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Synthetic Cannabinoid Hydroxypentyl Metabolites Retain Efficacy at Human Cannabinoid Receptors

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ABSTRACT

Synthetic cannabinoids (SCs) are novel psychoactive substances that are easily acquired, widely abused as a substitute for cannabis, and associated with cardiotoxicity and seizures. Although the structural bases of these compounds are scaffolds with known affinity and efficacy at the human cannabinoid type-1 receptor (hCB_1), upon ingestion or inhalation they can be metabolized to multiple chemical entities of unknown pharmacological activity. A large proportion of these metabolites are hydroxylated on the pentyl chain, a key substituent that determines receptor affinity and selectivity. Thus, the pharmacology of SC metabolites may be an important component in understanding the in vivo effects of SCs. We examined nine SCs (AB-PINACA, 5F-AB-PINACA, ADB/MDMB-PINACA, 5F-ADB, 5F-CUMYL-PINACA, AMB-PINACA, 5F-AMB, APINACA, and 5F-APINACA) and their hydroxypentyl (either 4-OH or 5-OH) metabolites in [^{3}H]CP55,940 receptor binding and the [^{35}S]GTPγS functional assay to determine the extent to which these metabolites retain activity at cannabinoid receptors. All of the SCs tested exhibited high affinity ($<$10 nM) and efficacy for hCB_1 and hCB_2. The majority of the hydroxypentyl metabolites retained full efficacy at hCB_1 and hCB_2, albeit with reduced affinity and potency, and exhibited greater binding selectivity for hCB_2. These data suggest that phase I metabolites may be contributing to the in vivo pharmacology and toxicology of abused SCs. Considering this and previous reports demonstrating that metabolites retain efficacy at the hCB_1 receptor, the full pharmacokinetic profiles of the parent compounds and their metabolites need to be considered in terms of the pharmacological effects and time course associated with these drugs.

Introduction

Despite the efforts of governments and law enforcement agencies to curb the sale and use of novel psychoactive substances (NPS), the method of reactionary drug scheduling has been met with an unrelenting effort by clandestine chemists to modify chemical structures in order to circumvent the law (Trecki et al., 2015). Among the NPS, synthetic cannabinoids have emerged as a robust market probably as a result of: 1) the widespread use of cannabis, 2) lack of knowledge or consideration regarding the safety of synthetic cannabinoid products, and 3) its potential to serve as a cannabis replacement to avoid detection in drug testing (Every-Palmer, 2011; Berry-Cabán et al., 2012; Gunderson et al., 2012; Vandrey et al., 2012). Historically, synthetic cannabinoids were developed for pharmacological interrogation of biologic systems (Wiley et al., 2011), including the study of their cognate $G_{i/o}$ protein-coupled receptors and cannabinoid type 1 (CB_1) and type-2 receptors (CB_2) [for reviews see Svízenská et al. (2008), Kendall and Yudowski (2017), Thomas, 2017)]. Therefore, little is known regarding their toxicological effects and how these relate to either the parent compound, its thermal degradants (Thomas et al., 2017), or its metabolites. Further, a focus on the metabolic fate of these compounds (Fantegrossi et al., 2014) has only recently become the subject of scientific inquiry as reports of human use and adverse health events become more prevalent, and analytical methods for the detection of synthetic cannabinoid use are developed.

Although the majority of abused synthetic cannabinoids are high affinity and high efficacy cannabinoid receptor agonists, only a few studies have examined the pharmacology of their metabolites (Brents et al., 2011, 2012; Chimalakonda et al., 2012; Rajasekaran et al., 2013; Cannaert et al., 2016, 2017;

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ABBREVIATIONS: AM2201, 1-[(5-fluoropentyl)-1*H*-indol-3-yl]-(naphthalen-1-yl)methanone; GDP, guanosine diphosphate [(2*R*,3*S*,4*R*,5*R*)-5-(2-amino-6-oxo-3*H*-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl phosphono hydrogen phosphate; GTPγS, [(2*S*,3*R*,4*S*,5*S*)-5-(2-amino-6-oxo-3*H*-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl dihydroxyphosphinothioyl hydrogen phosphate; hCB_1, human cannabinoid type-1 receptor; hCB_2, human cannabinoid type-2 receptor; [^{3}H]SR141716 (5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; HEK293, human embryonic kidney-293 cells; JWH-018, naphthalen-1-yl-(1-pentylindol-3-yl)methanone; JWH-073, naphthalen-1-yl-(1-butylindol-3-yl)methanone; MN-18, *N*-(naphthalen-1-yl)-1-pentyl-1*H*-indazole-3-carboxamide; Δ^9-THC, [(−)-(6a*R*,10a*R*)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6*H*-benzo(c)chromen-1-ol].

Longworth et al., 2017). Data suggest that seizure activity of the abused synthetic cannabinoids JWH-018 (Malyshevskaya et al., 2017), and AM2201 (Funada and Takebayashi-Ohsawa, 2018) is CB_1-dependent; thus, metabolites with activity at these receptors may contribute to the observed pharmacology and toxicity associated with synthetic cannabinoids.

Synthetic cannabinoids are metabolized via cytochrome P450 enzymes, resulting in phase I hydroxylated metabolites (Tai and Fantegrossi, 2017). An alkyl side chain, when present, appears as if it would undergo hydroxylation at several positions. Compounds fluorinated at the 5-position are also susceptible to oxidative defluorination and hydroxylation (Wohlfarth et al., 2015; Kusano et al., 2018). Metabolism of CUMYL-PICA as assessed by rat and human hepatocyte incubations revealed 18 metabolites, with hydroxylation at the terminal position of the pentyl chain being the greatest in abundance (Kevin et al., 2017). Analysis of 5F-MN-18 metabolism by human hepatocytes also revealed terminal hydroxylation of the pentyl chain as the most abundant metabolite (Carlier et al., 2018). Metabolism of AB-PINACA by human liver microsomes suggested hydroxylation occurred primarily on the pentyl chain (Takayama et al., 2014). Importantly, hydroxypentyl metabolites detected from metabolism experiments with pooled human liver microsomes were also detected in urine samples from two individuals who had been suspected of consuming AB-PINACA (Wohlfarth et al., 2015), demonstrating that these metabolic products occur in humans. 5F-ADB/5F-MDMB-PINACA, which was implicated in four deaths in Japan of people who had been in possession of a product called "Heart Shock BLACK" (Usui et al., 2018) and others (Hasegawa et al., 2015; Kusano et al., 2018), can also be metabolized to form hydroxylated metabolites, including at the 5 position of the pentyl chain (Barcelo et al., 2017). Metabolism of other synthetic cannabinoids, including AMB, 5F-AMB (Andersson et al., 2016), and EG-018 (Mogler et al., 2018), have been reported to lead to hydroxypentyl metabolites.

The *n*-pentyl side chain is a common feature of phyto- and endocannabinoids and is a key determinant of affinity, potency, and selectivity at cannabinoid receptors [for review, see Thakur et al. (2005)]. Considering the importance of the alkyl substituent and its likelihood to undergo metabolic hydroxylation, pharmacological impact of this biotransformation on abused synthetic cannabinoids is an important consideration regarding the in vivo effects of these compounds. Therefore, synthetic cannabinoids AB-PINACA, 5F-AB-PINACA, ADB/MDMB-PINACA, 5F-ADB/5F-MDMB-PINACA, 5F-AMB/5F-AMB-PINACA, AMB/AMB-PINACA, APINACA/AKB-48, 5F-APINACA/5F-AKB-48, 5F-CUMYL-PINACA and their hydroxylated metabolites (at the 4- or 5- position of the pentyl chain) were synthesized (McKinnie et al., 2018; unpublished) and tested in studies of receptor affinity and function using human CB_1 (hCB_1) and human CB_2 (hCB_2) expressing human embryonic kidney (HEK293) cells. Pharmacological properties were then compared between the metabolite and parent to determine changes regarding the ligand's affinity, potency and efficacy. These systematic studies were conducted to determine what impact pentyl hydroxylation would have across a range of abused synthetic cannabinoid structures (Fig. 1).

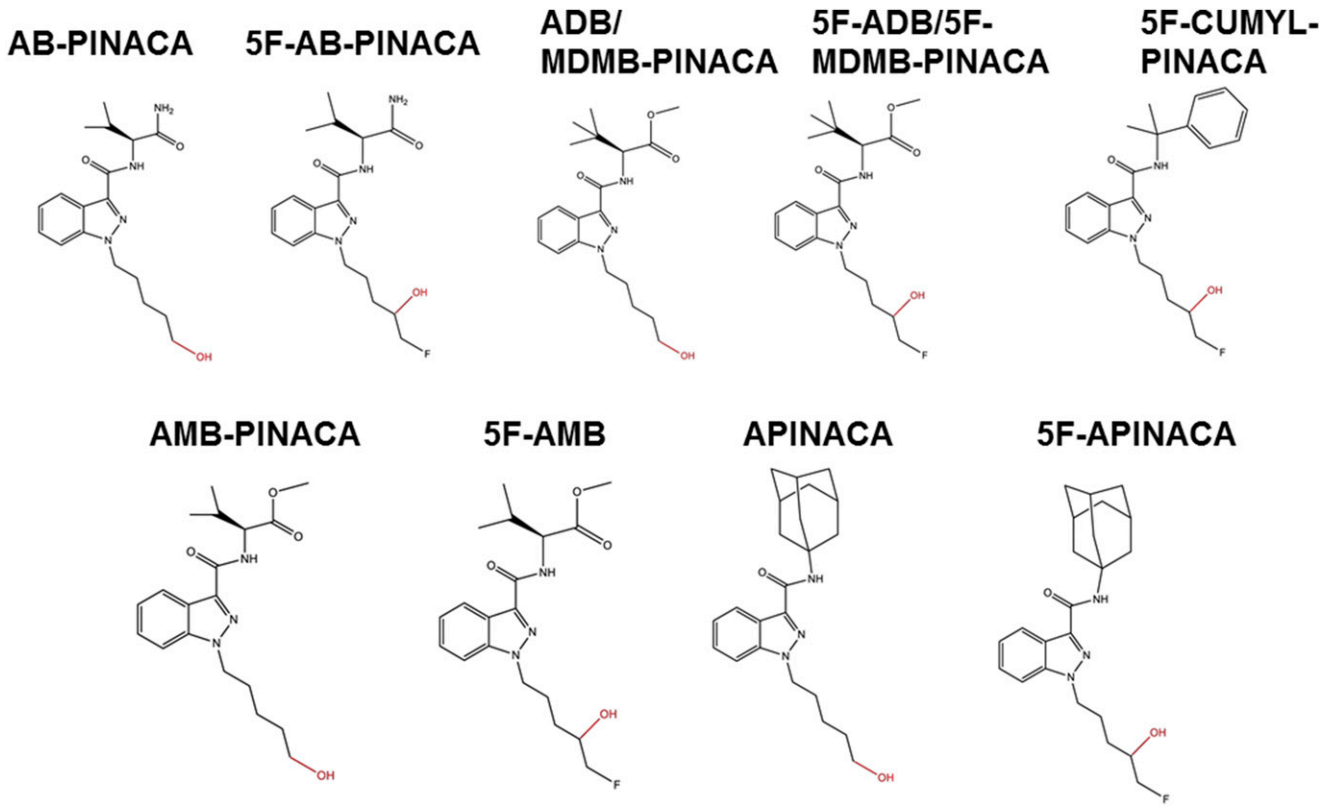

Fig. 1. Structures of synthetic cannabinoids and location of metabolite hydroxy group depicted in red.

Materials and Methods

Chemicals. For these studies, Δ^9-THC [(−)-(6a*R*,10a*R*)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo(c)chromen-1-ol], CP55,940 (5-(1,1-dimethylheptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol), [^{3}H]SR141716 (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; 24 Ci/mmol), [^{3}H]CP55,940 (81.1 Ci/mmol) and unlabeled SR141716 were obtained from the National Institute on Drug Abuse (NIDA; North Bethesda, MD) and dissolved in absolute ethanol. All synthetic cannabinoids were synthesized in the laboratory of Dr. M. L. Trudell and were dissolved in 100% dimethyl sulfoxide. All drugs were stored at −80°C as 10 mM stocks. Guanosine diphosphate (GDP; [(2R,3S,4R,5R)-5-(2-amino-6-oxo-3*H*-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl phosphono hydrogen phosphate; MilliporeSigma, St. Louis, MO), unlabeled guanosine 5′-O-[gamma-thio]triphosphate (GTPγS; [(2S,3R,4S,5S)-5-(2-amino-6-oxo-3H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl dihydroxyphosphinothioyl hydrogen phosphate; MilliporeSigma), and [^{35}S]GTPγS (1250 Ci/mmol; PerkinElmer, Waltham, MA) were dissolved in distilled water, aliquoted and stored at −80°C.

Receptor Binding and Agonist-Stimulated [^{35}S]GTPγS Binding. HEK293 cells stably expressing either the human CB_1 or CB_2 receptor (PerkinElmer) were grown in Dulbecco's modified Eagle's medium/F12 (10-092-CV; Corning Cellgro, Manassas, VA) with 10% fetal bovine serum (FBS-BBT; Rocky Mountain Biological Laboratory, Crested Butte, CO), 50 IU/ml penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA) in multilayer flasks to 90% confluence. Cells were detached using 1 mM EDTA in phosphate buffered saline (PBS; MilliporeSigma), pelleted in PBS at 200*g* for 6 minutes, then suspended in fractionation buffer (50 mM Tris base, 320 mM sucrose, 1 mM EGTA, pH 7.4), and homogenized by dounce. Cell homogenates were centrifuged at 1600*g* for 10 minutes at 4°C, the supernatant was collected, and the pellet was homogenized again and centrifuged at 1600*g* for 10 minutes at 4°C. The supernatants were pooled and spun at 40,000*g* for 1 hour at 4°C resulting in a P2 pellet. The P2 pellet was resuspended in membrane buffer (50 mM Tris base, 1 mM EGTA, 3 mM $MgCl_2$, pH 7.4), the protein amount was quantified by the Bradford method, and the membrane preparations were diluted to 1 mg/ml, snap-frozen in liquid nitrogen, and stored at −80°C until the day of the experiment. For receptor binding, reactions were carried out in assay buffer [50 mM Tris base, 125 mM NaCl, 3 mM $MgCl_2$, and 6.25 mg/ml of bovine serum albumin (BSA)] into which membranes (10 μg protein) were added in a volume of 100 μl, bringing the final reaction volume to 500 μl. This resulted in a final assay buffer containing 50 mM Tris base, 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA and 5 mg/ml BSA. Reactions were carried out for 90 minutes at 30°C with 1 nM [^{3}H]CP55,940 (hCB_1 K_d = 1.2 nM; hCB_2 K_d = 1.2 nM) and varying concentrations of synthetic cannabinoids. [^{3}H]CP55,940 saturation binding was conducted prior to competition binding experiments to determine K_d values for CP55,940 at hCB_1 and hCB_2 receptors using nominal concentrations of 0.01, 0.032, 0.1, 0.32, 0.6, 1, 3.2, and 6 nM. Amount of radioligand added for each experiment was determined by pipetting 50 μl of each nominal concentration stock, adding 20 ml of Ultima Gold scintillation cocktail, and analyzing on a Packard TriCarb 2300TR scintillation counter. Nonspecific binding was determined by addition of excess cold ligand (1 μM). Total bound [^{3}H]CP55,940 was less than 10% of total added (minimal ligand depletion). For receptor signaling, membranes (10 μg protein) were incubated for 60 minutes at 30°C with 30 μM GDP and 0.10–0.12 nM [^{35}S]GTPγS, and nonspecific binding was determined by adding 30 μM unlabeled GTPγS. Binding was terminated by vacuum filtration through a PerkinElmer GF/C filter plate using a PerkinElmer FilterMate.

Data Analysis. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). [^{35}S]GTPγS data were normalized to maximal stimulation by CP55,940 and were fit to three parameter nonlinear regression. pEC_{50} and E_{max} values were considered significantly different when 95% confidence intervals (CI) did not overlap. For saturation binding, data were fit to "One site – Specific Binding" using GraphPad Prism to determine radioligand K_d. For competition radioligand binding data, K_i values to displace 1 nM [^{3}H]SR141716 for hCB_1 or 1 nM [^{3}H]CP55,940 were determined using "One site – fit K_i" in Prism 6.0. Each data point represents the mean and S.E. of at least $N = 3$ experiments performed in duplicate.

Results

Receptor Binding. All compounds tested exhibited affinity for both hCB_1 and hCB_2 receptors as determined by displacement binding of the high-affinity cannabinoid agonist [^{3}H]CP55,940 (Fig. 2; Table 1). The control compound, unlabeled CP55,940, exhibited a K_i value of 1.25 nM at hCB_1 and 1.15 nM at hCB_2, consistent with the K_d values of 1.26 ± 0.399 nM at hCB_1 and 1.24 ± 0.377 nM at hCB_2 determined from separate [^{3}H]CP55,940 saturation binding experiments (data not shown). Parent compounds all exhibited high affinity at hCB_1 receptors in the nanomolar range, with a few compounds (i.e., ADB, 5F-ADB, and 5F-CUMYL-PINACA) exhibiting subnanomolar affinities (Fig. 2, A–C). Rank order affinities (high to low) for the parent compounds at hCB_1 were: 5F-CUMYL-PINACA = 5F-ADB = ADB > 5F-APINACA > AMB-PINACA = 5F-AMB-PINACA = 5F-AB-PINACA = AB-PINACA = APINACA. Parent compounds all exhibited high affinity at hCB_2 receptors in the nanomolar range, with a few compounds (i.e., ADB, 5F-ADB, and 5F-APINACA) exhibiting subnanomolar affinities (Fig. 2, D–F). Rank order affinities for the parent compounds at hCB_2 were: 5F-APINACA = ADB = 5F-ADB > 5F-CUMYL-PINACA = APINACA = AB-PINACA = AMB-PINACA = 5F-AMB-PINACA = 5F-AB-PINACA.

Synthetic cannabinoid hydroxypentyl metabolites all displaced [^{3}H]CP55,940 binding but exhibited marked reductions in affinity as determined by calculated K_i values from displacement curves. Changes in affinity for the majority of metabolites tested varied from 10- to 80-fold lower than the parent compounds for both receptors with the exception of AB-PINACA, which exhibited the largest reduction in affinity for hCB_1 and hCB_2, approximately 260- and 110-fold respectively. There was a positive correlation for K_i selectivity ratios between the parent and metabolite ($r(9) = 0.982$, $P < 0.0001$), suggesting that hydroxylation had little or no effect on receptor binding selectivity. Selectivity for hCB_1 versus hCB_2 was modest for all parent compounds tested, with 5F-CUMYL-PINACA and 5F-APINACA exhibiting the largest fold-difference in selectivity, a 5-fold greater affinity for hCB_1 and hCB_2, respectively. Pentyl-hydroxylation appeared to affect affinity at hCB_1 receptors to a greater extent than at hCB_2 receptors, as fold-changes in affinity for metabolite/parent were mostly greater for hCB_1 than hCB_2. In other words, in addition to reducing affinity, hydroxylation produced a modest increase in selectivity for hCB_2 over hCB_1 for all compounds except for 5F-APINACA, which retained a 5-fold greater affinity for hCB_2 following hydroxylation. AMB-PINACA, 5F-ADB, and ADB exhibited the greatest disparity in effects on hCB_1 versus hCB_2 affinity with approximate 6-, 4-, and 3.5-fold differences in relative shifts in hCB_1 affinity compared with hCB_2, respectively (determined by dividing the hCB_1 K_i metabolite/parent ratio with that of the hCB_2 ratio).

Agonist-Stimulated [^{35}S]GTPγS Binding in hCB_1- and hCB_2-Expressing HEK293 Cell Membranes. Synthetic cannabinoid parent and metabolites all exhibited

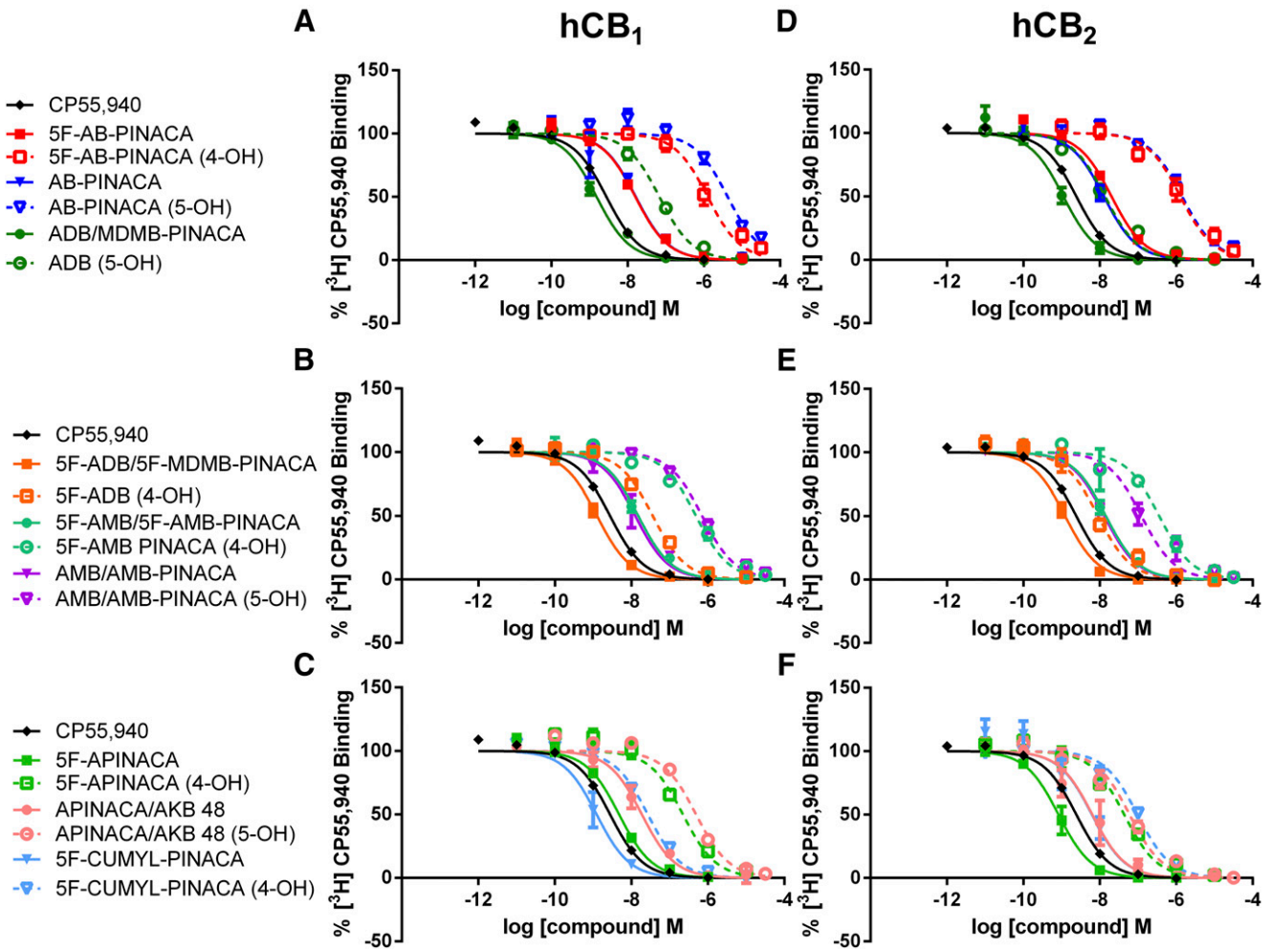

Fig. 2. Displacement of [^{3}H]CP55,940 binding in hCB_1 (A–C) and hCB_2 (D–F) expressing HEK293 cell membranes. Membranes were incubated with 1 nM [^{3}H]CP55,940 for 1 hour at 30°C. Parent compound displacement curves are depicted as solid lines and their hydroxylated metabolites are depicted as dashed lines of the same color. Displacement curves were calculated with top constrained to 100 and bottom constrained to 0. Each data point represents the mean and S.E. of at least $N = 3$ experiments performed in duplicate.

similar efficacy as CP55,940 (E_{max} = 94.5 ± 3.23) at hCB_1 receptors except for AB-PINACA, which exhibited greater efficacy (E_{max} = 122 ± 7) than CP55,940 as determined by nonoverlapping 95% confidence intervals (Fig. 3, A–C; Table 2). Likewise, all compounds exhibited similar efficacy compared with CP55,940 (E_{max} = 92.4 ± 3.27) at hCB_2 receptors except for AMB-PINACA (E_{max} = 131 ± 11.4), which exhibited greater efficacy than CP55,940 (Fig. 3, D–F; Table 3). Over half of the parent compounds tested exhibited subnanomolar potency at hCB_1 receptors including ADB, 5F-ADB, 5F-APINACA, 5F-CUMYL-PINACA, and AMB-PINACA. The remaining parent compounds, 5F-AMB, 5F-AB-PINACA, APINACA, and AB-PINACA exhibited potencies in the nanomolar range.

Consistent with the receptor binding data in which metabolites exhibited reduced affinity for both receptors, metabolites also exhibited reduced potency to stimulate [^{35}S]GTPγS binding in both hCB_1 and hCB_2 membranes (Fig. 3; Tables 2 and 3). Most synthetic cannabinoid parent compounds exhibited marginal selectivity for either receptor, with an approximate 2-fold difference in EC_{50} values on average. 5F-AB-PINACA, 5F-ADB, and AMB-PINACA exhibited roughly 2-fold greater potency at hCB_1 versus hCB_2, whereas AB-PINACA and 5F-APINACA exhibited approximately 2- to 3-fold greater potency at hCB_2 versus hCB_1. APINACA/AKB48 and 5F-CUMYL-PINACA had slightly greater selectivity with 10-fold greater potency at hCB_2 versus hCB_1. Notably, 5F-CUMYL-PINACA was very potent at stimulating hCB_2 receptors, with an EC_{50} value of 63 pM.

Although hydroxylation of the pentyl chain appeared to produce a greater reduction of potency at hCB_2 receptors (123 ± 138.1) versus hCB_1 receptors (54.4 ± 47.5), as determined by averaging the fold-changes in potency at hCB_1 and hCB_2 (Tables 2 and 3), this was not significant ($t = 1.42$, $P = 0.18$). Changes in potency following hydroxylation did vary, as shifts toward hCB_1 or hCB_2 selectivity were split almost equally (Table 3), with ratios of fold change for hCB_2 over hCB_1 being less than 1 (i.e., greater reduction in potency for hCB_1) for ADB (0.7), 5F-ADB (0.1), and AMB-PINACA (0.2) and greater than 1 (i.e., greater reduction in potency for hCB_2) for AB-PINACA (3.5), 5F-AMB-PINACA (6.4), 5F-APINACA (3.3), APINACA/AKB48 (2.3), and 5F-CUMYL-PINACA (15.1). 5F-AB-PINACA exhibited no change in selectivity (1.0).

In contrast to the binding data in which hydroxylation predominantly increased selectivity for hCB_2 over hCB_1, there was no correlation between the hCB_2/hCB_1 EC_{50} selectivity ratio for parent and metabolite ($r = -0.135$, $P = 0.73$),

TABLE 1
[^{3}H]CP55,940 competition binding affinities of synthetic cannabinoids and metabolites to hCB_1 and hCB_2 receptors

Compound	hCB_1 Binding			hCB_2 Binding			hCB_2/ hCB1 K_i	hCB_1 K_i Metabolite/ Parent	hCB_2 K_i Metabolite/ Parent
	pKi ± S.E.	pKi 95% CI	K_i	pK_i ± S.E.	pK_i 95% CI	K_i			
			nM			*nM*			
CP 55,940	8.90 ± 0.0435	8.82–8.99	1.25	8.94 ± 0.0387	8.86–9.02	1.15	0.9		
5F-AB-PINACA	8.06 ± 0.0449	7.97–8.16	8.72	7.96 ± 0.0554	7.84–8.07	11.1	1.3	78.8	63.3
5F-AB-PINACA (4-OH)	6.16 ± 0.079	6–6.33	687	6.15 ± 0.092	5.96–6.35	703	1.0		
AB-PINACA	8.05 ± 0.122	7.79–8.31	8.89	8.15 ± 0.0737	8–8.31	7.02	0.8	258.7	111.1
AB-PINACA (5-OH)	5.64 ± 0.0853	5.46–5.82	2300	6.11 ± 0.0842	5.93–6.29	780	0.3		
ADB/MDMB-PINACA	9.08 ± 0.0586	8.95–9.2	0.836	9.22 ± 0.0919	9.02–9.41	0.61	0.7	44.4	12.3
ADB/MDMB-PINACA (5-OH)	7.43 ± 0.0591	7.31–7.55	37.1	8.13 ± 0.0611	8–8.25	7.49	0.2		
5F-ADB/5F-MDMB-PINACA	9.16 ± 0.0614	9.03–9.29	0.692	9.17 ± 0.0596	9.04–9.3	0.677	1.0	28.6	7.3
5F-ADB (4-OH)	7.7 ± 0.0396	7.62–7.79	19.8	8.31 ± 0.085	8.13–8.48	4.95	0.3		
5F-AMB/5F-AMB-PINACA	8.08 ± 0.0597	7.96–8.21	8.29	8.1 ± 0.0586	7.98–8.22	7.93	1.0	32.2	24.8
5F-AMB PINACA (4-OH)	6.57 ± 0.0589	6.45–6.7	267	6.71 ± 0.109	6.48–6.93	197	0.7		
AMB/AMB-PINACA	8.16 ± 0.0808	7.99–8.33	6.91	8.14 ± 0.0405	8.06–8.22	7.25	1.0	55.9	9.3
AMB/AMB-PINACA (5-OH)	6.41 ± 0.0547	6.3–6.53	386	7.17 ± 0.0887	6.99–7.36	67.6	0.2		
5F-APINACA	8.58 ± 0.0591	8.46–8.71	2.61	9.34 ± 0.0725	9.19–9.5	0.454	0.2	46.7	52.4
5F-APINACA (4-OH)	6.92 ± 0.0939	6.72–7.11	122	7.62 ± 0.08	7.46–7.79	23.8	0.2		
APINACA/AKB 48	7.99 ± 0.074	7.83–8.14	10.3	8.46 ± 0.121	8.21–8.71	3.48	0.3	26.0	9.5
APINACA/AKB 48 (5-OH)	6.57 ± 0.0666	6.43–6.71	268	7.48 ± 0.0838	7.31–7.66	32.9	0.1		
5F-CUMYL-PINACA	9.17 ± 0.0883	8.99–9.36	0.674	8.47 ± 0.142	8.17–8.77	3.41	5.1	21.8	15.5
5F-CUMYL-PINACA (4-OH)	7.83 ± 0.0439	7.74–7.92	14.7	7.28 ± 0.0497	7.17–7.38	52.9	3.6		

meaning the parent compound's selectivity did not predict that of the metabolite (Table 2). In addition, there was no correlation between binding and functional data when hCB_2/hCB_1 selectivity ratios were calculated for metabolite/parent ($r(9) = 0.412$, $P = 0.271$); i.e., the fold change in selectivity following hydroxylation did not correlate between K_i and EC_{50} values, suggesting that relative shifts in binding selectivity did not translate into shifts in relative potencies. Indeed, hCB_2/hCB_1 EC_{50} selectivity ratios appeared to flip for the hydroxylated metabolite for a number of compounds (Table 2), including 5F-ADB (parent ratio: 1.6, metabolite ratio: 0.2), 5F-AMB (parent ratio: 0.2, metabolite ratio: 1.3), AMB-PINACA (parent ratio: 2.1, metabolite ratio: 0.5), 5F-APINACA (parent ratio: 0.4, metabolite ratio: 1.4), and 5F-CUMYL-PINACA (parent ratio: 0.1, metabolite ratio: 2.2). In contrast, binding selectivity (hCB_2/hCB_1) ratios (Table 1) were: 5F-ADB (parent ratio: 1.0, metabolite ratio: 0.3), 5F-AMB (parent ratio: 1.0, metabolite ratio: 0.7), AMB-PINACA (parent ratio: 1.0, metabolite ratio: 0.2), 5F-APINACA (parent ratio: 0.2, metabolite ratio: 0.2), and 5F-CUMYL-PINACA (parent ratio: 5.1, metabolite ratio: 3.6).

Discussion

A large proportion of metabolic products for synthetic cannabinoids are hydroxylated on the alkyl chain when it is present (Takayama et al., 2014; Castaneto et al., 2015; Andersson et al., 2016; Berg et al., 2016; Barcelo et al., 2017; Richter et al., 2017; Carlier et al., 2018). To examine the potential for these products to contribute to the overall pharmacological response in humans, we examined nine abused synthetic cannabinoids and their hydroxypentyl metabolites. These compounds were assessed for their pharmacological properties at the human CB_1 and CB_2 receptors to determine their binding affinities and their potencies and efficacies to stimulate receptor activation as measured by [^{35}S]GTPγS binding.

The parent compounds all exhibited high affinity binding to both cannabinoid receptors, with K_i values in the nanomolar to subnanomolar range, which were lower than the previously determined K_i values for THC of 16 and 23 nM at hCB_1 and hCB_2, respectively (Gamage et al., 2018). This is consistent with previous reports for these and other synthetic cannabinoids, which typically exhibit very high affinity for both receptors [for review, see Banister and Connor (2018)]. Notably, all the hydroxypentyl metabolites exhibited reductions in binding affinity for both cannabinoid receptors. A trend for the metabolites to exhibit a greater reduction in affinity for hCB_1 versus hCB_2 was observed, as most hCB_2/hCB_1 K_i ratios went down, except for 5F-APINACA, which did not differ from its 5-OH metabolite. Most metabolites retained the same magnitude of efficacy as the parent compounds, except for AMB-PINACA, which had a small but significant reduction in calculated E_{max} for hCB_2 receptors. These data suggest that even though the pharmacokinetic profiles of synthetic cannabinoids may reflect reductions in levels of the parent compound, the potential contribution of metabolites to the observed behavioral and physiologic effects cannot be discounted.

JWH-018 (Brents et al., 2011; Chimalakonda et al., 2012) and JWH-073 (Brents et al., 2012) were reported to exhibit similar reductions in CB_1 binding affinity following hydroxylation at the 5- position of the pentyl chain. Hydroxylation at the 4- position of AM-2201 also reduced its affinity at CB_1 (Chimalakonda et al., 2012). The JWH-018 (5-OH) metabolite was reported to exhibit a 20- (Brents et al., 2011) to 27-fold (Chimalakonda et al., 2012) reduction in affinity for CB_1 compared with the parent (Brents et al., 2011), and the JWH-073 (5-OH) metabolite exhibited an approximately 17-fold reduction in affinity (Brents et al., 2012). These data are consistent with the present study, which observed 26- to 56-fold reductions in affinity at hCB_1 receptors for most of the 5-OH metabolites except for AB-PINACA (5-OH), which exhibited a marked 260-fold reduction in affinity for hCB_1.

Overall, shifts in affinity for hCB_2 receptors were less than those observed for hCB_1 receptors for all compounds except 5F-APINACA, which exhibited roughly equivalent shifts for both receptors. Likewise, reductions in affinity for CB_2

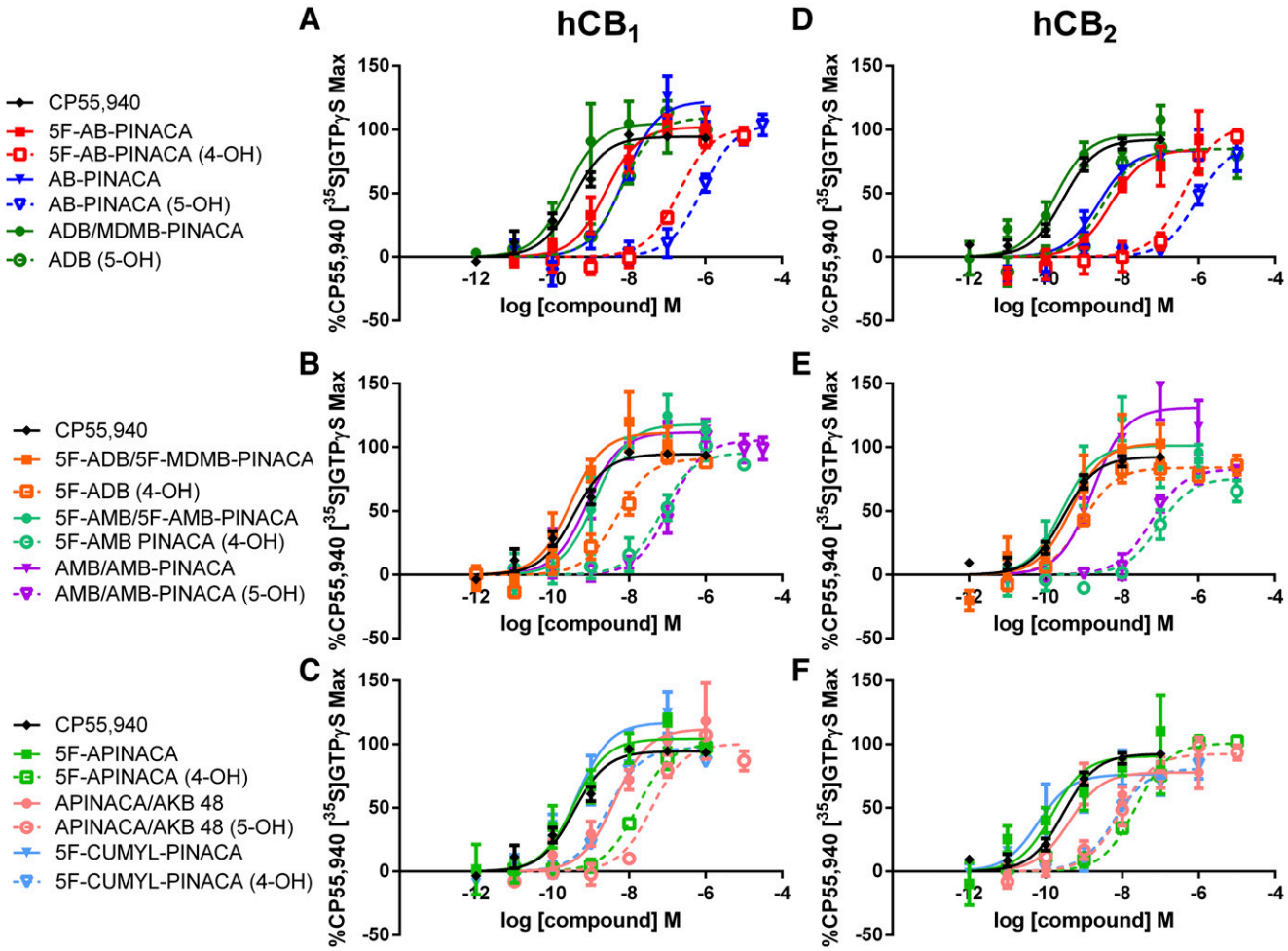

Fig. 3. Stimulation of [^{35}S]GTPγS binding in hCB_1 (A–C) and hCB_2 (D–F) expressing HEK293 cell membranes. Membranes were incubated with 30 μM GDP and 0.1–0.12 nM [^{35}S]GTPγS for 1 hour at 30°C. Parent compound displacement curves are depicted as solid lines and their hydroxylated metabolites are depicted as dashed lines of the same color. Stimulation curves were calculated with bottom constrained to 0. Each data point represents the mean and S.E. of at least $N = 3$ experiments performed in duplicate.

receptors for 4-OH and 5-OH pentyl chain metabolites of JWH-018 and JWH-073 were also reported (Rajasekaran et al., 2013). Specifically, the JWH-018 (5-OH) metabolite exhibited an 8-fold rightward shift and the JWH-018 (4-OH) metabolite exhibited a 15-fold rightward shift in affinity for CB_2 receptors. The JWH-073 (5-OH) metabolite exhibited a 10-fold shift, whereas only a 3-fold shift was observed for the JWH-073 (4-OH) metabolite, suggesting that the location of the hydroxy group on the pentyl chain does not confer changes in affinity for CB_2 equally across different structures.

Most of the parent compounds exhibited efficacy equal to that of CP55,940 with a few compounds exhibiting greater efficacy at hCB_1 (AB-PINACA) or hCB_2 (AMB-PINACA). This is in contrast to THC which was previously reported to exhibit partial agonism (less efficacy than CP55,940) at both hCB_1 and hCB_2 under the same assay conditions (Gamage et al., 2018). Except for AMB-PINACA (5-OH), all the hydroxylated metabolites retained the same level of efficacy as the parent compound in [^{35}S]GTPγS binding at hCB_1 and hCB_2. It had been previously reported that the JWH-073 (5-OH) hydroxypentyl metabolite had reduced efficacy compared with the parent compound, with an approximately 50% reduction in [^{35}S]GTPγS binding (Brents et al., 2012). However, the JWH-018 (5-OH) hydroxypentyl metabolite was reported to retain the same level of efficacy in [^{35}S]GTPγS binding (Brents et al., 2011). Further, 4-OH and 5-OH pentyl metabolites of APICA and ADB-PINACA retained efficacy at CB_1 and CB_2 receptors (Longworth et al., 2017). Therefore, hydroxylation does not seem to impact the efficacy of most synthetic cannabinoids. Considering the metabolites retained efficacy equal to that of CP55,940, and previously THC had been shown to exhibit less efficacy than CP55,940 and other synthetic cannabinoids in the same cannabinoid receptor HEK293 membranes (Gamage et al., 2018), these data suggest that synthetic cannabinoid metabolites could continue to exert effects greater than those of THC.

Although there was strong positive correlation between the parent and metabolite CB_2/CB_1 K_i selectivity ratios (i.e., selectivity for hCB_2 increased for all but one hydroxylated compound), it was not observed for CB_2/CB_1 EC_{50} selectivity ratios ($P = 0.73$), suggesting that the effects of hydroxylation on potency between hCB_1 and hCB_2 were less systematic. Additionally, when averaging the relative shifts in potency at hCB_1 and hCB_2, there was a trend for hydroxylation to produce greater reductions in potency at hCB_2 receptors in comparison with hCB_1 receptors. This was not statistically

TABLE 2
Potency and efficacy of synthetic cannabinoids and metabolites in agonist-stimulated [^{35}S]GTPγS binding in hCB_1 HEK293 membranes

Compound	pEC_{50} ± S.E.	pEC_{50} 95% CI	EC_{50}	E_{max} ± S.E.	E_{max} 95% CI	hCB_2/hCB_1 EC_{50}	Metabolite/Parent EC_{50}
			nM				
CP55,940	9.45 ± 0.0999	9.25–9.64	0.359	94.5 ± 3.23	88.1–101	0.8	
5F-AB-PINACA	8.61 ± 0.173	8.24–8.98	2.45	102 ± 6.55	88.3–116	2.1	82.9
5F-AB-PINACA (4-OH)	6.69 ± 0.135	6.41–6.97	203	102 ± 6.34	88.7–115	2.0	
AB-PINACA	8.12 ± 0.134	7.83–8.4	7.63	122 ± 7	108–137	0.3	107.1
AB-PINACA (5-OH)	6.09 ± 0.153	5.78–6.4	817	105 ± 7.34	89.7–120	1.1	
ADB/MDMB-PINACA	9.63 ± 0.27	9.06–10.2	0.235	105 ± 10.5	82.8–127	0.8	27.5
ADB/MDMB-PINACA (5-OH)	8.19 ± 0.13	7.92–8.46	6.46	110 ± 5.91	97.2–122	0.5	
5F-ADB/5F-MDMB-PINACA	9.53 ± 0.22	9.07–10	0.294	111 ± 9.14	92–131	1.6	16.2
5F-ADB (4-OH)	8.32 ± 0.157	8–8.64	4.77	90.9 ± 5.65	79.4–102	0.2	
5F-AMB/5F-AMB-PINACA	8.89 ± 0.201	8.46–9.31	1.3	118 ± 8.7	99.4–136	0.2	51.5
5F-AMB PINACA (4-OH)	7.18 ± 0.2	6.77–7.58	66.9	95.6 ± 8.02	79.3–112	1.3	
AMB/AMB-PINACA	9.1 ± 0.169	8.74–9.45	0.804	112 ± 6.73	97.4–126	2.1	143.0
AMB/AMB-PINACA (5-OH)	6.94 ± 0.146	6.64–7.24	115	106 ± 5.91	93.4–118	0.5	
5F-APINACA	9.44 ± 0.212	9–9.89	0.359	104 ± 7.1	89.4–119	0.4	45.7
5F-APINACA (4-OH)	7.79 ± 0.0929	7.59–7.98	16.4	101 ± 4.29	92–110	1.4	
APINACA/AKB 48	8.37 ± 0.235	7.88–8.87	4.24	112 ± 10.2	90.1–133	0.1	9.3
APINACA/AKB 48 (5-OH)	7.4 ± 0.123	7.15–7.66	39.4	100 ± 4.77	90.6–110	0.2	
5F-CUMYL-PINACA	9.37 ± 0.188	8.97–9.77	0.428	117 ± 8.54	98.8–135	0.1	6.6
5F-CUMYL-PINACA (4-OH)	8.55 ± 0.107	8.33–8.77	2.83	97.4 ± 4.09	88.9–106	2.2	

significant ($P = 0.18$) and was largely driven by two compounds, AB-PINACA and 5F-AMB. In contrast to the binding data, in which there was a modest increase in receptor selectivity for hCB_2, compounds were roughly evenly split when the effects of pentyl-hydroxylation on potency for hCB_1 were compared with hCB_2. Previously, the ADB-PINACA (5-OH) metabolite exhibited greater selectivity for CB_2 (11-fold) compared with the parent (0.5-fold) in functional studies (Longworth et al., 2017). In the present study, although most compounds exhibited greater reductions in potency at hCB_2 receptors in comparison with hCB_1 receptors, 5F-ADB exhibited a 1.6-fold selectivity for CB_1, whereas the metabolite (4-OH) was 5-fold more selective for hCB_2 in the functional assay. Thus, pentyl hydroxylation does not affect all structures in the same way.

In contrast to the pharmacological properties of synthetic cannabinoid metabolites, their toxicological properties remain less well characterized, though some work has been done. An hydroxypentyl metabolite of JWH-018 was reported to reduce cell viability—an effect that was not observed for the parent compound—via a noncannabinoid mechanism (Couceiro et al., 2016). Therefore, while assessment of synthetic cannabinoid metabolite pharmacology in the current study provides information regarding the potential for active metabolites to retain activity at cannabinoid receptors and contribute to the overall cannabinoid pharmacological profile in vivo, questions remain regarding how toxicity is mediated by noncannabinoid receptor mechanisms for these compounds and/or their metabolites. Numerous synthetic cannabinoids have now been implicated in deaths, including those characterized in the

TABLE 3
Potency and efficacy of synthetic cannabinoids and metabolites in agonist-stimulated [^{35}S]GTPγS binding in hCB_2 HEK293 membranes

Compound	pEC_{50} ± S.E.	pEC_{50} 95% CI	EC_{50} (nM)	E_{max} ± S.E.	E_{max} 95% CI	Metabolite/Parent EC_{50}	hCB_1 Selectivity Shift[a]
CP55,940	9.53 ± 0.0947	9.34–9.72	0.294	92.4 ± 3.27	85.8–98.9		
5F-AB-PINACA	8.28 ± 0.265	7.73–8.83	5.22	84.5 ± 9	65.8–103	79.3	1.0
5F-AB-PINACA (4-OH)	6.38 ± 0.224	5.92–6.85	414	104 ± 12.7	77.2–130		
AB-PINACA	8.62 ± 0.172	8.26–8.98	2.4	83.7 ± 5.34	72.7–94.8	372.5	3.5
AB-PINACA (5-OH)	6.05 ± 0.192	5.64–6.46	894	88.9 ± 10.2	67.3–111		
ADB/MDMB-PINACA	9.74 ± 0.2	9.32–10.2	0.183	96.4 ± 7.09	81.6–111	18.2	0.7
ADB/MDMB-PINACA (5-OH)	8.48 ± 0.227	8–8.95	3.33	84.9 ± 6.17	72–97.8		
5F-ADB/5F-MDMB-PINACA	9.33 ± 0.32	8.67–9.99	0.469	103 ± 13	76–130	2.0	0.1
5F-ADB (4-OH)	9.04 ± 0.108	8.81–9.26	0.92	83.7 ± 2.94	77.7–89.8		
5F-AMB/5F-AMB-PINACA	9.57 ± 0.315	8.91–10.2	0.272	101 ± 10.1	80.2–122	330.5	6.4
5F-AMB PINACA (4-OH)	7.05 ± 0.199	6.63–7.46	89.9	75.6 ± 6.58	62–89.3		
AMB/AMB-PINACA	8.77 ± 0.24	8.27–9.27	1.7	131 ± 11.4	107–155	33.3	0.2
AMB/AMB-PINACA (5-OH)	7.25 ± 0.155	6.93–7.57	56.6	82.7 ± 5.22	71.8–93.5		
5F-APINACA	9.83 ± 0.322	9.16–10.5	0.149	90.6 ± 10.8	68.3–113	152.3	3.3
5F-APINACA (4-OH)	7.64 ± 0.13	7.38–7.91	22.7	101 ± 5.09	90.3–111		
APINACA/AKB 48	9.38 ± 0.249	8.87–9.9	0.414	77.8 ± 6.32	64.7–91	21.3	2.3
APINACA/AKB 48 (5-OH)	8.06 ± 0.155	7.74–8.38	8.8	92.3 ± 5.36	81.2–103		
5F-CUMYL-PINACA	10.2 ± 0.294	9.59–10.8	0.063	76.3 ± 7.75	60.2–92.3	100.2	15.1
5F-CUMYL-PINACA (4-OH)	8.2 ± 0.175	7.84–8.56	6.31	81 ± 5.88	68.8–93.2		

[a](Metabolite/parent EC_{50} hCB_1 ratio)/(metabolite/parent EC_{50} hCB_2 ratio). Less than 1 = greater reduction in potency for hCB_1, greater than 1 = greater reduction in potency for $_{hCB2}$.

present study, e.g., 5F-AMB (Shanks and Behonick, 2016), 5F-ADB (Hasegawa et al., 2015; Angerer et al., 2017; Kusano et al., 2018), and 5F-APINACA (Hess et al., 2015). Despite detection and implication in these deaths and others, the contribution of the parent and/or metabolite is unknown as are the mechanisms.

Characterization of enzymes involved in synthetic cannabinoid metabolism and how that may relate to their toxicity is currently being investigated. Major cytochrome P450 isoforms involved in metabolism of JWH-018 and AM2201 include CYP2C9 and CYP1A2 (Chimalakonda et al., 2012). CYP3A4 was reported to be the major enzyme mediating oxidative metabolism of AKB-48 (Holm et al., 2015). Variation in metabolism of synthetic cannabinoids by polymorphisms in cytochrome P450 enzymes (e.g., CYP2C9) has been offered as a possible explanation for variance in toxicological effects of synthetic cannabinoids, specifically JWH-018 (Patton et al., 2018). In some cases, the metabolite exhibits toxicity not observed with the parent (Couceiro et al., 2016). Further, toxicity may not even involve cannabinoid receptor mechanisms, as metabolism of CUMYL-4CN-BINACA has been reported to liberate cyanide (Åstrand et al., 2018; Kevin et al., 2018). Thus synthetic cannabinoids could produce toxicity by a multitude of ways. Glucuronidation is the next step in biologic inactivation of synthetic cannabinoids leading to their excretion in urine (Möller et al., 2011). It was reported that glucuronidation of the 5-hydroxypentyl JWH-018 metabolite retains affinity, albeit much lower, for the CB_1 receptor, but acts as an antagonist rather than an agonist (Seely et al., 2012). It may be then that intermediate metabolic oxidative products contribute to pharmacological effects but following glucuronidation lose their agonist activity, though this has yet to be established for other synthetic cannabinoids.

In summary, pentyl hydroxylation reduces the affinity of the synthetic cannabinoids at both hCB_1 and hCB_2 receptors. The greater reduction in affinity at hCB_1 effectively increases the binding selectivity for hCB_2 receptors. Importantly, the synthetic cannabinoid hydroxypentyl metabolites retain the same level of efficacy, which is greater than THC's (Gamage et al., 2018). These metabolites probably contribute to the observed in vivo pharmacology of synthetic cannabinoids and the differences in subjective intensity compared with that of cannabis (Griffiths et al., 2010; Barratt et al., 2013). Further studies exploring the toxicological properties of synthetic cannabinoids and their metabolites are needed to better understand the mechanisms through which they are producing life-threatening effects.

Authorship Contributions

Participated in research design: Gamage, Farquhar, McKinnie, Trudell, Wiley, Thomas.

Conducted experiments: Gamage, Farquhar.

Contributed new reagents or analytic tools: Trudell, McKinnie.

Performed data analysis: Gamage, Farquhar.

Wrote or contributed writing to the manuscript: Gamage, Farquhar, McKinnie, Kevin, McGregor, Trudell, Wiley, Thomas.

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