results showed similar tendencies to 5F-EMB-PINACA and AMB-CHMICA [18]. Therefore, a rapid recovery is expected when smoking these SCs.

The degrees of the suppression of sound, touch, and pain responses as well as righting, pinna, corneal, and tendon reflexes also corresponded to the functional activity at the CB_1 receptor. However, AMB-CHMICA excited these responses and reflexes contrary to the other SCs. Especially, the SC excited the pain response at 30 min and 1 h after combustion. Antinociception is known as one of the tetrad of cannabinoid actions in addition to hypothermia, catalepsy, and suppression of locomotion. AMB-CHMICA may have induced the enhancement of nociception rather than antinociception.

SCs are generally abused in the form of smoking [15–17]. In this study, the influence of smoking SCs was investigated with the equipment in which mice inhaled the smoke from burned SCs; hence, we could evaluate the biological effects considering how they are consumed and thermal degradation. It has been reported that some SCs produce degradation products during smoking [29–34]. Furthermore, a very small amount of 5F-EMB-PINACA and AMB-CHMICA was also degraded by combustion (see the Supplementary Material). Therefore, the equipment allows us to accurately evaluate the biological effects when smoking SCs.

Conclusion

The [^{35}S]GTPγS binding assays were performed to evaluate the activities of three carboxamide-type SCs (5F-MDMB-PICA, 5F-EMB-PINACA, and AMB-CHMICA) for the CB_1 and CB_2 receptors. 5F-MDMB-PICA had the strongest functional activity at the CB_1 receptor. Considering other reports on the functional activity of these SCs,

5F-MDMB-PICA possessed the equivalent functional activity of 5F-MDMB-PINACA, which has caused many fatal cases in Japan, so 5F-MDMB-PICA can also cause many fatalities.

The biological effects of smoking the SCs were also evaluated using inhalation exposure tests. The equipment produces an accurate evaluation considering the way of inhalation and thermal degradation. The degrees of various behaviors corresponded to the functional activity at the CB_1 receptor. 5F-MDMB-PICA possesses stronger biological activity than 5F-EMB-PINACA and AMB-CHMICA, and equivalent activity to 5F-MDMB-PINACA. 5F-MDMB-PICA is expected to cause intoxication accompanied with fatalities, as with the expectation indicated by the functional activity at a CB_1 receptor. On the other hand, 5F-MDMB-PICA can reduce muscle tone, but 5F-MDMB-PINACA can conversely enhance it. We expect that when smoking these SCs, 5F-MDMB-PICA can cause traffic accidents unlike 5F-MDMB-PINACA, which induces severe muscle rigidity; thus, abusers are not able to drive cars. AMB-CHMICA induced some biological effects, particularly sound, touch, and pain responses and righting, pinna, corneal and tendon reflex, opposite of the other tested SCs. As per the results, it seems that AMB-CHMICA induces the enhancement of nociception rather than antinociception, which is known as one of the tetrad of cannabinoid actions, including hypothermia, catalepsy, and suppression of locomotion. However, further studies are needed to interpret the results. As this study reveals that the biological effects of SCs could be complicated, we consider that there is a good reason for the presence of regulatory acts not only in Japan but also worldwide.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal experiments performed in this study were reviewed and approved by the Animal Experiment Committee of Tokyo Metropolitan Institute of Public Health. The animal experiments were performed in accordance with the code of practice for animal experiment, and the standards of conduct and the standard operation procedure for experimental animal facility of Tokyo Metropolitan Institute of Public Health based on the "Act on Welfare and Management of Animals" (Act No. 105 of 1973), "Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals" (Notice of The Ministry of the Environment No. 88 of 2006), "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Research Institutions under the jurisdiction of the ministry of Health, Labor and Welfare" (Notification of the Ministry of Health, Labor and Welfare of 1st June, 2006), "Standards relating to Methods of Destruction of Animals" (Notice of the Ministry of the Environment No. 105 of 2007), "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan of 1st June, 2006), and "Tokyo Metropolitan Act for Welfare and Management of Animals" (Act No. 81 of 1979).

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