Evaluation of carboxamide-type synthetic cannabinoids as CB₁/CB₂ receptor agonists: difference between the enantiomers

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Abstract Recently, carboxamide-type synthetic cannabinoids have been distributed globally as new psychoactive substances (NPS). Some of these compounds possess asymmetric carbon, which is derived from an amide moiety composed of amino acid derivatives (i.e., amides or esters of amino acids). In this study, we synthesized both enantiomers of synthetic cannabinoids, \(\text{N}-(1\text{-amino-3-methyl-1-oxobutan-2-yl})\text{-}1\text{-}(2\text{-fluorobenzyl})\text{-}1\text{H-indazole-3-carboxamide} \) (AB-FUBINACA 2-fluorobenzyl isomer), \(\text{N}-(1\text{-amino-1-oxo-3-phenylpropan-2-yl})\text{-}1\text{-}(\text{cyclohexylmethyl})\text{-}1\text{H-indazole-3-carboxamide} \) (APP-CHMINACA), ethyl \[1\text{-}(5\text{-fluoropentyl})\text{-}1\text{H-indazole-3-carbonyl}]\text{valinate} \) (5F-EMB-PINACA), ethyl \[1\text{-}(4\text{-fluorobenzyl})\text{-}1\text{H-indazole-3-carbonyl}]\text{valinate} \) (EMB-FUBINACA), and methyl \[2\text{-}[1\text{-}(4\text{-fluorobenzyl})\text{-}1\text{H-indole-3-carboxamido}]\text{-}3,3\text{-dimethylbutanoate} \) (MDMB-FUBICA), which were reported as NPS found in Europe from 2014 to 2015, to evaluate their activities as CB₁/CB₂ receptor agonists. With the exception of \((\text{R})\text{-MDMB-FUBICA}\), all of the tested enantiomers were assumed to be agonists of both CB₁ and CB₂ receptors, and the EC₅₀ values of the \((\text{S})\)-enantiomers for the CB₁ receptors were about five times lower than those of \((\text{R})\)-enantiomers. \((\text{R})\text{-MDMB-FUBICA}\) was shown to function as an agonist of the CB₂ receptor, but lacks CB₁ receptor activity. To the best of our knowledge, this is the first report to show that the \((\text{R})\)-enantiomers of the carboxamide-type synthetic cannabinoids have the potency to activate CB₁ and CB₂ receptors. The findings presented here shed light on the pharmacological properties of these carboxamide-type synthetic cannabinoids in forensic cases.

Keywords Carboxamide-type synthetic cannabinoid · Enantiomeric difference in activity · CB₁/CB₂ receptor agonist · AB-FUBINACA 2-fluorobenzyl isomer · APP-CHMINACA · 5F-EMB-PINACA

Introduction

In recent years, several new psychoactive substances (NPS) have been newly identified from illicit drug products, one after another, and have been abused as legal alternatives to scheduled drugs [1]. One of the most popular classes of NPS is synthetic cannabinoids, which are recreationally used as substitutes for Cannabis sativa. Forensic cases of synthetic cannabinoid use have increased recently; the adverse effects of indazole-carboxamide-type synthetic cannabinoids, especially, present severe social problems [2].

In a previous study, we developed a method to separate enantiomers of synthetic cannabinoids by liquid chromatography–mass spectrometry (LC–MS) [3]. In the case of NPS classified into cathinone and phenethylamine groups, several reports have concluded that \((\text{S})\)-enantiomers are more potent than \((\text{R})\)-enantiomers [4–7]. Among the carboxamide-type synthetic cannabinoids, some compounds possess asymmetric carbon, which is derived from an amide moiety composed of amino acid derivatives (i.e., amides or esters of amino acids). These classes of compounds were first developed by Pfizer as potential therapeutic drugs; however, their patent only includes \((\text{S})\)-enantiomers, even though the structures of these compounds have chiral centers [8]. Some previous
studies reported the cannabimimetic activities of the chiral synthetic cannabinoids, but they all investigated only the (S)-enantiomers, and thus the pharmaceutical activities of the (R)-enantiomers remain unknown [9].

In this study, we synthesized both enantiomers of N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(2-fluorobenzyl)-1H-indazole-3-carboxamide (AB-FUBINACA 2-fluorobenzyl isomer), N-(1-amino-1-oxo-3-phenylpropan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide (APP-CHMINACA), ethyl [1-(5-fluoropentyl)-1H-indazole-3-carbonyl]valinate (5F-EMB-PINACA), ethyl [1-(4-fluorobenzyl)-1H-indazole-3-carbonyl]valinate (EMB-FUBINACA), and methyl 2-[1-(4-fluorobenzyl)-1H-indole-3-carboxamido]-3,3-dimethylbutanoate (MDMB-FUBICA) to evaluate their potency to activate CB$_1$ and CB$_2$ cannabinoid receptors.

Materials and methods

Reagents

Methyl 1H-indazole-3-carboxylate and (bromomethyl)cyclohexane were purchased from Sigma-Aldrich (St. Louis, MO, USA); 1-bromo-5-fluoropentane from Fluorochem Ltd. (Hadfield, UK); L-valine ethyl ester hydrochloride, L-valine ethyl ester hydrochloride, and D-valine amide from Wako Chemical Industries Ltd. (Hiroshima, Japan). All other reagents used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded using an ECZS-400 spectrometer (JEOL Resonance, Tokyo, Japan) with dimethyl sulfoxide (DMSO)-d$_6$ as the solvent. The chemical shifts $\delta$ were recorded in ppm relative to tetramethylsilane ($^1$H: $\delta = 0$ ppm, $^{13}$C: $\delta = 0$ ppm) or the solvent ($^{13}$C: $\delta = 39.5$ ppm) as an internal standard. The compounds were assigned by $^1$H NMR, $^{13}$C NMR, distortionless enhancement by polarization transfer, $^1$H-$^{13}$C heteronuclear multiple quantum coherence, $^1$H-$^{13}$C heteronuclear multiple-bond correlation, and $^1$H-$^1$H correlation spectroscopy.

Chiral chromatography

Chirality of the compounds was confirmed by LC–MS as described previously [3]. A CHIRALPAK AZ-3R column (3.0 µm particle size, 150 × 2.1 mm i.d.) (Daicel Corporation, Osaka, Japan) was used, and the mobile phase was composed of H$_2$O/acetonitrile (55:45, v/v) under isocratic conditions. The flow rate of the mobile phase was 0.3 mL/min, and the injection volume was 1 µL. The column temperature was 40 °C. The stock standard solutions were prepared as described previously [3]. When necessary, the sample solutions and the stock standard solutions were diluted by the mobile phase for the liquid chromatography–high-resolution-mass spectrometry (LC–HR-MS) analysis [3].

Chemical synthesis

The synthetic pathway of these enantiomers is shown in Fig. 1. All of the target compounds were made from methyl 1H-indazole-3-carboxylate (compound 1) or methyl 1H-indole-3-carboxylate (compound 2). The enantiomers of the target compounds were synthesized by a slightly modified version of a previously described method [3, 10]. Compound 1 or 2 was N-alkylated by alkyl halide under basic conditions and yielded compounds that were deprotected by hydrolysis of the methyl group. The target compounds were synthesized by chlorination of the carboxylic acids by oxalyl chloride and then amidation with the amino acid derivatives. The chemical structures of the compounds dealt with in this study are shown in Fig. 2.

N-Aklylation of methyl 1H-indazole/indole-3-carboxylate

Compound 5 was synthesized as described previously [3]. Compounds 3, 4, 6, and 7 were prepared in the same manner, except for the starting compounds. To a solution of compound 1 or 2 in tetrahydrofuran (THF), potassium tert-butoxide (t-BuOK) (1.2 equivalent) was added. To the flask containing the solution, alkyl halide (1.5 equivalent) was added and stirred at room temperature for more than 12 h. Ethyl acetate and distilled water were added to the solution, and the organic layer was separated from the aqueous layer. The organic layer was then washed with saturated aqueous NaCl and dried with anhydrous MgSO$_4$. The solution was filtered, and the solvent was removed under a vacuum. The residue was purified by an Isolute Speckt System (Biotage, Tokyo, Japan) on a SNAP Ultra column (Biotage) with ethyl acetate in n-hexane as the mobile phase. The solvent of the obtained fraction was removed under a vacuum to yield compounds 3, 4, 6 or 7.

Hydrolysis of methyl N-alkyl-1H-indazole-3-carboxylate

Synthesis of compound 10 was performed as described previously [3]. Compounds 8, 9, and 11 were synthesized similarly, except for the starting compounds. To a flask containing compounds 3, 4, or 6, THF, ethanol, and 1 M NaOH$_{aq}$ (approximately 2.5 mL/1 mmol of the material compounds)
were added and stirred at room temperature for more than 16 h. After the reaction, the organic solvents were removed under a vacuum. Ethyl acetate and 20% Na₂CO₃ in H₂O were added to the solution and stirred, and the solution was allowed to separate. The organic layer was extracted by 20% Na₂CO₃ in H₂O again, and the aqueous layers were combined. The aqueous layer was extracted twice with 1 M NaOH in H₂O. The organic layer was washed twice with brine. Anhydrous MgSO₄ was added to the organic layer and removed under a vacuum to yield compound 12.

Hydrolysis of methyl N-alkyl-1H-indole-3-carboxylate

Compound 12 was synthesized by the hydrolysis of compound 7. To a flask containing compound 7, THF, ethanol, methanol, and 10 M NaOH (aq) (approximately 2 mL/ 1 mmol of compound 7) were added and stirred at room temperature for 2.5 h. After the reaction, the organic solvents were removed under a vacuum. Ethyl acetate was added to the solution and mixed well, and the solution was allowed to separate. The organic layer was extracted twice with 1 M NaOH in H₂O, and the aqueous layers were then combined. The aqueous layer was neutralized with HCl and extracted with ethyl acetate twice. The organic layer was then washed twice with brine. Anhydrous MgSO₄ was added to the combined organic layer and removed under a vacuum to yield compound 12.

(R)-N-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(2-fluorobenzyl)-1H-indazole-3-carboxamide (13S)

To a flask containing compound 8 in dichloromethane (20 mL/1 mmol of compound 8), N,N-dimethylformamide (20 μL/1 mmol) and oxalyl chloride (2.5 equivalent) were added and stirred at room temperature for 30 min. The solvent was then removed under a vacuum to obtain 1-(2-fluorobenzyl)-1H-indazole-3-carbonyl chloride as a hazy oily residue. The residue was dissolved in dichloromethane (10 mL), and L-valine amide hydrochloride (1.1 equivalent) was added. Triethylamine (1 mmol) was added to the mixture and then the resulting solution was stirred at room temperature for 90 min. The solvent was removed under reduced pressure and dissolved in ethyl acetate. The solvent was separated with water and washed with 0.1 M HCl, saturated NaHCO₃, and saturated NaCl. Drying with MgSO₄ and removal of the solvent afforded compound 13S as a fibrous white solid.

¹H NMR (DMSO-d₆): δ 8.19 (1H, dd, J = 8.0, 1.0 Hz), 7.77 (1H, d, J = 8.5 Hz), 7.69 (1H, d, J = 9.0 Hz), 7.65 (1H, s), 7.48 (1H, t-like), 7.43–7.34 (1H, m), 7.30 (1H, dd, J = 8.0, 7.0 Hz), 7.26–7.18 (2H, m), 7.17–7.09 (2H, m), 5.84 (2H, s), 4.42 (1H, dd, J = 9.0, 6.0 Hz), 2.09 (1H, m), 0.94 (3H, d, J = 7.0 Hz), 0.89 (3H, d, J = 7.0 Hz). ¹³C NMR (DMSO-d₆): δ 172.5, 161.1, 159.9 (d, J = 246 Hz), 140.8, 137.2, 130.1 (d, J = 8 Hz), 129.7 (d, J = 4 Hz), 127.0, 124.7 (d, J = 4 Hz), 123.5 (d, J = 14 Hz), 122.7, 122.1, 121.8, 115.5 (d, J = 21 Hz), 110.4, 56.8, 46.5 (d, J = 3 Hz), 31.2, 19.3, 17.9.

Fig. 1 Procedures for synthesizing the enantiomers dealt with in this study. tBu-OK potassium tert-butoxide, THF tetrahydrofuran, DMF N,N-dimethylformamide, (COCl)₂ oxalyl chloride, TEA triethylamine, MeOH methanol
7.28–7.20 (2H, m), 7.18–7.10 (2H, m), 5.84 (2H, s), 4.42 (1H, dd, J = 9.0, 6.0 Hz), 2.10 (1H, m), 0.94 (3H, d, J = 7.0 Hz), 0.89 (3H, d, J = 7.0 Hz). $^{13}$C NMR (DMSO-d$_6$): $\delta$ 172.5, 161.2, 159.9 (d, J = 247 Hz), 140.8, 137.2, 130.2 (d, J = 9 Hz), 129.8 (d, J = 4 Hz), 127.0, 124.7 (d, J = 3 Hz), 123.6 (d, J = 15 Hz), 122.7, 122.1, 121.8, 115.6 (d, J = 21 Hz), 110.5, 56.8, 46.5 (d, J = 3 Hz), 31.2, 19.4, 18.0.

Fig. 2 Chemical structures of the target compounds in this study

$^{13}$C NMR (DMSO-d$_6$): $\delta$ 172.5, 161.2, 159.9 (d, J = 247 Hz), 140.8, 137.2, 130.2 (d, J = 9 Hz), 129.8 (d, J = 4 Hz), 127.0, 124.7 (d, J = 3 Hz), 123.6 (d, J = 15 Hz), 122.7, 122.1, 121.8, 115.6 (d, J = 21 Hz), 110.5, 56.8, 46.5 (d, J = 3 Hz), 31.2, 19.4, 18.0.
Subjecting compound 9 to a procedure similar to that for 13S, but substituting \( \text{t-} \)valine ethyl ester hydrochloride for \( \text{t-} \)valine amide, yielded 14S as a clear oily residue.

\[ ^{1}H \text{ NMR (DMSO-}d_{6}) : \delta 8.15 \ (1H, d, J = 8.5 \text{ Hz}), \ 8.12 \ (1H, d, J = 8.5 \text{ Hz}), \ 7.80 \ (1H, d, J = 8.5 \text{ Hz}), \ 7.46 \ (1H, d, J = 8.5 \text{ Hz}), \ 7.29 \ (1H, d, J = 8.5 \text{ Hz}) \]

Subjecting compound 10 to a procedure similar to that for 13S, but substituting \( \text{p-} \)valine ethyl ester hydrochloride for \( \text{t-} \)valine amide, yielded 15R as a clear oily residue.

\[ ^{1}H \text{ NMR (DMSO-}d_{6}) : \delta 8.11 \ (1H, d, J = 8.0 \text{ Hz}), \ 7.99 \ (1H, d, J = 8.0 \text{ Hz}), \ 7.81 \ (1H, d, J = 8.5 \text{ Hz}), \ 7.47 \ (1H, d, J = 8.5 \text{ Hz}) \]

Subjecting compound 11 to a procedure similar to that for 13S, but substituting \( \text{L-} \)phenylalanine amide hydrochloride for \( \text{L-} \)phenylalanine amide hydrochloride, yielded 16S as a crystalline white solid.

\[ ^{1}H \text{ NMR (DMSO-}d_{6}) : \delta 8.11 \ (1H, d, J = 8.0 \text{ Hz}), \ 7.96 \ (1H, d, J = 8.5 \text{ Hz}), \ 7.47 \ (1H, d, J = 8.5 \text{ Hz}) \]

Subjecting compound 12 to a procedure similar to that for 16S, but substituting \( \text{p-} \)valine ethyl ester hydrochloride for \( \text{L-} \)phenylalanine amide hydrochloride, yielded 17R as a crystalline white solid.

\[ ^{1}H \text{ NMR (DMSO-}d_{6}) : \delta 8.11 \ (1H, d, J = 8.0 \text{ Hz}), \ 7.96 \ (1H, d, J = 8.5 \text{ Hz}), \ 7.47 \ (1H, d, J = 8.5 \text{ Hz}) \]
Subjecting compound 12 to a procedure similar to that for 13S, but substituting L-tert-leucine methyl ester hydrochloride for L-valine amide yielded 17S as a white powder.

\(^1\)H NMR (DMSO-d\(_6\)): \(\delta = 8.50 (1H, s), 8.11 (1H, d, J = 8.0 Hz), 7.71 (1H, d, J = 8.5 Hz), 7.52 (1H, d, J = 8.5 Hz), 7.34–7.31 (2H, m), 7.20–7.11 (4H, m, overlapped), 5.47 (2H, s), 4.50 (1H, d, J = 8.5 Hz), 3.66 (3H, s), 1.04 (9H, s). 13C NMR (DMSO-d\(_6\)): \(\delta = 172.0, 164.3, 161.6 (d, J = 244 Hz), 136.0, 133.8 (d, J = 3 Hz), 132.1, 129.2 (d, J = 8 Hz), 126.9, 122.2, 121.3, 120.9, 115.5 (d, J = 22 Hz), 110.6, 109.3, 60.0, 51.4, 48.8, 33.8, 26.8.

**Methyl (R)-2-[(4-fluorobenzyl)-1H-indole-3-carboxamido]-3,3-dimethylbutanoate (17R)**

Subjecting compound 12 to a procedure similar to that for 17S, but substituting D-tert-leucine methyl ester hydrochloride for L-tert-leucine methyl ester hydrochloride, yielded 17R as a white powder.

\(^1\)H NMR (DMSO-d\(_6\)): \(\delta = 8.50 (1H, s), 8.11 (1H, d, J = 7.0 Hz), 7.71 (1H, d, J = 8.5 Hz), 7.53 (1H, d, J = 8.0 Hz), 7.30–7.31 (2H, m), 7.20–7.13 (4H, m, overlapped), 5.47 (2H, s), 4.50 (1H, d, J = 8.5 Hz), 3.66 (3H, s), 1.04 (9H, s). 13C NMR (DMSO-d\(_6\)): \(\delta = 172.0, 164.3, 161.6 (d, J = 244 Hz), 136.0, 133.7 (d, J = 3 Hz), 132.1, 129.2 (d, J = 8 Hz), 126.9, 122.2, 121.2, 120.9, 115.5 (d, J = 21 Hz), 110.6, 109.3, 60.0, 51.4, 48.7, 33.8, 26.8.

**In vitro assays to evaluate the CB\(_1\)/CB\(_2\) receptor activities**

For the evaluation of CB\(_1\)/CB\(_2\) cannabinoid receptor activity, \(^{[35]}\)S[GTPyS binding assays were performed. These assays were performed at ADME and Tox. Research Institute, Sekisui Medical Co., Ltd. (Tokai-mura, Ibaraki, Japan). The assay conditions were as described previously [11], except for the tested concentration levels of the compounds ranging from 1 \(\times\) 10\(^{-11}\) to 1 \(\times\) 10\(^{-4}\) M. Agonistic activities (EC\(_{50}\) value; concentration showing 50% response) of the test compound to the cannabinoid receptors CB\(_1\) and CB\(_2\) were measured.

**Results and discussion**

In this study, we synthesized both (S)- and (R)-enantiomers of synthetic cannabinoids to evaluate their pharmacological properties. All target compounds were reported in an EMCDDA-Europol joint report to be NPS sold and recreationally used in Europe [12, 13]. NMR data for the enantiomers were similar, as shown in the section on materials and methods above, and the results are in good agreement with the values expected for their structures.

Figure 3 shows the extracted ion chromatograms for the enantiomers of the target compounds (1 \(\mu\)g/mL solution for both enantiomers) obtained by LC–HR-MS using the method that we developed previously [3]. By the previously developed method, all of the enantiomers of the tested compounds were separated, and no enantio-impurities were found in their chromatograms, except for (S) APP-CHMINACA. Approximately less than 2% of (R) APP-CHMINACA was confirmed in the extracted ion chromatogram of (S) APP-CHMINACA as shown in Fig. 3. However, because (R)-enantiomer was much less effective than (S)-enantiomer to both CB\(_1\) and CB\(_2\) receptors, it is suspected that the effect of contaminated (R)-enantiomer on the EC\(_{50}\) value was almost negligible.

Table 1 shows the results of the \(^{[35]}\)S[GTPyS binding assays performed in this study. Each compound functioned as both a CB\(_1\) and a CB\(_2\) receptor agonist, except for (R) MDMB-FUBICA. The EC\(_{50}\) values of (S)-enantiomers for the CB\(_1\) receptors were at least one-fifth of or less than those of (R)-enantiomers, which indicates that (S)-enantiomers are more potent CB\(_1\) receptor agonists than (R)-enantiomers. The (S)-enantiomer of APP-CHMINACA could activate CB\(_1\) at a concentration 134 times lower than that for the (R)-enantiomer. For MDMB-FUBICA, the CB\(_1\) receptor activity was not confirmed for the (R)-enantiomer even at the maximum tested concentration, but the EC\(_{50}\) of the (S)-enantiomer was 9.72 \(\times\) 10\(^{-9}\) M; the difference in the EC\(_{50}\) levels between the enantiomers of MDMB-FUBICA to activate the CB\(_1\) receptor was more than 10,000-fold, which indicates that the activities for the CB\(_1\) receptor are markedly different depending on the chirality of the compounds. In contrast, we could not observe a clear relevance between EC\(_{50}\) values for the CB\(_2\) receptor and chirality of the compounds. (R) APP-CHMINACA and (R) MDMB-FUBICA lacked the potential to be CB\(_1\) receptor agonists, but both of their EC\(_{50}\) values for the CB\(_2\) receptor were less than 10\(^{-6}\) M; all of the target compounds were strong agonists for the CB\(_2\) receptor, irrespective of their chirality.

As for the CB\(_1\)/CB\(_2\) selectivity, (R)-enantiomers tended to be CB\(_2\)-selective and were at least 20-fold more active than for CB\(_1\) for all of the tested compounds. Interestingly, (R) MDMB-FUBICA functioned only on the CB\(_2\) receptor as an agonist, while showing no CB\(_1\) activity as mentioned above, although (S) MDMB-FUBICA could activate both of these receptors at nanomolar levels. In several preclinical studies, compounds such as JWH-133 and HU308 were used as CB\(_2\)-selective ligands [14]. More recently, the synthesis and evaluation of novel CB\(_2\)-selective agonists have been repeatedly reported. In these pharmacological or
medicinal chemistry studies seeking CB$_2$ agonists, the most selective compound showed a selective index of CB$_2$/CB$_1$ of only 100- to 1000-fold. In this study, (R) MDMB-FUBICA was shown to have potency to activate the CB$_2$ receptor signal with an EC$_{50}$ value of 3.1 $\times$ 10$^{-9}$ M, but had no [${}^{35}$S]GTP$_{S}$ binding activity for the CB$_1$ receptor, even at 1.0 $\times$ 10$^{-4}$ M, which corresponds to a selective index of more than 3.2 $\times$ 10$^{4}$-fold.

The metabolism of MDMB-FUBICA enantiomers has not been reported yet; thus it remains unclear whether
(R) MDMB-FUBICA can mimic the function of the CB$_2$ receptor agonist when applied to an animal model to evaluate the effect on the central nervous system. Recently, it was reported that the (S)-enantiomer was mainly detected in herbal products containing chiral carboxamide-type synthetic cannabinoids [3, 15]. The cannabimimetic
activities of each enantiomer presented in this study may be helpful to precisely recognize forensic cases associated with these synthetic cannabinoids when evaluated with the quantitative data of a specimen analyzed after enantiomeric separation.

Conclusions

The activities of the enantiomers of five synthetic cannabinoids (AB-FUBINACA 2-fluorobenzyl isomer, APP-CHMINACA, 5F-EMB-PINACA, EMB-FUBINACA, and
MDMB-FUBICA) were evaluated by the \([^{35}\text{S}]{\text{GTP}}_\gamma\text{S}\) binding assay. We also confirmed that these enantiomers could be clearly differentiated by chiral-LC–MS. These findings should contribute to better understanding of the forensic cases associated with these synthetic cannabinoids.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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