

**Detection and activity profiling of synthetic cannabinoids and metabolites
with a newly developed bio-assay**

Annelies Cannaert¹, Jolien M. Storme¹, Florian Franz², Volker Auwärter², Christophe P. Stove¹

¹Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium

²Institute of Forensic Medicine, Forensic Toxicology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Albertstr. 9, 79104 Freiburg, Germany

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Supplementary Data S-1

Chemicals and reagents

Dulbecco's modified eagle's medium (DMEM), Opti-MEM[®] I Reduced Serum Medium, penicillin/streptomycin (10.000 IU/ml and 10.000µg/ml), amphotericin B (250µg/ml), glutamine (200mM), the restriction enzymes *Xho*I and *Eco*RI and the DNA polymerase (Phusion polymerase, a polymerase with proofreading activity) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). The transfection reagent FuGENE[®] HD and the Nano-Glo Live Cell reagent were purchased from Promega (Madison, WI, USA). Primers were procured from Eurofins Genomics (Ebersberg, Germany). JWH-122 ((4-methyl-1-naphthyl)-(1-pentyl-1*H*-indol-3-yl)methanone), JWH-210 ((4-ethyl-1-naphthyl)-(1-pentyl-1*H*-indol-3-yl)methanone), PB-22 (1-pentyl-1*H*-indole-3-carboxylic acid 8-quinolinyl ester), their 5-fluoro analogues MAM-2201, EAM-2201, 5F-PB-22, their metabolites and all deuterated standards were supplied by Cayman Chemical (Ann Arbor, MI, USA), except JWH-018 (naphthyl(1-pentyl-1*H*-indol-3-yl)methanone) was obtained from LGC (Wesel, Germany) and 5-OH-pentyl-JWH-018 and N-pentanoic acid JWH-018 were purchased from Sigma Aldrich. Fetal bovine serum (FBS), poly-D-lysine, formic acid (Rotipuran[®] ≥ 98 %, p.a.), potassium hydrogen phosphate (≥ 99 %, p.a.), 2-propanol (Rotisol[®] ≥ 98 %, p.a.), acetonitrile (LC-MS grade), ammonium formate 10 M (99,995 %) and potassium hydroxide (puriss. p.a. ≥ 86 % (T) pellets) were from Sigma Aldrich (Steinheim, Germany). Roche Diagnostics (Mannheim, Germany) supplied the β -glucuronidase (*E. coli* K 12). Deionized water was prepared using a Medica[®] Pro deionizer from ELGA (Celle, Germany). Blank urine samples were donated by one volunteer and tested for the absence of synthetic cannabinoid metabolites prior to use. Mobile phase A (0.2 % formic acid and 2 mM ammonium formate in water) was freshly prepared prior to LC-MS/MS analysis. Mobile phase B was pure acetonitrile.

Plasmids and constructs

Plasmids containing the human *CNR1* (NM_016083) and *ARRB2* (NM_004313) coding sequences were purchased from Origene Technologies (Rockville, MD, USA). A plasmid containing the human *CNR2* (NM_001841) coding sequence and the expression vectors, NB MCS-1, NB MCS-2, NB MCS-3 and NB MCS-4 were kindly provided by respectively, Atwood *et al.* and Promega (Madison, WI, USA). The expression vectors contain the sequences encoding the subunits of the NanoLuc[®] luciferase (LgBiT or SmBiT) and the flexible linker (GSSGGGGSGGGGSSG). All expression plasmids were constructed by cloning PCR products, flanked by a unique restriction site, into the respective vectors, as described below. All constructs were sequence-verified.

To generate the constructs, specific primers were used to PCR-amplify the coding sequence of interest, flanked by *XhoI* or *EcoRI* restriction sites (see Table S-1a). PCR was performed on 100 pg of plasmid DNA using the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Pittsburg, PA, USA) according to the manufacturer's instructions. Reactions were done in a Mastercycler™ Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 98°C for 30s, 35 cycles of 98°C for 10s (denaturation), T_m for 30s (annealing), and 72°C for 1min (elongation), followed by a final extension at 72°C for 5min. The resulting amplification products were purified using E.Z.N.A.® MicroElute Cycle-Pure kit (VWR International, Radnor, PA, USA). Both the vector and the amplification products were digested with either *XhoI* or *EcoRI* restriction enzymes (Thermo Fisher Scientific) and purified using E.Z.N.A.® MicroElute Gel Extraction kit (VWR International). The digested PCR products were ligated into the corresponding dephosphorylated (TSAP Thermosensitive Alkaline Phosphatase, Promega), digested vector (see Table S-1b). After transformation of One Shot® Mach1™ T1 Phage-Resistant Chemically Competent E. coli (Thermo Fisher Scientific) with the ligated product, the ampicillin-resistant clones were screened by PCR using primers complementary to sequences within the insert and sequences of the vector surrounding the insert. The integrity of all constructs was confirmed by DNA sequencing. Positively screened colonies were grown and used for plasmid isolation, using E.Z.N.A.® Plasmid DNA Mini kit (VWR International).

Vector	POI	Primers	T_m (°C)
NB MCS-1	CB1	Forward ACTCAA <u>CTCGAG</u> ACC ATGAAGTCGATCC	69.6
NB MCS-2		Reverse ACTCAA <u>CTCGAG</u> CC CAGAGCCTCGGC	
	CB2	Forward ACTCAA <u>CTCGAG</u> CC CAGAGCCTCGGC	71.0
		Reverse ACTCAA <u>CTCGAG</u> CC GCAATCAGAGAGG	
	βarr2	Forward ACTCAA <u>GAATTC</u> ACC ATGGGGGAGAAAACCCGGGACC	71.1
		Reverse ACTCAA <u>GAATTC</u> CC GCAGAGTTGATCATCATAGTCG	
NB MCS-3	βarr2	Forward ACTCAA <u>GAATTC</u> A ATGGGGGAGAAAACCCGGGACC	69.7
NB MCS-4		Reverse ACTCAA <u>GAATTC</u> TCA GCAGAGTTGATCATCATAGTCG	

Table S-1a. Primers used to clone the protein of interest (POI) in the expression plasmids. Six extra nucleotides precede the restriction site (underlined). In some primers, extra nucleotides were added to correct the reading frame. Also the Kozak sequence or stop codon (bold) were added, if necessary. The nucleotides in italics are the coding sequences of the POI.

Vector	Fusion protein	POI	Restriction enzyme
NB MCS-1	POI - Linker - LgBiT	CB1	<i>XhoI</i>
		CB2	<i>XhoI</i>
		βarr2	<i>EcoRI</i>
NB MCS-2	POI - Linker - SmBiT	CB1	<i>XhoI</i>
		CB2	<i>XhoI</i>
		βarr2	<i>EcoRI</i>
NB MCS-3	LgBiT - Linker - POI	βarr2	<i>EcoRI</i>
NB MCS-4	SmBiT - Linker - POI	βarr2	<i>EcoRI</i>

Table S-1b. Lay-out of the expression vectors and restriction enzymes used for each protein of interest (POI).

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were routinely maintained at 37°C, 5% CO₂, under humidified atmosphere in DMEM (high glucose) supplemented with 10% heat-inactivated FBS, 2mM of glutamine, 100IU/ml of penicillin, 100µg/ml of streptomycin and 0.25µg/ml of amphotericin B. For experiments, HEK 293T cells were plated in 6-well plates at 5x10⁵ cells/well. The next day, cells were transiently transfected using FuGENE[®] HD reagent according to the manufacturer's protocol (optimal ratio of FuGENE:DNA 3:1). Transfection mixes contained 1.65µg of each of the plasmids of interest. On the third day, cells were plated on poly-D-lysine-coated 96-well plates at 5x10⁴ cells/well and incubated overnight.

Supplementary Data S-2

To evaluate the activity of the different SCs and their metabolites

To determine the activity of a compound, the absolute signals were corrected for solvent controls and for inter-well variability. First, the absolute raw signals (colored lines) were baseline-corrected by subtracting the average of the signals from vehicle control samples (black lines) (A to B). Next the signals were corrected for the inter-well variability by forcing the curve through 0 (B to C). The AUC were calculated and normalized by the AUC of the reference compound, JWH-018.

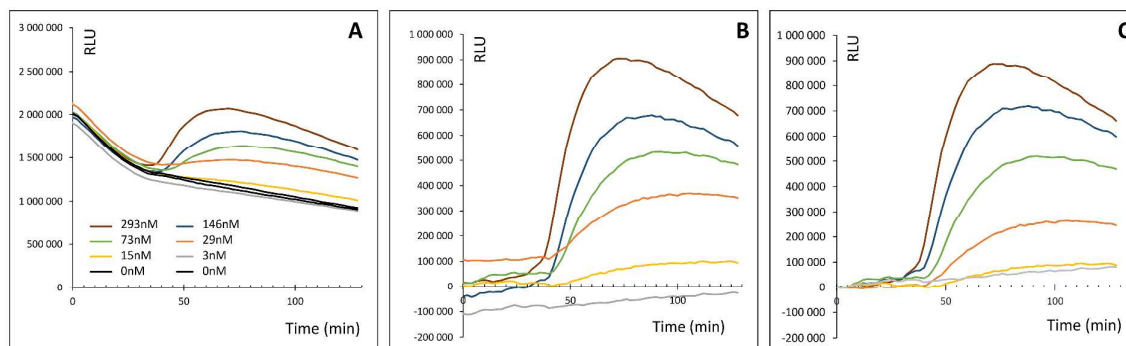


Figure S-2a: Example of solvent control and inter-well correction.

To assess the activity of the natural cannabinoids and the urinary samples

Here the absolute raw signals were only corrected for the inter-well variability. The absolute signals were corrected by forcing all the curves through the same starting point at time point 0 (A to B).

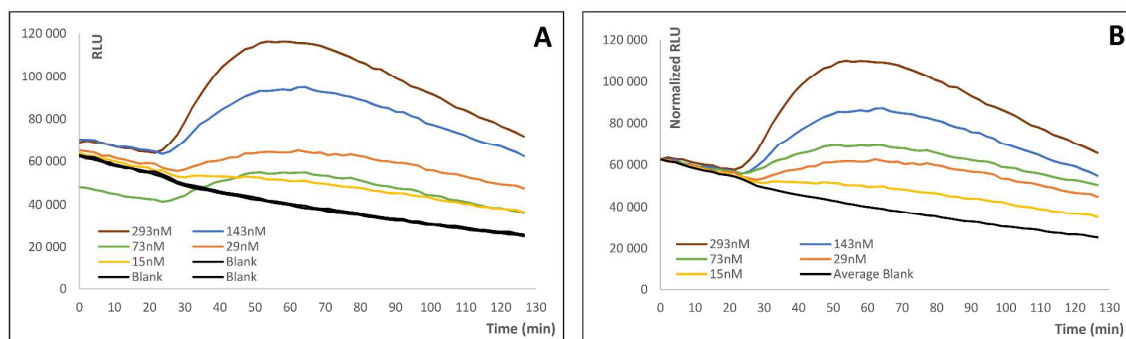


Figure S-2b: Example of inter-well correction.

Supplementary Data S-3

Supporting table showing MRM transitions and optimized MS parameters for the analytes detected in the urine samples and the used internal standards.

Analyte	Q1	Q3	DP	EP	CE	CXP	Internal standard
	[amu]	[amu]	[V]	[V]	[V]	[V]	
JWH-018 4-OH-pentyl	358	155	150	5	30	15	D5-JWH-018 4-OH-pentyl
	358	127	150	5	65	15	
JWH-018 5-OH-pentyl	358	155	150	5	30	15	D5-JWH-018 5-OH-pentyl
	358	127	150	5	65	15	
JWH-122 4-OH-pentyl	372	169	160	7	30	12	D5-JWH-122 5-OH-pentyl
	372	141	160	7	57	16	
JWH-122 5-OH-pentyl	372	169	160	7	30	12	D5-JWH-122 5-OH-pentyl
	372	141	160	7	57	16	
JWH-122 <i>N</i> -pentanoic acid	386	169	190	4	35	13	D4-JWH-018 <i>N</i> -pentanoic acid
	386	141	190	4	61	11	
MAM-2201 4-OH-pentyl	390	169	160	11	35	20	D5-JWH-018 5-OH-pentyl
	390	141	160	11	65	22	
JWH-210 4-OH-pentyl	386	183	160	5	31	15	D7-JWH-018 6-OH-indole
	386	153	160	5	50	13	
JWH-210 5-OH-indole	386	183	180	4	32	16	D5-JWH-250
	386	230	180	4	32	18	
JWH-210 5-OH-pentyl	386	183	160	5	31	15	D7-JWH-018 6-OH-indole
	386	153	160	5	50	13	
JWH-210 <i>N</i> -pentanoic acid	400	183	160	11	35	14	D7-JWH-073 6-OH-indole
	400	155	160	11	55	20	
PB-22 3-carboxyindole	232	132	130	3	29	16	D7-JWH-073 6-OH-indole
	232	118	130	3	30	14	
PB-22 4-OH-pentyl	375	144	110	5	35	23	D5-JWH-073 <i>N</i> -butanoic acid
	375	69	110	5	55	15	
PB-22 5-OH-pentyl	375	230	110	6	45	15	D5-JWH-073 <i>N</i> -butanoic acid
	375	144	110	5	35	23	
PB-22 <i>N</i> -pentanoic acid	389	244	140	4	30	16	D5-JWH-073 <i>N</i> -butanoic acid
	389	144	140	4	48	16	
5F-PB-22 3-carboxyindole	250	206	110	3	22	16	D5-JWH-250 4-OH-pentyl 1
	250	118	110	3	26	13	
D5-JWH-018 4-OH-pentyl	363	155	180	4	25	11	---
D5-JWH-018 5-OH-pentyl	363	155	165	10	33	12	---
D7-JWH-018 6-OH-indole	367	155	180	4	35	12	---
D4-JWH-018 <i>N</i> -pentanoic acid	376	155	170	10	35	7	---
D7-JWH-073 6-OH-indole	351	155	170	6	35	12	---
D5-JWH-073 <i>N</i> -butanoic acid	363	155	190	5	35	11	---
D5-JWH-122 5-OH-pentyl	377	169	185	5	29	14	---
D5-JWH-250	341	121	175	10	30	8	---
D5-JWH-250 4-OH-pentyl	357	121	150	8	31	15	---

Q1 *m/z* of the precursor ion
Q2 *m/z* of the fragment ion
DP declustering potential
EP entrance potential
CE collision energy
CXP collision cell exit potential

Supplementary Data S-4

Graphs show the concentration-dependent interaction of CB1 (A) and CB2 (B) with β arr2 upon stimulation with different SCs. AUC, area under the curve. Data are given as mean AUC \pm SEM (n=5-6).

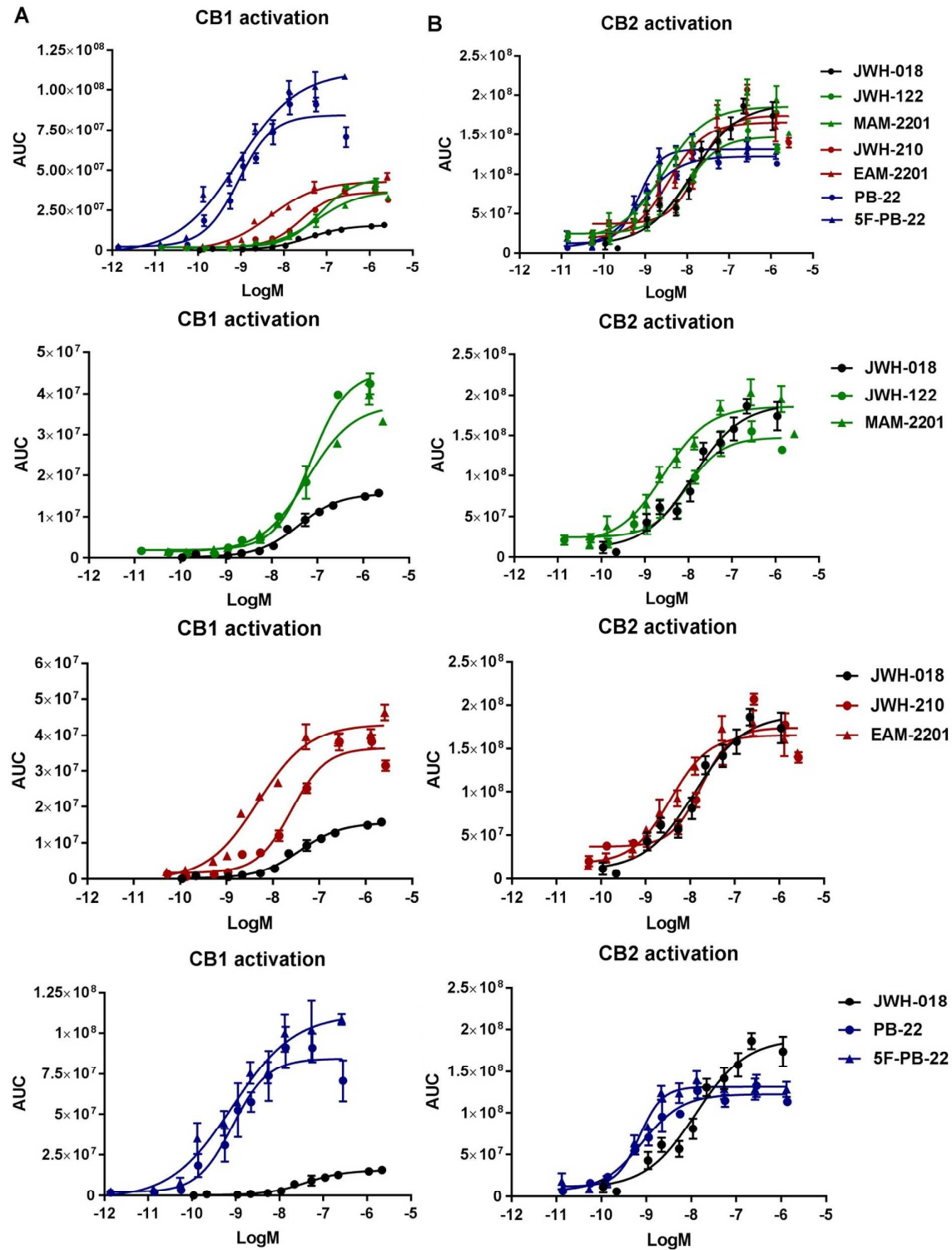


Figure S-4. The concentration-dependent interaction of CB1 (A) and CB2 (B) with β arr2 upon stimulation with different SCs. AUC, area under the curve. Data are given as mean AUC \pm SEM (n=5-6).

Supplementary Data S-5

Table S-5 shows the potency (EC₅₀) of Δ⁹THC and JWH-018, determined by different functional assays.

Drug	Functional assay	CB1 EC ₅₀ (nM)	CB2 EC ₅₀ (nM)	Reference
Δ ⁹ THC	GTPγS binding	81 ± 34	-	Breivogel et al. (2001) ¹
		167 ± 84.7	-	Brents et al. (2011) ²
		77.0 ± 29.9	-	Brents et al. (2012) ³
	FLIPR membrane potential assay	250	1157	Banister et al. (2015a) ⁴
		172	-	Banister et al. (2015b) ⁵
	Adenylate cyclase inhibition	-	57.9 ± 19.8	Rajaskaran et al. (2013) ⁶
JWH-018	GTPγS binding	36	-	Nakajima et al. (2011) ⁷
		6.8 ± 2.5	-	Brents et al. (2011) ²
		20.2 ± 1.3	-	De Luca et al. (2013) ⁸
	FLIPR membrane potential assay	102	133	Banister et al. (2015a) ⁴
		18	22	Banister et al. (2015b) ⁵
	Adenylate cyclase inhibition	-	3.6 ± 2.2	Rajaskaran et al. (2013) ⁶
	Internalization assay	2.8	-	Atwood et al. (2010) ⁹
		10.1	-	Atwood et al. (2011) ¹⁰

Table S-5. Potency of Δ⁹THC and JWH-018.

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Supplementary Data S-6

Drug/metabolite	Relative potential of CB1 activation at 1 μ M	Relative potential of CB2 activation at 1 μ M
JWH-018	100.0 \pm 10.6 (20)	100 \pm 17.6 (20)
4-OH-pentyl JWH-018	90.3 \pm 10.9 (4)	102.3 \pm 9.5 (4)
5-OH-pentyl-JWH-018	20.7 \pm 5.3 (4)	85.1 \pm 13.2 (4)
5-OH-indole-JWH-018	73.1 \pm 18.3 (3)	70.8 \pm 24.0 (4)
6-OH-indole-JWH-018	24.1 \pm 4.1 (4)	75.7 \pm 4.3 (4)
N-pentanoic acid JWH-018	0.53 \pm 1.77 (4)	15.9 \pm 12.6 (4)
JWH-122	173.4 \pm 17.6 (4)	94.0 \pm 7.5 (4)
4-OH-pentyl-JWH-122	165.2 \pm 13.0 (3)	99.4 \pm 4.1 (4)
MAM-2201	174.7 \pm 31.2 (3)	97.4 \pm 23.0 (3)
5-OH-pentyl-JWH-122	99.5 \pm 5.9 (4)	96.3 \pm 10.9 (4)
4-OH-pentyl-MAM-2201	173.0 \pm 20.2 (4)	87.6 \pm 13.5 (4)
N-pentanoic acid JWH-122	1.4 \pm 3.7 (4)	3.6 \pm 6.2 (3)
JWH-210	197.9 \pm 13.3 (4)	84.5 \pm 12.0 (4)
4-OH-pentyl-JWH-210	179.3 \pm 19.0 (3)	115.4 \pm 22.2 (4)
5-OH-pentyl-JWH-210	157.8 \pm 12.3 (4)	101.4 \pm 12.7 (4)
5-OH-indole-JWH-210	100.3 \pm 13.4 (3)	90.9 \pm 18.9 (4)
EAM-2201	239.7 \pm 10.5 (4)	92.4 \pm 25.3 (4)
N-pentanoic acid JWH-210	1.9 \pm 1.6 (3)	54.8 \pm 7.8 (4)
PB-22	287.0 \pm 29.6 (4)	137.6 \pm 13.8 (3)
3-carboxyindole PB-22	1.3 \pm 3.6 (4)	1.6 \pm 4.3 (3)
4-OH-pentyl PB-22	208.0 \pm 21.2 (4)	144.6 \pm 33.0 (4)
5F-PB-22	278.8 \pm 9.2 (3)	131.9 \pm 9.3 (4)
3-carboxyindole 5F-PB-22	3.1 \pm 5.0 (4)	4.6 \pm 13.1 (4)
5-OH-pentyl PB-22	171.0 \pm 6.9 (3)	142.3 \pm 13.3 (4)
N-pentanoic acid PB-22	25.8 \pm 3.4 (4)	43.5 \pm 5.3 (4)

Table S-6. Comparison of relative potential to activate CB1 and CB2 at 1 μ M. The data are presented as the % CB activation (relative to the receptor activation of JWH-018) \pm SEM (number of replicates).

Supplementary Data S-7

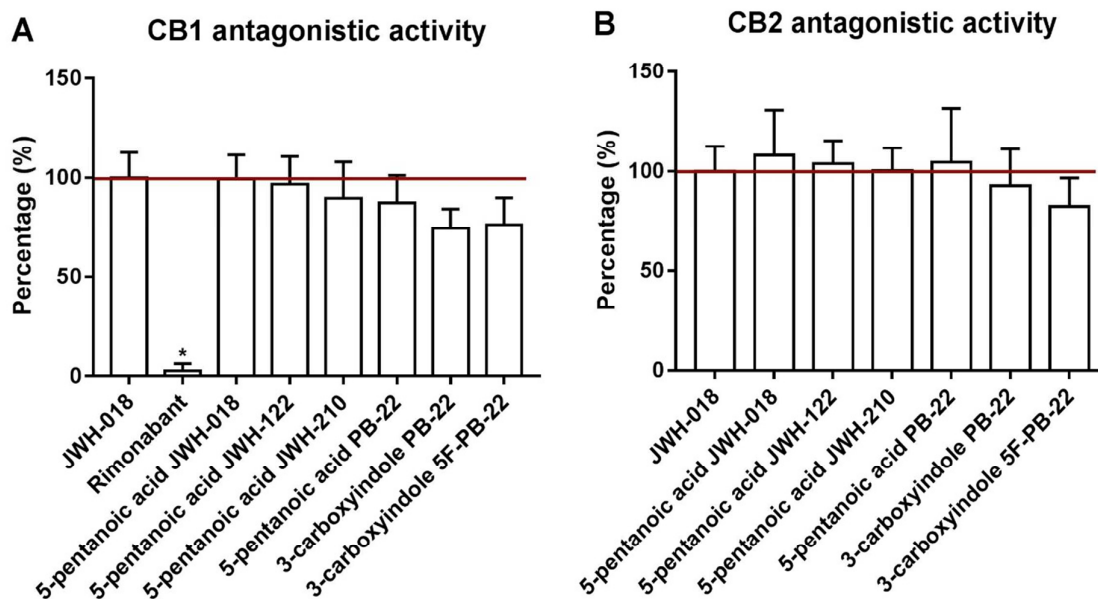


Figure S-7. Evaluation of antagonistic properties of several SC metabolites. The cells were stimulated with 1 μ M of the test compound and were incubated for 5 min before adding a fixed concentration of JWH-018 (ED80 concentration). The bar 'JWH-018' marks the response when no antagonist is present. The bar 'Rimonabant' was taken as a positive control for CB1 antagonism. Bars assigned with an (*) are not significantly different from basal levels. All test compounds did not show antagonistic activity.