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In vitro activity-profiling of Cumyl-PEGACLONE variants at the CB_1 receptor: fluorination *versus* isomer exploration

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ABSTRACT

Synthetic cannabinoid receptor agonists (SCRAs) are one of the largest groups of new psychoactive substances (NPS) monitored in Europe. SCRAs are known to typically exert higher cannabinoid activity than THC from cannabis, therefore entailing a greater health risk. Both Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE were not controlled by the national legislation upon their first detection in Germany in 2016 and 2017, respectively, and have been linked to several fatalities. In this study, the CB_1 receptor activity of these compounds, together with two newly synthesized structural isomers (Cumyl-PEGACLONE ethylbenzyl isomer and n-propylphenyl isomer) was assessed using two different *in vitro* receptor-proximal bio-assays, monitoring the recruitment of either β-arrestin2 or a modified G protein (mini-$G\alpha_i$) to the activated CB_1 receptor. Both in terms of potency and relative efficacy, Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE were found to exert strong CB_1 activation, with sub-nanomolar EC_{50} values, and efficacy values exceeding those of the reference agonist JWH-018 >3 fold (β-arrestin2 assay) or almost 2-fold (mini-$G\alpha_i$ assay). The ethylbenzyl and n-propylphenyl isomers showed a strongly reduced CB_1 activity (EC50 values >100 nM; efficacy <40% relative to JWH-018), which is hypothesized to originate from steric hindrance in the ligand binding pocket. Therefore, their abuse potential seems less likely. None of the evaluated compounds showed significant biased agonism. In conclusion, the functional assays applied here allowed

us to demonstrate that 5-fluorination of Cumyl-PEGACLONE is not linked to an intrinsically higher CB_1 activation potential, and that the ethylbenzyl and n-propylphenyl isomers yield a strongly reduced CB_1 activitation.

KEYWORDS

Cumyl-PEGACLONE, 5F-Cumyl-PEGACLONE, Isomers, Bio-assay, CB_1 Cannabinoid receptor, Synthetic cannabinoid receptor agonists, New psychoactive substances

INTRODUCTION

Synthetic cannabinoid receptor agonists (SCRAs) are a class of designer drugs that form one of the largest group of new psychoactive substances (NPS) monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [1]. Their synthesis for recreational purposes created legal alternatives to cannabis, mimicking the effect of the main psychotropic constituent Δ^9-tetrahydrocannabinol (THC). However, in comparison to the partial agonist THC, SCRAs are often much more potent at the CB_1 cannabinoid receptor, which is mainly responsible for the psychotropic effects. This more pronounced activity of SCRAs poses a higher risk for overdoses and serious adverse health effects, even though they may be marketed as 'safe and legal' alternatives to cannabis [2–4]. Considering the plethora of health consequences, several national and/or international legislations have tried to include these NPS to prohibit their use/possession/trafficking, via the implementation of for example generic legislations that encompass all 'variants' of a certain core structure. However, although these legislations are able to ban clusters of NPS in advance, the regulation of these drugs is a never-ending challenge for national authorities. Novel compounds with either related or even completely divergent structures continue to be synthesized and sold on the internet, circumventing existing legislations, in line with the popular term 'legal highs' that is sometimes used to refer to these substances.

The SCRA Cumyl-PEGACLONE (pentyl-2-(2-phenylpropan-2-yl)-2,5-dihydro-1H-pyrido[4,3-b]indol-1-one) was first identified in December 2016 in Germany within the framework of a profiling project for online monitoring of smokeable herbal mixtures containing SCRAs [5]. The tricyclic γ-carboline-1-one core structure of this compound was rather unconventional and was not included in the German generic legislation (NpSG) of November 2016 to control 'cannabimimetics/synthetic cannabinoids' by prohibiting all substances with core structures consisting of an indole, indazole or benzimidazole combined with specific side groups (Figure 1). This circumvention of the NpSG legislation was postulated as the reason for the synthesis and sale in the first place. Cumyl-PEGACLONE initially appeared on the market in the form of

herbal blends and e-liquids and became available as a pure 'research chemical' in July 2017 [6]. Since then, several research groups have characterized this new compound, its metabolization, and, to a lesser extent, its cannabinoid activity. The psychoactive effects, most desired by (ab)users, but also several adverse effects, mainly stem from CB_1 receptor activation. Angerer *et al*. reported a high binding affinity of Cumyl-PEGACLONE to the CB_1 receptor (K_i = 1.37 ± 0.24 nM) and scored the compound as a full agonist via a cAMP assay [5]. We recently reported on the ultrahigh potency of this compound, using two distinct bio-assays, assessing the recruitment of either a modified G protein (mini-$G\alpha_i$) or β-arrestin2 (β-arr2) to the activated CB_1 receptor. With EC_{50} values of 0.07 nM (95% confidence interval (CI) 0.02−0.22 nM) and 0.09 nM (95% CI 0.05−0.13 nM) in the respective bio-assays, Cumyl-PEGACLONE was the most potent SCRA of all the compounds tested in a previous study, encompassing over 20 representatives of different SCRA classes [7].

In response to the emergence of Cumyl-PEGACLONE, it was included in 2018 in the annex of the German narcotics law (BtMG), again incentivizing sellers to quickly look for replacement analogues. The result was that 5F-Cumyl-PEGACLONE (2,5-Dihydro-2-(1-methyl-1-phenylethyl)-5-(5-fluoropentyl)-1H-pyrido[4,3-b]indol-1-one) entered the recreational drug market as a new non-scheduled SCRA (Figure 1). Following the identification of this compound in November 2017, the EU Early Warning System of the EMCDDA sent out a formal notification for this compound late 2017. Not surprisingly, identification and case reports for both Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE have emerged since then [6,8–12]. Initially, SCRAs containing a γ-carbolinone core structure were hypothesized to be relatively safe, as no lethalities were observed in cases with (over)consumption of Cumyl-PEGACLONE. However, several fatalities have been reported during the last few years [13]. Also for 5F-Cumyl-PEGACLONE several fatalities have been reported [10,13]. Hence, the relative 'safety' of γ-carbolinones can be questioned [6,10,13]. As it is known that fluorination can have a beneficial effect on a compound's activity, resulting in a higher potency, it could be hypothesized that this would lead to a higher toxicity of 5F-Cumyl-PEGACLONE [7,14,15]. However, no activity data are available to confirm this hypothesis.

In the context of this report, two new Cumyl-PEGACLONE isomers (ethylbenzyl and n-propylphenyl) were synthesized. These compounds differ in their regulatory status in Singapore: whereas Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE, as well as their phenylpropyl isomers, are listed as scheduled substances, this is not the case for their ethylbenzyl isomers [16]. Neither of these isomeric analogues has been identified in drug seizures or patient samples (yet), nor is anything known about (a potential difference in) these isomers' pharmacological/toxicological profile.

As the toxicological profile of a compound is at least partially linked to its intrinsic receptor activation potential, Cumyl-PEGACLONE and its fluorinated variant, as well as the two above-mentioned isomers, were explored in this study. The activity at the CB_1 receptor was appraised using two different *in vitro* receptor-proximal bio-assays, assessing the recruitment of either β-arrestin2 (β-arr2) or a modified G protein (mini-$G\alpha_i$) to the activated receptor. The use of two recruitment bio-assays also allowed the assessment of possible biased agonism at CB_1 of these synthetic drugs [7,17]. This might be relevant, as some SCRAs may display a certain preference, which might have biological and/or toxicological implications. More specifically, we previously observed that Cumyl-PEGACLONE did not exhibit biased agonism, while EG-018, which has another 3-ring core structure (carbazole instead of γ-carbolinone) showed a 10-fold preference towards G protein over β-arr2 recruitment (Figure 1) [7]. Overall, these functional assays allow us to gain better insight into the (possibly) structurally related 'functional' activity of these compounds. As most CB_1-agonists still provoke psychotropic effects, their broad therapeutic utility remains limited. While the research on biased agonism amongst SCRAs is still in its infancy, outcomes on signaling pathway-selective agonists could aid the development of therapeutics diminishing on-target adverse effects.

MATERIALS & METHODS

Materials and reagents

Dulbecco's modified eagle's medium (DMEM, GlutaMAX™), Opti-MEM® I Reduced Serum Medium, penicillin/streptomycin (10.000 IU/ml and 10.000µg/ml), amphotericin B (250µg/ml), glutamine (200mM) and trypsin-EDTA (0.05%) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). Fetal bovine serum (FBS) was from Biochrom AG (Berlin, Germany). The Nano-Glo Live Cell reagent was procured from Promega (Madison, WI, USA). Cumyl-PEGACLONE (2,5-Dihydro-2-(1-methyl-1-phenylethyl)-5-pentyl-1H-pyrido[4,3-b]indol-1-one) (purity >99%), Cumyl-PEGACLONE ethylbenzyl isomer (purity >85%), Cumyl-PEGACLONE n-propylphenyl isomer (purity >99%) and 5F-Cumyl-PEGACLONE (2,5-Dihydro-2-(1-methyl-1-phenylethyl)-5-(5-fluoropentyl)-1H-pyrido[4,3-b]indol-1-one) (purity >99%) were synthesized (information below) by Chiron AS (Trondheim, Norway). Despite several attempts, the grade of purity of the reference standard for the ethylbenzyl isomer was only >85%, owing to technical difficulties in the purification. However, throughout this article we handled it in the same way as the n-propylphenyl isomer, meaning that the potency values for the ethylbenzyl isomer are likely a slight underestimation due to the presence of an impurity. JWH-018 (naphthyl(1-pentyl-1H-indol-3-yl)methanone) was obtained from LGC (Wesel, Germany). Poly-D-lysine was supplied by Sigma Aldrich (Steinheim, Germany) and absolute methanol from Biosolve B.V.

(Valkenswaard, The Netherlands). The white 96-well plates were obtained from Greiner Bio-One (Kremsmünster, Austria).

Synthesis of Cumyl-PEGACLONE & variants

Details on the chemicals used for the synthesis are provided in Supplementary Data.

1. *Synthesis of 1-substituted 1H-indole-2-methyl-3-carboxylic acids* (Figure 2)[18]

Sodium hydride (60% in mineral oil, 2.29 g, 57.17 mmol) was added to a solution of 2-methylindole (5.00 g, 38.12 mmol) in anhydrous DMF (30 mL) at 0 °C and the resulting mixture was stirred at this temperature for 20 min, before 1-bromoalkane (41.93 mmol, 1.1 eq.) was added. The mixture was stirred at 55 °C for 2 h, before being re-cooled to 0 °C and trifluoroacetic anhydride (13.25 mL, 95.29 mmol) was added. The mixture was stirred at 55 °C for 2 h and then poured into vigorously stirred ice-water (500 mL), washing once with DMF (5 mL). The red precipitate was collected by filtration and dried under high vacuum. This precipitate was dissolved in methanol (75 mL) and solid KOH (7.49 g, 133.40 mmol) was added. This mixture was heated to reflux for 16 h, before being cooled to room temperature and concentrated in vacuo. 2 M Hydrochloric acid (50 mL) and ethyl acetate (50 mL) were added and the aqueous phase was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated. The residue was re-crystallised from iPrOH to provide acid 1 (5.56 g, 22.68 mmol) as a pale brown crystalline solid.

2. *Amidation of 1-substituted 1H-indole-2-methyl-3-carboxylic acids* (Figure 2)

The appropriate amine ($R2NH_2$, 1.2 eq.) was added to a stirred solution of acid 1 (1.0 eq.), EDC hydrochloride (1.5 eq.), 1-hydroxybenzotriazole hydrate (1.5 eq.) and N,N-diisopropylethylamine (4.0 eq.) in DMF (25 mL). The solution was heated to 55 °C and stirred at this temperature for 16 h. The reaction mixture was diluted with ethyl acetate (75 mL) and water (75 mL), the layers were separated and the aqueous layer was further extracted with ethyl acetate (2 × 75 mL). The combined organic extracts were washed with water (75 mL), 5% aq. citric acid solution (2 × 75 mL), saturated aqueous $NaHCO_3$ solution (2 × 75 mL) and brine (2 × 75 mL), before being dried over $MgSO_4$, filtered and concentrated. Flash column chromatography (SiO_2, 15% ethyl acetate-petrol) provided the amide 2 as an oil.

3. *Synthesis of Cumyl-PEGACLONE derivatives* (Figure 2)[19]

n-Butyllithium (2.3 M in hexane, 3.0 eq.) was added dropwise to a stirred solution of amide 2 (1.0 eq.) in anhydrous THF (20 mL) at −30 °C and the stirred solution was allowed to warm naturally to 0 °C over 45 min. The solution was re-cooled to −30 °C and anhydrous DMF (4.0

eq.) was added dropwise. The resulting solution was stirred at −30 °C for 15 min and then at room temperature for 90 min. 2 M Hydrochloric acid (degassed by bubbling Argon with stirring for 20 mins, 15 mL) was added and the mixture was stirred vigorously at 55 °C for 16 h. The mixture was cooled to room temperature, diluted with CH_2Cl_2 and the pH of the aqueous phase was adjusted to pH 10 with 2 M NaOH solution. The aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic extracts were dried over $MgSO_4$, filtered and concentrated. Flash column chromatography (SiO_2, 15% ethyl acetate-petrol) provided a yellow oil which was further purified, if necessary, by preparative HPLC to provide the corresponding Cumyl-PEGACLONE derivative. Details about the characterization of the end products are provided in Supplementary Data.

Cell culture

The *in vitro* cannabinoid activity of all compounds was assessed by two previously reported live cell-based CB_1 reporter assays, based on the NanoLuc Binary Technology (Promega) [7,20]. Different cell lines of human embryonic kidney (HEK) 293T cells, stably transduced with one of two cannabinoid reporter systems (CB_1 with β-arr2 or CB_1 with mini-$G\alpha_i$ protein), were routinely maintained at 37°C, 5% CO2, under humidified atmosphere (passaged at confluence of 80-90%). They were cultured in DMEM (containing high glucose levels) supplemented with 10% heat-inactivated FBS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. For the assays, the cells were seeded on a poly-D-lysine coated 96-well plate at a concentration of $5x10^4$ cells/well and incubated overnight. The next day, the cells were washed twice with 150 µL Opti-MEM I Reduced Serum Medium to remove any remaining proteins (present in FBS) and 100 µL Opti-MEM I Reduced Serum Medium was added to each well. Subsequently, the Nano-Glo Live Cell reagent (Promega) was prepared by diluting the Nano-Glo Live Cell substrate 1 to 20 with Nano-Glo LCS Dilution buffer. Following addition of 25 µL of this nonlytic detection reagent to each well, the plate was read during equilibration of the signal (10-15 min) in the TriStar² LB 942 Multimode Microplate Reader (Berthold Technologies GmbH & Co., Germany). After this equilibration period, 10 µL of the freshly prepared 13.5× stock solutions (50% methanol in Opti-MEM I Reduced Serum medium) of the agonists was added in order to reach the reported in-well-concentrations and luminescence was continuously monitored for 120 min. Solvent controls were taken along to control for the amount of methanol present in the stock solutions. No problematic effect on the cells was observed for the present concentration of methanol in the wells (3.7%), probably given the short readout time of the assay [14]. In addition to the panel of Cumyl-PEGACLONE variants and solvent controls, JWH-018 was tested in each individual experiment as reference compound.

Data analysis & Statistical Analysis.

As FACS-sorted (Fluorescence-Activated Cell Sorting) stably transduced cells were used, there inherently is less variability in these assays. Hence, data were obtained in minimally three independent experiments, each performed in duplicate. Following the equilibration period, a baseline-correction was made of the absolute signals to correct for the inter-well variability. The final corrected luminescence measurements were obtained by subsequently subtracting the signals of the vehicle control samples from those of the experimental samples. These corrected signals were used to calculate the area under the curve (AUC) as measure for cannabinoid activity. All data were evaluated with the Grubbs test to detect outliers, before further statistical analysis.

Curve fitting and statistical analysis were performed using GraphPad Prism software (San Diego, CA, USA). The results are represented as normalized mean area under the curve (AUC) ± standard error of mean (SEM). EC_{50} values as measure of potency and E_{max} values as measure of efficacy were determined for all compounds by sigmoidal curve fitting the concentration−effect curves via nonlinear regression. A three parametric logistic fit was used for all compounds to comply to the prerequisite for calculating the bias factor in further data analysis (hill slope equals 1). The activity of the different SCRAs was evaluated in comparison with the reference compound JWH-018. The resulting cannabinoid activity is represented as the percentage (%) CB_1 activation of a compound relative to the maximum receptor activation (E_{max}) of JWH-018.

Bias calculation & Statistical analysis

For the evaluation and quantification of biased agonism, we used the relative activity-based method by calculating the bias factor using eq1 as previously described [21].

$$\beta = Log\left(\frac{RA_i^{\beta-arrestin2}}{RA_i^{mini-G_{\alpha i}}}\right) \quad \text{(eq1)}$$

This bias factor calculation entails the logarithm of the ratio of the intrinsic relative activity (RA_i) value of a compound for recruitment of β-arr2 to the value for recruiting mini-$G\alpha_i$. Therefore, the intrinsic relative activity (RA_i) was calculated for each compound of the tested panel for their response in both bioassays using eq2. Herein, $E_{max,i}$ and $EC_{50,i}$ represent the efficacy and potency, respectively, of the tested Cumyl-PEGACLONE variants. $E_{max,JWH\text{-}018}$ and $EC_{50,JWH\text{-}018}$ represent the efficacy and potency for the reference compound JWH-018, which, as in previous work, is considered unbiased [7].

$$RA_i = \frac{E_{max,i} \times EC_{50,JWH-018}}{EC_{50,i} \times E_{max,JWH-018}} \quad \text{(eq2)}$$

Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA) to detect statistical differences in the calculated bias factors of all compounds in comparison to the non-biased reference compound JWH-018. Statistical significance ($P < 0.05$) was determined using a non-parametric (Kruskal-Wallis) one-way ANOVA test, followed by post hoc analysis using Dunn's multiple comparison test.

RESULTS

In vitro CB_1 receptor activity

For the *in vitro* evaluation of the intrinsic receptor activation potential, all γ-carbolinones were compared to the reference SCRA, JWH-018. All Cumyl-PEGACLONE variants were able to activate the CB_1 receptor in the two separate bio-assays, measuring β-arr2 or mini-$G\alpha_i$ recruitment (Figure 3 & Supplementary Figure 1). EC_{50} and E_{max} values were derived as measures of potency and relative efficacy, respectively (Table 1).

Both in terms of potency and relative efficacy, Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE were found to be much more active compared to JWH-018 in both CB_1 activation bio-assays. The data obtained for Cumyl-PEGACLONE (β-arr2: EC_{50} = 0.23 nM and E_{max} = 344%; mini-$G\alpha_i$: EC_{50} = 0.17 nM and E_{max} = 194%) are in line with what we previously observed [7]. 5F-Cumyl-PEGACLONE hadn't been tested in these settings before and gave similar activity profiles compared to the non-fluorinated parent compound, for both the recruitment of β-arr2 (EC_{50} = 0.58 nM and E_{max} = 356%) and mini-$G\alpha_i$ (EC_{50} = 0.22 nM and E_{max} = 174%). Hence, while in addition to Cumyl-PEGACLONE, also 5F-Cumyl-PEGACLONE now ranks amongst the most potent SCRAs evaluated by these *in vitro* bioassays so far, no beneficial effect of fluorination could be observed in terms of intrinsic CB_1 receptor activation potential. Efficacy-wise, both compounds also classify as highly efficacious SCRAs, although even higher efficacy values have been measured in previous studies (e.g. for AB-CHMINACA in the β-arr2 assay) [22–24].

Interestingly, a very divergent activity profile was observed for both isomeric variants of Cumyl-PEGACLONE (ethylbenzyl isomer and n-propylphenyl isomer). The concentration-response curves were clearly shifted to the right, resulting in higher EC_{50} and lower E_{max} values (EC_{50} = 123-170 nM and E_{max} = 25.8-38.0%). The very high activity of Cumyl-PEGACLONE, therefore, seems to (at least partially) rely on an adequate linker. The relatively low activity of the isomers was observed in both CB_1 activation bio-assays. No difference in EC_{50} and E_{max} values was observed for the two isomers, although one should be aware that the reference standard for the ethylbenzyl isomer was only >85% pure (as discussed in Materials & Methods).

Biased agonism at the CB_1 receptor

As all compounds were tested in the two different bio-assays, this also allowed the assessment of possible biased agonism by calculating the compounds' bias factor β (Table 1). Although the relative activity-based approach, taking into account both potency and efficacy, did not reveal a significant bias of any of the tested SCRAs, compared to the non-biased JWH-018, both isomers did cluster together, with a trend differing from (5F-)Cumyl-PEGACLONE (Figure 3 & Supplementary Figure 2). Given this apparent different trend, statistical significance was analyzed for a difference in biased agonism between the isomers and Cumyl-PEGACLONE itself, but no statistically significant bias was detected.

DISCUSSION

The synthesis of structurally very diverse SCRAs seems to be driven by the will to circumvent structure-based legislation via scaffold hopping, isomer exploration and so on. When first characterizing the new SCRA Cumyl-PEGACLONE in 2017, structural similarities were observed with Cumyl-PICA (Figure 1) [5]. Hereby, Cumyl-PEGACLONE exhibits scaffold-hopping to a new tricyclic γ-carbolinone core structure. We and others have already reported Cumyl-PEGACLONE as a very potent and full agonist of the CB_1 receptor. In general, higher binding affinity and lower EC_{50} values have been reported than those for Cumyl-PICA, albeit in different experimental settings, which hampers easy comparison of EC_{50} values [5,7,25]. Regardless of the differences in assay set-ups, the scaffold hopping to a γ-carbolinone core can be considered successful in terms of creating a SCRA with ultrahigh activation potential of the CB_1 receptor. It can thus be hypothesized that the use of Cumyl-PEGACLONE could therefore entail a greater risk of overdosing and inducing severe adverse effects.

On the contrary, the activity-profiling of Cumyl-PEGACLONE ethylbenzyl and n-propylphenyl isomers revealed that structural isomer exploration of Cumyl-PEGACLONE did not yield analogues with similar or higher activity but, instead, led to compounds with a strongly reduced intrinsic receptor activation potential. Based on insights obtained from molecular docking of Cumyl-PEGACLONE in the cryo-EM structure of the CB_1-$G\alpha_i$ complex, possible steric hindrance can be hypothesized at the orthosteric CB_1 binding site [7]. The presence of a more bulky substituent on the linker or an increased linker length could hamper a hydrogen bonding network with $S383^{7.39}$ and water molecules in the pocket, possibly explaining the observed lower CB_1 activation potential.

Initially, 5F-Cumyl-PEGACLONE was thought to be associated with a higher toxicity than Cumyl-PEGACLONE, although also several fatalities, involving mono-intoxications, have been observed with the latter [13]. At this point, it is thus unclear whether there actually is a difference in toxicity *in vivo*. Interestingly, fluorination of Cumyl-PEGACLONE did not provide the postulated beneficial effect on the intrinsic cannabinoid activity. Both the potency and efficacy of the parent compound and the 5-fluorinated analogue were similar regarding recruitment of both β-arr2 and mini-$G\alpha_i$. Hence, these compounds demonstrate similar intrinsic CB_1 receptor activation potential. This finding is not in line with other reports where the fluorinated SCRA usually exerts a higher potency than the unfluorinated analog [15,26]. Possibly there is a limit at the potency that can be reached by this type of SCRAs, and potentially Cumyl-PEGACLONE (and hence also its 5F analog) has reached that limit.

Earlier, the presumed relatively low toxicity of Cumyl-PEGACLONE was postulated to originate from a more homogenous distribution and low concentrations of the compound in products that were sold, as this SCRA initially appeared in herbal blends and e-liquids only [6]. However, even when the pure substance became available in July 2017 as a research chemical, there apparently wasn't an increase in severe or lethal intoxication cases. So, at the time, the most plausible explanation for the lack of severe intoxication or death cases with Cumyl-PEGACLONE remained an intrinsic relatively low toxicity of the compound, when compared to other SCRAs on the recreational drug market. In line with this low toxicity hypothesis, a group of related carboline derivatives was described as a possible medical treatment for respiratory and non-respiratory diseases in a US patent of Leftheris *et al.* [27]. In addition, Cheng *et al.* suggested a group of γ-carbolines as a class of SCRAs combining water solubility and low CNS penetration, which could explain both medical potential and (previously postulated) low (central) toxicity [28]. However, contrasting with these reports are a number of fatalities reported in Australia, in which even sub-ng/ml concentrations of Cumyl-PEGACLONE in blood were considered as highly probable to have contributed to death [13]. Similarly, for 5F-Cumyl-PEGACLONE, low blood concentrations have been linked to fatalities [10,13]. Hence, whether there is a difference in toxicity between Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE is currently not clear. For cathinones, another class of NPS, it has been suggested that fluorination can increase the compound's ability to cross the Blood-Brain-Barrier (BBB) (Fabregat-Safont et al., submitted). In a similar scenario, higher 5F-Cumyl-PEGACLONE concentrations might be reached at the target sites in the brain - only *in vivo* experiments can provide a definitive answer to this. Furthermore, besides a compound's intrinsic receptor activation potential and BBB penetrability, additional factors will determine the eventual toxicity of a compound *in vivo*. Amongst these are potential differential off-target effects, differences in resorption, metabolic stability, or the existence of active metabolites that may contribute to

the overall *in vivo* cannabinoid activity. Differences in metabolites between Cumyl-PEGACLONE and the 5-fluorinated analogue have been identified, but no information on their activity is available yet [29]. Neither has the *in vivo* metabolic stability of these γ-carbolinones been reported so far. Only consideration of all contributing factors in combination with further *in vivo* examinations can provide an adequate comparison of both compound's toxicological profile.

Reported concentrations of Cumyl-PEGACLONE ranged from 0.38 nM to 34.9 nM (0.14 to 13 ng/ml) in serum from clinical samples and from 0.32 nM to 24.2 nM (0.12 to 9 ng/ml) in femoral post mortem blood samples [6,13]. Cases of 5F-Cumyl-PEGACLONE included a concentration range of 0.24 nM – 1.2 nM (0.09 to 0.45 ng/ml) [10,13]. The latter concentrations, derived from post mortem samples, should be interpreted with caution for different reasons, amongst which post mortem redistribution and non-availability of the exact methods that were applied for quantification. Thus, for both compounds, observed *in vivo* concentrations correspond to *in vitro* concentrations around or above the EC_{50}, representing a pronounced CB_1 activation, certainly taking into account the high efficacy of these compounds.

As to biased agonism, no statistical difference in preference towards recruitment of either β-arr2 or mini-$G\alpha_i$ was detected in comparison to the non-biased reference compound JWH-018. Keeping in mind that varying levels of signal amplification across assays can confound the interpretation of biased agonism, especially in the case of low-efficacy agonists like the evaluated isomers in this study, it is important to note that no signal amplification (like in for example cAMP assays) occurs in the bio-assays applied here, which were designed to maximally ensure an equally effective engagement of both signaling molecules. It is relevant to mention that *in vivo* active metabolites could exert divergent activity profiles from the parent compound. Accordingly, these can possibly contribute to overall or delayed *in vivo* biased agonism, which was not studied here [30].

CONCLUSION

Both Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE intoxications have been linked to fatalities. Currently, the relatively limited number of cases does not allow to judge whether the fluorinated derivative is more toxic than Cumyl-PEGACLONE, which was originally thought to be relatively safe. This study clearly demonstrates that both compounds do not differ in *in vitro* intrinsic CB_1 receptor activation potential. Whether these compounds differ in BBB permeability or whether improved metabolic stability or active metabolites play a role in intoxications was not evaluated here, but could be the topic of future work. Furthermore, Cumyl-PEGACLONE ethylbenzyl and n-propylphenyl isomers were found to have a strongly reduced CB_1 receptor

activation potential, both in terms of potency and efficacy, notwithstanding a potential underestimation for the ethylbenzyl isomer due to the lower (>85%) purity level. Finally, no statistically significant biased agonism was observed in this *in vitro* study for any of the evaluated γ-carbolinones. In conclusion, Cumyl-PEGACLONE and 5F-Cumyl-PEGACLONE rank amongst the most potent SCRAs tested in these assays. Their strong activity is seemingly highly dependent on an adequate linker. In the future, evaluation of other newly emerging γ-carbolinones analogs with varying substitutions (e.g. Cumyl-CH-MEGACLONE with varying tail) could shed more light on the structure activity relationship of this group of SCRAs.

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REFERENCES

1. EMCDDA. European Drug Report: Trends and Developments. Published online 2019.

2. Abouchedid R, Hudson S, Thurtle N, et al. Analytical confirmation of synthetic cannabinoids in a cohort of 179 presentations with acute recreational drug toxicity to an Emergency Department in London, UK in the first half of 2015. *Clin Toxicol*. 2017;55(5):338-345. doi:10.1080/15563650.2017.1287373

3. Fantegrossi WE, Moran JH, Radominska-Pandya A, Prather PL. Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ9-THC: Mechanism underlying greater toxicity? *Life Sci*. 2014;97(1):45-54. doi:10.1016/j.lfs.2013.09.017

4. Tait RJ, Caldicott D, Mountain D, Hill SL, Lenton S. A systematic review of adverse events arising from the use of synthetic cannabinoids and their associated treatment. *Clin Toxicol*. 2016;54(1):1-13. doi:10.3109/15563650.2015.1110590

5. Angerer V, Mogler L, Steitz J-P, et al. Structural characterization and pharmacological evaluation of the new synthetic cannabinoid CUMYL-PEGACLONE. *Drug Test Anal*. 2018;10(3):597-603. doi:10.1002/dta.2237

6. Halter S, Angerer V, Röhrich J, et al. Cumyl-PEGACLONE: A comparatively safe new synthetic cannabinoid receptor agonist entering the NPS market? *Drug Test Anal*. 2019;11(2):347-349. doi:10.1002/dta.2545

7. Wouters E, Walraed J, Robertson MJ, et al. Assessment of Biased Agonism among Distinct Synthetic Cannabinoid Receptor Agonist Scaffolds. *ACS Pharmacol Transl Sci*. 2020;3(2):285-295. doi:10.1021/acsptsci.9b00069

8. Ernst L, Brandhorst K, Papke U, et al. Identification and quantification of synthetic cannabinoids in ‘spice-like’ herbal mixtures: Update of the German situation in early 2017. *Forensic Sci Int*. 2017;277:51-58. doi:10.1016/j.forsciint.2017.05.019

9. Ernst L, Langer N, Bockelmann A, Salkhordeh E, Beuerle T. Identification and quantification of synthetic cannabinoids in ‘spice-like’ herbal mixtures: Update of the German situation in summer 2018. *Forensic Sci Int*. 2019;294:96-102. doi:10.1016/j.forsciint.2018.11.001

10. Giorgetti A, Mogler L, Halter S, et al. Four cases of death involving the novel synthetic cannabinoid 5F-Cumyl-PEGACLONE. *Forensic Toxicol*. Published online December 24, 2019. doi:10.1007/s11419-019-00514-w

11. Aknouche F, Ameline A, Richeval C, et al. Testing for SGT-151 (CUMYL-PEGACLONE) and its Metabolites in Blood and Urine after Surreptitious Administration. *J Anal Toxicol*. 2019;44(1):75-80. doi:10.1093/jat/bkz011

12. Halter S, Mogler L, Auwärter V. Quantification of Herbal Mixtures Containing Cumyl-PEGACLONE—Is Inhomogeneity Still an Issue? *J Anal Toxicol*. 2019;44(1):81-85.

doi:10.1093/jat/bkz028

13. United Nations Office on Drugs and Crimes (UNODC). UNODC Early Warning Advisory - Tox Portal. https://www.unodc.org/tox/#/login

14. Cannaert A, Storme J, Franz F, Auwärter V, Stove CP. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. *Anal Chem*. 2016;88(23):11476-11485. doi:10.1021/acs.analchem.6b02600

15. Banister SD, Stuart J, Kevin RC, et al. Effects of Bioisosteric Fluorine in Synthetic Cannabinoid Designer Drugs JWH-018, AM-2201, UR-144, XLR-11, PB-22, 5F-PB-22, APICA, and STS-135. *ACS Chem Neurosci*. 2015;6(8):1445-1458. doi:10.1021/acschemneuro.5b00107

16. Pang Kin Keon. MISUSE OF DRUGS ACT (AMENDMENT OF FIRST, THIRD AND FIFTH SCHEDULES) ORDER 2019. Published April 25, 2019. Accessed May 26, 2020. https://sso.agc.gov.sg/SL-Supp/S354-2019/Published/20190430?DocDate=20190430

17. Wouters E, Walraed J, Banister SD, Stove CP. Insights into biased signaling at cannabinoid receptors: synthetic cannabinoid receptor agonists. *Biochem Pharmacol*. 2019;169:113623. doi:10.1016/j.bcp.2019.08.025

18. Banister SD, Wilkinson SM, Longworth M, et al. The Synthesis and Pharmacological Evaluation of Adamantane-Derived Indoles: Cannabimimetic Drugs of Abuse. *ACS Chem Neurosci*. 2013;4(7):1081-1092. doi:10.1021/cn400035r

19. Wrobleski ST, Chen P, Hynes, J, et al. Rational Design and Synthesis of an Orally Active Indolopyridone as a Novel Conformationally Constrained Cannabinoid Ligand Possessing Antiinflammatory Properties. *J Med Chem*. 2003;46(11):2110-2116. doi:10.1021/jm020329q

20. Cannaert A, Franz F, Auwärter V, Stove CP. Activity-Based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples Using a Stable Cannabinoid Reporter System. *Anal Chem*. 2017;89(17):9527-9536. doi:10.1021/acs.analchem.7b02552

21. Rajagopal S, Ahn S, Rominger DH, et al. Quantifying Ligand Bias at Seven-Transmembrane Receptors. *Mol Pharmacol*. 2011;80(3):367-377. doi:10.1124/mol.111.072801

22. Noble C, Cannaert A, Linnet K, Stove CP. Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. *Drug Test Anal*. 2019;11(3):501-511. doi:10.1002/dta.2517

23. Antonides LH, Cannaert A, Norman C, et al. Enantiospecific Synthesis, Chiral Separation, and Biological Activity of Four Indazole-3-Carboxamide-Type Synthetic Cannabinoid Receptor Agonists and Their Detection in Seized Drug Samples. *Front Chem*. 2019;7:321. doi:10.3389/fchem.2019.00321

24. Wouters E, Mogler L, Cannaert A, Auwärter V, Stove C. Functional evaluation of carboxy metabolites of synthetic cannabinoid receptor agonists featuring scaffolds based on L-valine or L- *tert* -leucine. *Drug Test Anal*. 2019;11(8):1183-1191. doi:10.1002/dta.2607

25. Longworth M, Banister SD, Boyd R, et al. Pharmacology of Cumyl-Carboxamide Synthetic Cannabinoid New Psychoactive Substances (NPS) CUMYL-BICA, CUMYL-PICA, CUMYL-5F-PICA, CUMYL-5F-PINACA, and Their Analogues. *ACS Chem Neurosci*. 2017;8(10):2159-2167. doi:10.1021/acschemneuro.7b00267

26. Gurney S, Scott K, Kacinko S, Presley B, Logan B. Pharmacology, toxicology, and adverse effects of synthetic cannabinoid drugs. *Forensic Sci Rev*. 2014;(26):53-78.

27. Leftheris K, Wu H, Wrobleski S, Chen P, Hynes J, Tokarski P. Cannabinoid receptor modulators,their processes of preparation, and use of cannabinoid receptor modulators for treating respiratory and non-respiratory diseases. *US 6653304 B2*. Published online 2003.

28. Cheng Y-X, Pourashraf M, Luo X, et al. γ-Carbolines: A novel class of cannabinoid agonists with high aqueous solubility and restricted CNS penetration. *Bioorg Med Chem Lett*. 2012;22(4):1619-1624. doi:10.1016/j.bmcl.2011.12.124

29. Mogler L, Halter S, Wilde M, Franz F, Auwärter V. Human phase I metabolism of the novel synthetic cannabinoid 5F-CUMYL-PEGACLONE. *Forensic Toxicol*. 2019;37(1):154-163. doi:10.1007/s11419-018-0447-4

30. Gillis A, Gondin AB, Kliewer A, et al. Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Sci Signal*. 2020;13(625):eaaz3140. doi:10.1126/scisignal.aaz3140

Tables

Compound	β-arrestin2		mini- $G\alpha_i$		β ± SEM
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	
JWH-018	14.4	101	13.1	98.8	
	(7.76 – 27.8)	(92.3 – 110)	(5.72 -31.1)	(87.6 – 111)	
Cumyl-PEGACLONE	0.23	344	0.17	194	0.04 ± 0.15
	(0.13- 0.43)	(314 – 375)	(0.10 – 0.30)	(180 – 209)	
5F-Cumyl-PEGACLONE	0.58	356	0.22	174	-0.04 ± 0.10
	(0.32 – 1.00)	(323 – 389)	(0.15 – 0.33)	(165 – 184)	
Cumyl-PEGACLONE ethylbenzyl isomer	170	25.8	123	36.3	-0.20 ± 0.13
	(82.7 – 352)	(22.0- 29.8)	(43.5 – 329)	(30.2 – 42.6)	
Cumyl-PEGACLONE	170	27.1	123	38.0	0.26 ± 0.21
n-propylphenyl isomer	(87.1 – 321)	(24.3 – 30.0)	(44.3 - 340)	(32.1 – 44.2)	

TABLE 1. Overview of Potency (EC_{50}), Efficacy (E_{max}, relative to JWH-018) and bias factor (β) for the SCRAs[a]

[a] Each value is accompanied by the corresponding 95% confidence intervals (CI) or standard error of the mean (SEM)

Figures

Figure 1: Structures of all compounds: the ones evaluated in this study are displayed in black (Cumyl variants), the ones mentioned but not studied in this paper (EG-018, Cumyl-PICA) or used as reference (JWH-018) are displayed in light grey.

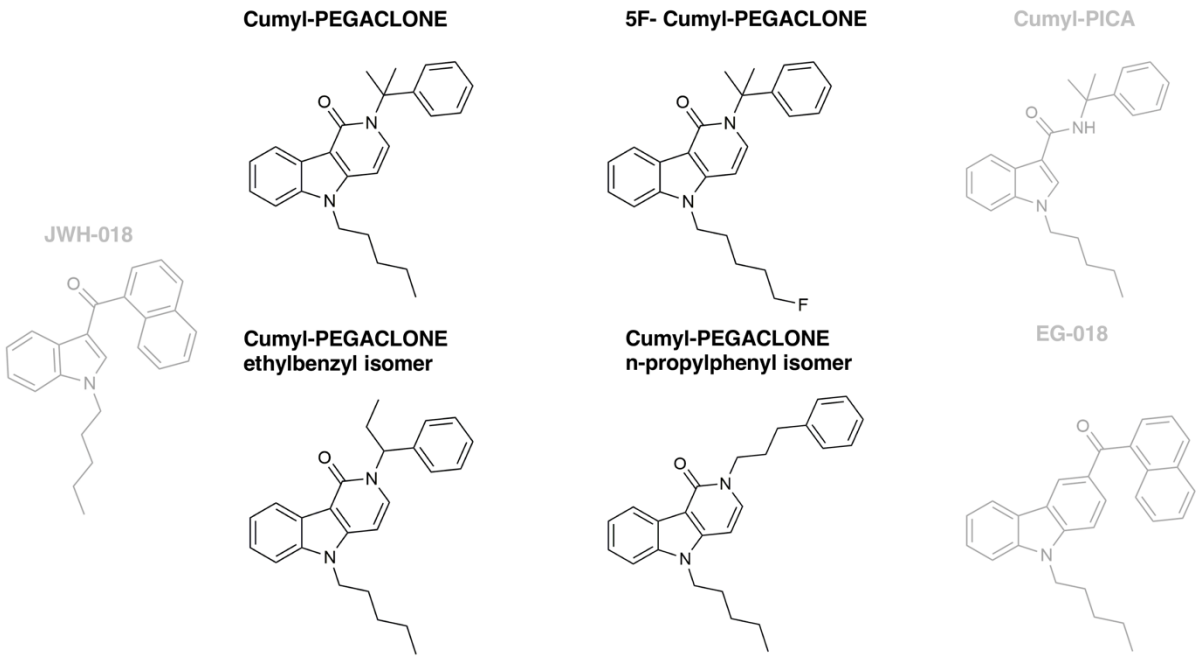


Figure 2: Chemical synthesis of Cumyl-PEGACLONE and variants. 1) Synthesis of 1-substituted 1H-indole-2-methyl-3-carxylic acids; 2) Amidation of 1-substituted 1H-indole-2-methyl-3-carxylic acids; 3) Synthesis of Cumyl-PEGACLONE derivatives.

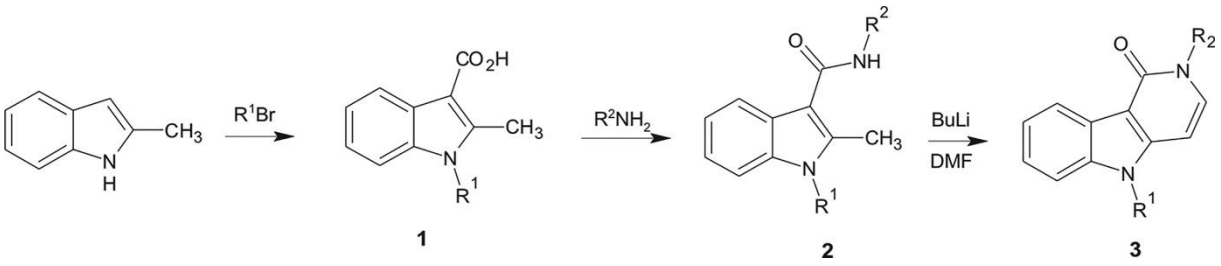
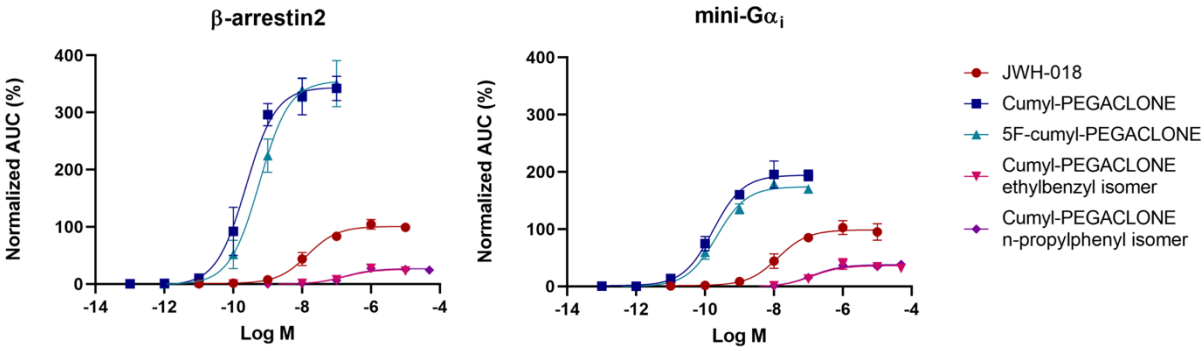
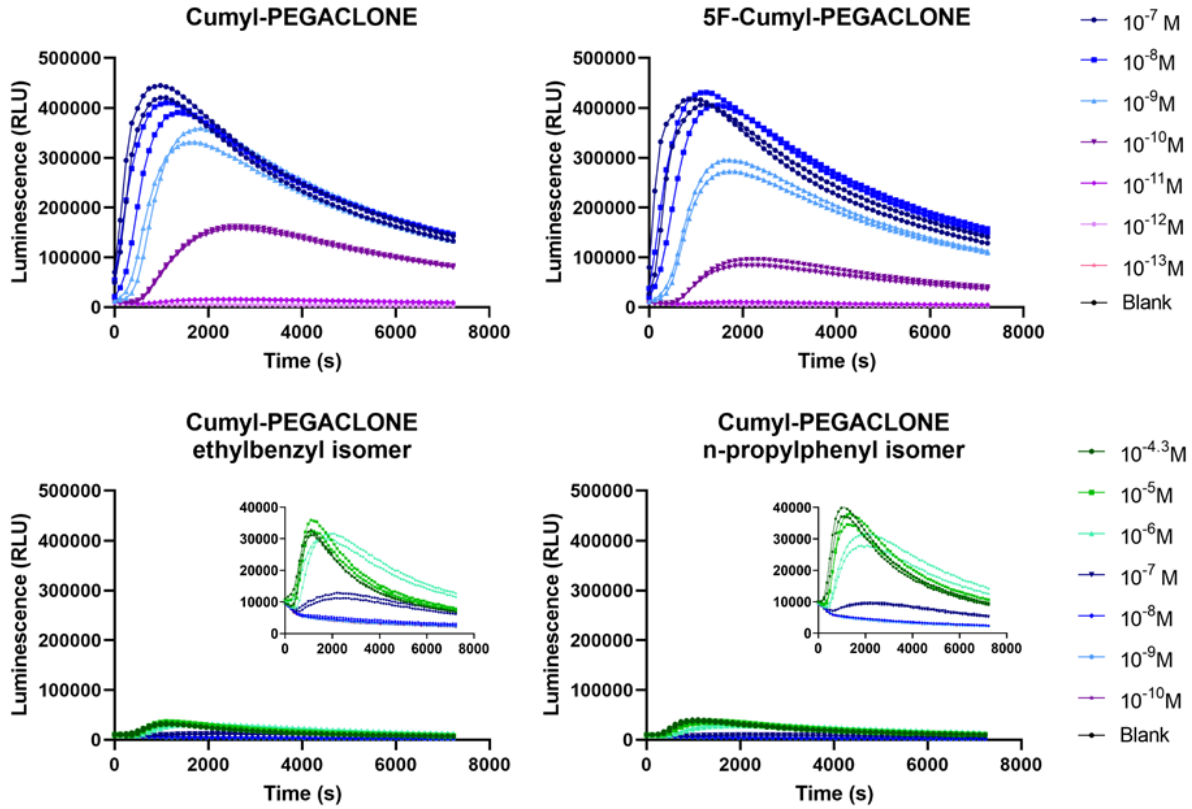


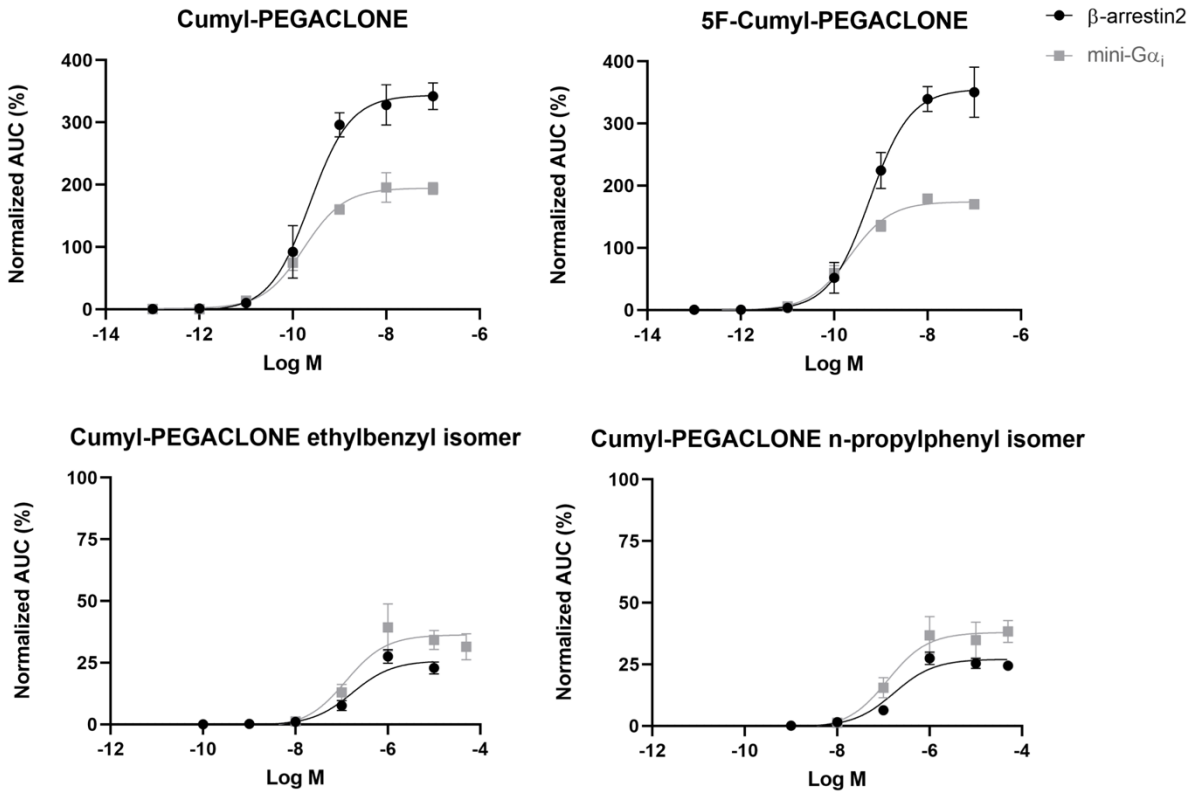
Figure 3: Graphical representation of the concentration-response curves, reflecting the activity of all compounds in the CB_1 bio-assays measuring recruitment of β-arrestin2 (left panel) or mini-$G\alpha_i$ (right panel). AUC: Area Under the Curve (normalized to the maximal receptor activation of JWH-018).



Supplementary Figure 1: CB_1 activation profiles of all tested compounds from one representative experiment in the β-arr2 assay. Note the difference in scale in the zoomed-in graphs of the isomers.



Supplementary Figure 2: Concentration-response curves in both the CB_1 bio-assay measuring recruitment of β-arrestin2 and the bio-assay measuring mini-$G\alpha_i$ recruitment, graphically represented per individual compound. Note the difference in scale between the upper and lower panels. AUC: Area Under the Curve (normalized to the maximal receptor activation of JWH-018).


Cumyl-PEGACLONE
5F-Cumyl-PEGACLONE
β-arrestin2
mini-Gαi
Normalized AUC (%)
Log M
Cumyl-PEGACLONE ethylbenzyl isomer
Cumyl-PEGACLONE n-propylphenyl isomer