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Cannabinoid receptor activation potential of the next generation, generic ban evading OXIZID synthetic cannabinoid receptor agonists

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Abstract:	In recent years, several nations have implemented various measures to control the surge of new synthetic cannabinoid receptor agonists (SCRAs) entering the recreational drug market. In July 2021, China put into effect a new generic legislation, banning SCRAs containing one of 7 general core scaffolds. However, this has driven manufacturers towards the synthesis of SCRAs with alternative core structures, exemplified by the recent emergence of "OXIZID SCRAs". Here, using in vitro β-arrestin2 recruitment assays, we report on the CB1 and CB2 potency and efficacy of five members of this new class of SCRAs: BZO-HEXOXIZID, BZO-POXIZID, 5-fluoro BZO-POXIZID, BZO-4en-POXIZID and BZO-CHMOXIZID. All compounds behaved as full agonists at CB1 and partial agonists at CB2. Potencies ranged from 84.6 – 721 nM at CB1 and 2.21 – 25.9 nM at CB2. Shortening the n-hexyl tail to a pentyl tail enhanced activity at both receptors. Fluorination of this pentyl analog did not yield a higher receptor activation potential, whereas an unsaturated tail resulted in decreased potency and efficacy at CB1. The cyclohexyl methyl analog BZO-CHMOXIZID was the most potent compound at both receptors, with EC50 values of 84.6 and 2.21 nM at CB1 and CB2, respectively. Evaluation of the activity of a seized powder containing BZO-4en-POXIZID suggested a high purity, in line with HPLC-DAD, GC-MS, LC-QTOF-MS and FTIR and NMR analysis. Furthermore, all tested compounds showed a preference for CB2, except for BZO-POXIZID. Overall, these findings inform public health officials, law enforcement agencies and clinicians on these newly emerging SCRAs.

Cannabinoid receptor activation potential of the next generation, generic ban evading OXIZID synthetic cannabinoid receptor agonists

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ABSTRACT

In recent years, several nations have implemented various measures to control the surge of new synthetic cannabinoid receptor agonists (SCRAs) entering the recreational drug market. In July 2021, China put into effect a new generic legislation, banning SCRAs containing one of 7 general core scaffolds. However, this has driven manufacturers towards the synthesis of SCRAs with alternative core structures, exemplified by the recent emergence of "OXIZID SCRAs". Here, using *in vitro* β-arrestin2 recruitment assays, we report on the CB_1 and CB_2 potency and efficacy of five members of this new class of SCRAs: BZO-HEXOXIZID, BZO-POXIZID, 5-fluoro BZO-POXIZID, BZO-4en-POXIZID and BZO-CHMOXIZID. All compounds behaved as full agonists at CB_1 and partial agonists at CB_2. Potencies ranged from 84.6 – 721 nM at CB_1 and 2.21 – 25.9 nM at CB_2. Shortening the n-hexyl tail to a pentyl tail enhanced activity at both receptors. Fluorination of this pentyl analog did not yield a higher receptor activation potential, whereas an unsaturated tail resulted in decreased potency and efficacy at CB_1. The cyclohexyl methyl analog BZO-CHMOXIZID was the most potent compound at both receptors, with EC_{50} values of 84.6 and 2.21 nM at CB_1 and CB_2, respectively. Evaluation of the activity of a seized powder containing BZO-4en-POXIZID suggested a high purity, in line with HPLC-DAD, GC-MS, LC-QTOF-MS and FTIR and NMR analysis. Furthermore, all tested compounds showed a preference for CB_2, except for BZO-POXIZID. Overall, these findings inform public health officials, law enforcement agencies and clinicians on these newly emerging SCRAs.

Keywords

OXIZID, BZO-HEXOXIZID, synthetic cannabinoid receptor agonists, bioassay, CB1 cannabinoid receptor, new psychoactive substances

INTRODUCTION

Synthetic cannabinoid receptor agonists (SCRAs) remain one of the most identified classes of new psychoactive substances (NPS) worldwide and their number continues to increase[1]. Eleven new SCRAs were reported in Europe for the first time in 2020, adding up to a total of 209 compounds being detected since 2008[2]. Although a decrease in the number of newly detected SCRAs has been noticed during the last years[1], monitoring SCRA use remains important in specific settings, for instance among homeless people and in prisons (in Europe), the latter usually to circumvent mandatory drug tests[3,4]. SCRAs exert their main sought-after psychoactive effects at the CB_1 cannabinoid receptor, thereby mimicking the effects of the phytocannabinoid Δ^9-tetrahydrocannabinol (THC), the primary psychoactive substance of cannabis. Their major threat lies in their often much higher potency and efficacy compared to THC[5–7], the variability in the composition of the marketed products and their easy accessibility via the Internet[8]. Additionally, there has been an increase in reports of cannabis adulterated with potent SCRAs, resulting in users being unaware of the potential harms they could be exposed to[2]. SCRAs have been associated with psychosis, agitation, hallucinations, seizures, respiratory failure, cardiovascular effects, coma and even death [9–13].

SCRAs and NPS in general are hard to put under legislative control, as unknown substances appear on the illicit drug market at a rapid pace. The stabilizing number of new NPS detected during recent years suggests the impact of regulatory steps taken by several nations, such as the introduction of generic legislations. These allow a

nation to ban a larger group of substances encompassing certain core structures[1,14]. However, the already complex recreational drug market remains a 'game of cat and mouse', as clandestine labs manage to find 'legal loopholes' by synthesizing structurally diverse compounds not covered by the current control measures. For instance, in May 2021, the Office of China National Narcotics Control Commission announced that as of July 1st 2021, a generic legislation would be in place to control synthetic cannabinoids, similar to the scheduling of fentanyl-related substances in 2019[15,16]. As opposed to the former individual listing of substances, which required the cumbersome identification and specification of each individual compound to be controlled, this measure allowed for a nation-wide ban of compounds structurally related to 7 general scaffolds (See Figure 1)[17]. This may have driven manufacturers towards the synthesis of compounds with new core structures, not covered by this legislation[18]. The first series of such compounds was recently reported by Liu *et al.*, who identified and characterized AD-18, 5F-MDA-19 and pentyl-MDA-19 in a seizure of powders and e-liquids[19]. As the term MDA (stemming from M.D. Anderson Cancer Center, the Institute where these compounds were first synthesized – see below) may be confused with the abbreviation of methylenedioxyamphetamine, a new, more 'SCRA-friendly' OXIZID-nomenclature, based on the chemical structure and IUPAC name, was developed by Cayman Chemical and the NPS Discovery program at the US-based Center for Forensic Science Research & Education (CFSRE)[18]. The term *OXIZID* refers to the OXoIndoline core attached to the aZIDe linker and will be used throughout this article. As these OXIZIDs introduce a new class of non-scheduled

compounds, it is anticipated that the number of OXIZID SCRAs may further increase in the near future.

Unlike CB_1, which is predominantly present in the central nervous system, CB_2 is primarily located in cells of the immune system and is involved in inflammatory processes[20,21]. As CB_2-selective agonists are believed to be devoid of the undesirable side effects correlated with CB_1 activation and, importantly, are not believed to be psychoactive, they are considered to be potentially interesting therapeutic tools[22–25]. It is in this context that the CB_2 agonist MDA-19 (BZO-HEXOXIZID) was developed in 2008 and was later pharmacologically characterized at the University of Texas M.D. Anderson Cancer Center[26,27]. It served as a lead compound in the development of therapeutics for the treatment of neuropathic pain, an often difficult-to-treat condition caused by trauma or disease of the somatosensory nervous system[28,29] as a result of e.g. diabetic neuropathy, multiple sclerosis, trigeminal neuralgia and postherpetic neuralgia[26,30]. The scarcely available literature also mentions *in vitro* antiproliferative potential of BZO-HEXOXIZID, as studied using melanoma, osteosarcoma and hepatocellular carcinoma cell lines[31–33].

In October 2016, BZO-HEXOXIZID was notified for the first time in Spain and reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)[34]. In October 2021, the CFSRE reported on the seizure of BZO-HEXOXIZID and two of its analogs, the truncated BZO-POXIZID (pentyl-MDA-19) and its fluorinated counterpart 5F-BZO-POXIZID (5F-MDA-19) in the US[35–37]. Shortly after, the first appearance on the recreational drug market in China of BZO-POXIZID and 5F-BZO-POXIZID was

reported by Liu *et al.*[19]. Only one month later the CFSRE reported on the identification in plant-like material of yet another analog, BZO-CHMOXIZID (CHM-MDA-19, with a cyclohexyl methyl moiety instead of the hexyl tail of MDA-19)[38]. Most recently, in December 2021, the Hungarian government announced that BZO-POXIZID, BZO-CHMOXIZID and another analog, BZO-4en-POXIZID, carrying an unsaturated tail seen in other commonly identified SCRAs such as MDMB-4en-PINACA and ADB-4en-PINACA[39–41], were to be added to the list of controlled NPS (Decree No 55/2014)[42,43]. Relatively little is known about the pharmacology and structure-activity relationship of these compounds.

The fact that BZO-HEXOXIZID and its related compounds have repeatedly been identified in seized materials suggests that these compounds might still possess the potential to activate CB_1 and may be used for their psychoactive properties. In this study, we therefore assessed the intrinsic receptor activation potential of a panel of 5 OXIZID SCRAs (BZO-HEXOXIZID, BZO-POXIZID, 5F-BZO-POXIZID, BZO-4en-POXIZID and BZO-CHMOXIZID) at both CB_1 and CB_2 by means of activity-based bioassays, monitoring β-arrestin2 (βarr2) recruitment to the activated receptor. Furthermore, these bioassays were also used to evaluate the $CB_{1/2}$ receptor activation potential of a powder that was intercepted by the Belgian Customs in November 2021, and which was confirmed to contain BZO-4en-POXIZID.

MATERIALS AND METHODS

Materials and Reagents

Dulbecco's Modified Eagle's medium (DMEM) (GlutaMAX™), Opti-MEM I Reduced Serum, penicillin, streptomycin and amphotericin B were procured from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) and poly-D-lysine were supplied by Sigma-Aldrich (Darmstadt, Germany). Methanol was purchased from Chem-Lab NV (Zedelgem, Belgium). BZO-HEXOXIZID, BZO-POXIZID, 5F-BZO-POXIZID, BZO-4en-POXIZID and BZO-CHMOXIZID were kindly provided by Cayman Chemical (Ann Arbor, MI, USA). The Nano-Glo® Live Cell reagent and the Nano-Glo® LCS Dilution buffer were obtained from Promega (Madison, WI, USA). A powder sample containing BZO-4en-POXIZID was seized in November 2021 by the Laboratory of the Belgian Customs and Excise services (Vilvoorde, Belgium). All reagents used for the analytical characterization were at least of high-performance liquid chromatography (HPLC) grade. LC-MS grade methanol and formic acid were purchased from Chem-Lab NV. Acetonitrile was procured from Biosolve (Valkenswaard, The Netherlands). Ammonium formate, ortho-phosporic acid (85%) and potassium dihydrogen phosphate were purchased from Sigma-Aldrich (Diegem, Belgium). For NMR analysis, all reagents were obtained from Sigma-Aldrich. For FTIR analysis, no solvents or reagents were used.

In Vitro CB_1 and CB_2 β-Arrestin2 Recruitment assays

To determine activity at CB_1 and CB_2, previously described live cell-based bio-assays monitoring agonist-induced recruitment of the intracellular βarr2 protein to the activated receptor were used. The concept is based on the NanoLuc Binary Technology (NanoBiT®, Promega), monitoring the interaction between one inactive subunit of a nanoluciferase fused to the receptor, and the other subunit, fused to βarr2. Receptor activation by a ligand results in the recruitment of βarr2, bringing the two subunits in close proximity, resulting in functional complementation of the enzyme. In the presence of the furimazine substrate, measurable bioluminescence is generated. The development of the system and the establishment of the stable cell lines used for these assays has been reported before[44–46].

Human embryonic kidney (HEK) 293T stably expressing the CB_1-βarr2 or CB_2-βarr2 system were routinely maintained at 37 °C, 5% CO_2 under humidified atmosphere in DMEM (GlutaMAX™), supplemented with 10% heat-inactivated FBS, 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B.

Stock solutions were made in MeOH. Working solutions were prepared by serial dilution in Opti-MEM containing 50% MeOH and were used within 24 hours upon preparation.

On the day prior to the assay, cells were seeded in poly-D-lysine coated white opaque-walled 96-well plates at approximately 50,000 cells per well and left to incubate overnight. Next, to remove residual traces of serum that could potentially interfere with

protein interactions during the assay, cells were rinsed twice with 150 µL of Opti-MEM I Reduced Serum and 100 µL of this medium was added to each well. The Nano-Glo® Live Cell reagent, a non-lytic cell reagent containing the furimazine substrate, was diluted 20-fold in LCS buffer and 25 µL of this mix was added to each well. The plate was then placed in the TriStar^2 LB 942 Multimode Microplate Reader (Berthold Technologies GmbH & Co., Germany) and luminescence was monitored for 10-15 min during an initial equilibration phase, which will later be used to correct for *inter-well* variability. Upon stabilization of the signal, 10 µL of a 13.5x concentrated stock solution was added and luminescence was measured during 2 hours. A concentration range of the reference compound CP55,940 and appropriate solvent controls for the analyzed compounds were included on each plate. CP55,940 was selected as a reference since it was previously used for the characterization of MDA-19 and structural analogs[26,27]. To allow for a better comparison with earlier work, JWH-018 was also taken along, as it has often served as a reference compound in the used bioassays[47–49]. All test concentrations were run in duplicate in minimally 3 independent experiments.

Data analysis and statistical analyses

Raw data was processed using Microsoft Excel 2019, followed by curve fitting and statistical analysis using the GraphPad Prism software (Version 9.3.0) (San Diego, CA, USA). Firstly, to correct for *inter-well* variability, a baseline correction was performed on the absolute luminescence values, using data generated during the equilibration period. Then, for each compound, the mean area under the curve (AUC) was calculated. A blank correction was performed by subtracting AUC values of the solvent

controls. Results represent the AUC ± standard error of mean (SEM) and were obtained by normalizing to the E_{max} of the reference compound CP55,940, arbitrarily set at 100%. Data points were consistently excluded for the highest concentration in case of a signal reduction of 20% or more compared to the next dilution, as this could potentially be a sign of cell toxicity or solubility issues at higher concentrations. Potency and efficacy were assessed by calculating pharmacological parameters EC_{50} and E_{max} by curve fitting the obtained concentration-response curves via nonlinear regression (three-parameter logistic fit). Outliers were detected using the Grubbs test and omitted from the dataset if applicable (p-value < 0.05; applicable for 1 out of 958 data points).

Receptor selectivity was evaluated and quantitated using an intrinsic relative activity-based method, commonly employed to calculate pathway bias[48,50–52]. For each test compound the intrinsic relative activity (RA_i) was calculated using Equation (1) for both CB_1 and CB_2, where "A" represents the compound and "CP" represents the reference compound CP55,940. The latter was appropriate to use for this selectivity determination as it is considered a non-selective cannabinoid agonist[53].

$$RA_i = \frac{E_{max,\,A} \times EC_{50,\,CP}}{EC_{50,\,A} \times E_{max,\,CP}} \quad (1)$$

Both RA_i values were incorporated in Equation (2), yielding a numerical 'receptor selectivity factor', calculated to assess a potential preference towards either cannabinoid receptor.

$$\text{Receptor selectivity} = \text{Log}\left(\frac{RA_i^{CB_1}}{RA_i^{CB_2}}\right)$$

(2)

Analytical characterization of the OXIZID standards and the seized BZO-4en-POXIZID

The reference standards of BZO-HEXOXIZID, BZO-POXIZID, 5F-BZO-POXIZID, BZO-4en-POXIZID and BZO-CHMOXIZID and the seized powder were analytically characterized via high-performance liquid chromatography coupled to diode-array detection (HPLC-DAD), gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to time-of-flight mass spectrometry (LC-QTOF-MS) as described before[54]. The obtained spectra are provided in Supplementary Data. For the BZO-4en-POXIZID powder also Fourier Transform Infrared Spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR) was carried out. A short summary of each technique is provided below.

High-Performance Liquid Chromatography Coupled to Diode-Array Detection (HPLC-DAD)

Reversed-phase separation of the sample was performed on a LaChrom HPLC system from Merck-Hitachi (Tokyo, Japan), using a Merck Purospher® Star RP-8 endcapped column (5 µm, 125 mm x 4.6 mm) with a Merck Purospher® Star RP-8 endcapped guard column (5 µm, 4 mm x 4 mm). A diode-array detector was used to monitor a wavelength from 220 to 350 nm with a slit of 1 nm, a spectral bandwidth of 1 nm, and a spectral interval of 200 ms. The selected wavelength, used to display the chromatographic trace, was 230 nm. A total of ~1 µg was injected onto the column (50 µL). For more detailed settings, the reader is referred to Supplementary Data.

Gas Chromatography – Mass Spectrometry (GC-MS)

One µL of a 1 mg/mL solution was injected on an Agilent 7890A GC system coupled to a 5975 XL mass-selective detector operated by MSD Chemstation software. A 30 m x 0.25 mm i.d. x 0.25 µm Agilent HP-5-MS column was used. Splitless injections were performed automatically at an injection temperature of 250 °C and purge time of 1 minute, with helium as a carrier gas at constant flow rate (1 mL/min). The temperature program started at 80 °C for 1 min, followed by an increase at 20 °C/min to 200 °C. The temperature was then raised by 4 °C/min to 260 °C and by 30 °C/min to 300 °C, which was held for an additional 8 min. Transfer line temperature and ion source temperature were set at 300 and 230 °C, respectively. The MS quadrupole temperature was set at 150 °C and an ionization energy of 70 eV was used. The mass spectrometer operated in SCAN-mode, scanning a range of 50 to 700 *m/z*.

Liquid Chromatography Coupled to Time-of-Flight Mass Spectrometry (LC-QTOF-MS)

Chromatographic separation was performed using an Agilent 1290 Infinity LC system equipped with a Phenomenex Kinetex C18-column (2.6 µm, 3 x 50 mm), maintained at 30 °C. The high resolution mass spectrometry (HRMS) system used was a 5600+ QTOF with an electrospray ionization (ESI) source (Sciex). Upon selection of the parent compound in the quadrupole (based on mass-to-charge ratio), fragmentation occurs in the collision cell (collision energy: 35 V). Sciex Analyst TF 1.7.1 software was used to steer the system. Exact settings were the same as reported before[54,55], and

resulted in a TOF-MS full scan combined with a data dependent acquisition of product ion spectra. For more detailed settings, the reader is referred to Supplementary Data.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR-analysis was performed directly on the powder as received, using an Alpha-FTIR instrument from Bruker (Billerica, MA, US), equipped with an attenuated total reflection (ATR)-unit. A series of 24 scans were recorded in the 400-4000 cm^{-1} wave number range, with a resolution of 4 cm^{-1}.

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR analyses were performed as previously described[56]. NMR spectra were acquired on a Bruker (Rheinstetten, Germany) spectrometer Avance II HD 600 (nominal proton frequency 600.13 MHz), equipped with a 5 mm QCI cryo-probe (^{1}H, ^{13}C, ^{15}N and ^{19}F), in DMSO-d_6 solvent at 300 K. ^{1}H and ^{13}C NMR chemical shifts are expressed in δ scale (ppm) and referenced to the solvent (DMSO-d_6) residuals, at 2.50 ppm and 39.52 ppm respectively. The seized BZO-4en-POXIZID powder was characterized by one-dimensional ^{1}H, ^{13}C and APT as well as $^{1}H/^{1}H$ COSY, $^{1}H/^{1}H$ TOCSY,$^{1}H/^{13}C$ HMBC and $^{15}N/^{1}H$ HMBC experiments.

RESULTS AND DISCUSSION

The activity of the novel SCRA BZO-HEXOXIZID and 4 structural analogs was evaluated using 2 similar bioassays based on the NanoBiT® technique, monitoring the recruitment of βarr2 to either CB_1 or CB_2, upon receptor activation. This event typically results in desensitization and internalization of the receptor, thereby preventing further G protein-mediated downstream signaling. Compared to other, commercialized, βarr2 recruitment assays, such as the PathHunter® assay (Discoverx) or Tango™ assay (ThermoFisher Scientific) which only allow for one end-point measurement, the NanoBiT® assay output covers a 2-hour luminescence measurement period, taking into account the complete receptor activation profile for further calculations[57]. EC_{50} values, representing potency, and E_{max} values, representing efficacy, are depicted in Table 2. Concentration-response curves of the compounds can be found in Figure 3.

Structure-activity relationship at CB_1 and CB_2

At CB_1, all tested compounds were found to be full agonists in comparison with the reference compound CP55,940, with E_{max} values exceeding 100%. On the other hand, all compounds behaved as partial agonists at CB_2, compared to the reference, with relative efficacies ranging from 35.0 to 69.2%.

In both assays, BZO-HEXOXIZID exhibited the lowest potency and efficacy of the analyzed set. The EC_{50} and E_{max} values found in the CB_1-βarr2 assay were 721 nM and 165%, respectively, while at CB_2, an EC_{50} of 25.9 nM and E_{max} of 35.0% was calculated. Shortening the n-hexyl tail to an n-pentyl tail resulted in a substantial increase in both potency and relative efficacy at CB_1, with BZO-POXIZID showing an

EC_{50} value of 244 nM and E_{max} value of 686%. Its terminally fluorinated counterpart 5F-BZO-POXIZID showed very similar activation profiles, with an EC_{50} of 226 nM and an E_{max} of 731%. Our data are in line with those reported by Diaz *et al.*, who, using a [^{35}S]GTP-γ-S assay, compared the CB_1 activation potential of BZO-HEXOXIZID with that of BZO-POXIZID and also observed a higher functional activity for the latter[26]. The absence of an impact of fluorination on intrinsic CB_1 activation potential is in line with previous findings observed for CUMYL-PEGACLONE and its 5F analog[48]. Looking at CB_2 activation, a slight increase in potency can be noticed for 5F-BZO-POXIZID (EC_{50} = 4.11 nM) relative to BZO-POXIZID (EC_{50} = 12.2 nM), although this difference is relatively minor. Relative efficacies at CB_2 of both analogs were also in the same order of magnitude, i.e. 51.7% for 5F-BZO-POXIZID and 59.8% for BZO-POXIZID.

Furthermore, the CB_1 activation data suggest that the presence of a double bond in the pentyl tail, as present in BZO-4en-POXIZID, negatively impacted both the potency and efficacy (EC_{50} = 532 nM, E_{max} = 399%), relative to BZO-POXIZID. Interestingly, at CB_2 this negative impact could not be demonstrated, with very similar EC_{50} and E_{max} values for BZO-4en-POXIZID and BZO-POXIZID (12.6 *vs.* 12.2 nM and 54.1% *vs.* 59.8%, respectively). However, this decrease in activity is not unequivocally reflected in literature. When comparing JWH-018 with its unsaturated analog JWH-022, the latter was found to be more potent *in vivo*, as demonstrated via monitoring of antinociception, hypomobility, hypothermia, catalepsy in mice and discriminative stimulus effects in rats, indicating that a pentenyl tail does not universally have a negative effect on cannabinoid activity[58]. Furthermore, using our CB_1-βarr2 assay, we

previously found that the unsaturated MDMB-4en-PICA had roughly the same potency and efficacy at CB_1 (EC_{50} = 3.70 nM, E_{max} = 289%) as its fluorinated, saturated analog 5F-MDMB-PICA (EC520 = 2.13 nM, E_{max} = 289%)[59].

The most potent OXIZID SCRA of this set in terms of both CB_1 and CB_2 activation was BZO-CHMOXIZID, with EC_{50} values of 84.6 nM and 2.21 nM, respectively. With an E_{max} of 716% compared to the reference, its efficacy at CB_1 lies within the same range of that of BZO-POXIZID and 5F-BZO-POXIZID. The E_{max} value obtained at CB_2 was 69.2%, which ranks it amongst the most efficacious SCRAs of this set. These findings align well with those of Diaz. *et al*, who reported that replacing the aliphatic tail of BZO-HEXOXIZID (MDA-19) by a cyclohexyl methyl resulted in an important increase in activity at both receptors in a [^{35}S]GTP-γ-S assay, yielding the most potent compound of the analyzed set[26]. Looking at other SCRAs, however, this is not a consistent finding. For instance, comparing the CB_1 activity of the assumed SCRA intermediate NNL-3 (HOBt-5F-P7AIC, carrying a fluoro pentyl tail), with its defluorinated (HOBt-P7AIC) or its cyclohexyl methyl (HOBt-CHM7AIC) analog, we noticed a dramatic decrease in activity for the HOBt-CHM7AIC, while both pentyl analogs had a quite similar activation profile[60]. Furthermore, when comparing 2 L-valine SCRAs at CB_1, replacement of the fluoro pentyl tail in 5F-AB-PINACA (EC_{50} = 55.4 nM) by a cyclohexyl methyl moiety in AB-CHMINACA (EC_{50} = 3.45 nM) did yield a more potent compound, while the *tert*-leucine analogs 5F-MDMB-PINACA (EC_{50} = 0.84 nM) and MDMB-CHMINACA (EC_{50} = 0.78 nM) had essentially the same potency at CB_1[49].

It is interesting to highlight that, although we found these compounds to exhibit a broad range of intrinsic activities at CB_1, Diaz *et al*. did not observe large differences in binding affinity of BZO-HEXOXIZID, BZO-POXIZID and BZO-CHMOXIZID for both cannabinoid receptors, as evaluated using radioligand binding assays[26]. This emphasizes the fact that the differences in activities, as demonstrated in our bioassays, are most likely not the consequence of different receptor affinities, but rather the result of other or better interactions with residues inside the binding pocket of the receptors. Compared to the efficacies obtained using the CB_1-βarr2 bioassay, E_{max} values for the CB_2 receptor are less divergent, which is in agreement with past analyses in which we have consistently noticed a more clustered profile for CB_2. To this day, the underlying reason for this has not been elucidated.

In summary, a general trend could be noticed regarding the impact of the tail of these OXIZID SCRAs on potency and relative efficacy at CB_1. BZO-HEXOXIZID, carrying a hexyl tail, had the lowest activity, followed by the 4-pentenyl analog BZO-4en-POXIZID. While the saturated BZO-POXIZID and its fluoro pentyl analog 5F-BZO-POXIZID were more potent and efficacious, the lowest EC_{50} value (and hence highest potency) was observed for BZO-CHMOXIZID, the only SCRA in this set carrying a cyclic tail. Overall, at CB_2, the same rank order in terms of potency was applicable, albeit less distinct.

Assessment of cannabinoid receptor selectivity

BZO-HEXOXIZID was originally selected as a potential lead compound in the search for new therapeutics for neuropathic pain, based on its potency at CB_2 (63.4 nM), its

CB_2 selectivity and its only moderate potency at CB_1, as assessed by means of a [^{35}S] guanosine-5'-triphosphate (GTP)-γ-S assay[26]. To further investigate the receptor preference (CB_1 *vs.* CB_2) of BZO-HEXOXIZID and its analogs, two methods were implemented. First, in line with the method applied by Banister *et al.*, the ratio of EC_{50} values (CB_1/CB_2) was calculated[61]. A higher value reflects a larger fold difference between both EC_{50} values, indicative of a more CB_2 selective compound. Second, and more elaborately, a numerical value, similar to a bias factor, was calculated. This calculation entails the relative intrinsic activity (RA_i), which takes into account both the EC_{50} (potency) and E_{max} (efficacy) value of a compound at the 2 cannabinoid receptors. As this method includes both pharmacological parameters (potency and efficacy) in the equation and therefore considers multiple aspects of CB_1 and CB_2 activation, it may be a more comprehensive and complete approach to evaluate receptor selectivity. Values below 0 indicate a preference towards CB_2 and therefore a potential CB_2 selectivity.

Implementing the EC_{50} ratio method, all SCRAs exhibited a clear CB_2 selectivity (20.0-55.0), compared to CP55,940 (1.41) and JWH-018 (3.53). The same conclusion could be drawn from the bias formula method, assigning a preference towards CB_2 activation for all compounds, except for the n-pentyl analog BZO-POXIZID. In fact, BZO-POXIZID was found to be the least CB_2 selective using both calculation methods. Taken together, the CB activation profile of most OXIZID compounds somewhat resembles that of XLR-11, a SCRA found in authentic urine samples of drug users in 2017, which also demonstrated a CB_2 preference in our bioassay[45]. Overall, the assessment of

receptor selectivity based on the formula also implemented for bias calculation seems to present a clearer view on the selective behaviour of these substances, as differences appear to be more pronounced compared to the somewhat clustered EC_{50} ratios.

Analytical characterization of a seized powder containing BZO-4en-POXIZID

A yellow powder, in which the presence of BZO-4en-POXIZID was demonstrated, was intercepted by the Belgian Customs in November 2021. This powder was characterized alongside the reference standard for BZO-4en-POXZID using HPLC-DAD, GC-MS and LC-QTOF-MS, as well as with FTIR and NMR, which confirmed the identity of the powder (Supplementary Data), with no impurities being detected. This powder was also analyzed alongside the BZO-4en-POXIZID reference standard for its activity in the CB_1 and CB_2 bioassays. Panel C and D of Figure 3 illustrate the similar activation profiles for the BZO-4en-POXIZID powder and the reference standard. Given the comparable potency and efficacy at both receptors, (EC_{50} 512 nM, E_{max} 318% for the powder *vs*. EC_{50} 532 nM, E_{max} 399% for the BZO-4en-POXIZID standard at CB_1), a high level of purity of the powder can be assumed, in line with the analytical characterization.

Analytical characterization of reference standards of a panel of OXIZID SCRAs

Similar to the analysis of the seized sample, reference standards of BZO-HEXOXIZID, BZO-POXIZID, 5F-BZO-POXIZID, BZO-4en-POXIZID and BZO-CHMOXIZID were characterized using HPLC-DAD, GC-MS and LC-QTOF-MS. Results were in line with findings reported by Liu et al.[19], who characterized BZO-POXIZID and 5F-BZO-POXIZID using GC-MS and QTOF-MS. For BZO-HEXOXIZID and BZO-CHMOXIZID,

results were in accordance with the analytical reports distributed by the CFSRE[35,38]. Chromatograms and mass spectra can be found in Supplementary Data.

Conclusion

This study is the first to report on the *in vitro* intrinsic receptor activation potential at CB_1 and CB_2 of the newly emerging SCRA BZO-HEXOXIZID and 4 structural analogs. Using two live cell-based βarr2 recruitment assays, all compounds were found to be full agonists at CB_1, with efficacies ranging from 165 – 731 % compared to CP55,940, and with potencies (EC_{50}) ranging from 84.6 nM (BZO-CHMOXIZID) to 721 nM (BZO-HEXOXIZID), all being less potent than CP55,940. The n-hexyl analog BZO-HEXOXIZID (also known in literature as MDA-19) had the lowest potency and efficacy, followed by the pentenyl analog BZO-4en-POXIZID. Shortening the n-hexyl tail resulted in an important increase in CB_1 activation potential. The pentyl and fluoro pentyl analogs BZO-POXIZID and 5F-BZO-POXIZID exhibited higher but quite similar potencies, demonstrating that the addition of a fluorine atom did not have a major impact on CB_1 activation. The most potent SCRA of the investigated set was the cyclohexyl methyl analog BZO-CHMOXIZID, which had a relative efficacy within the same range as that of BZO-POXIZID and 5F-BZO-POXIZID. Overall, the same general trend and rank order regarding potency was seen at CB_2, although differences were less pronounced. More specifically, the negative impact of an unsaturated hydrocarbon tail was not observed at CB_2. All OXIZIDs showed a clear preference for CB_2, compared to CP55,940. Given the rather moderate potencies found for these compounds at CB_1, it is premature to predict whether they will pose extensive

cannabinoid related toxicity. However, these findings may be of value for drug policy makers and health care workers, as they give an idea on the pharmacology of these newly emerging SCRAs and may hint at substances that could potentially appear in the future. Depending on multiple variables such as ease of synthesis, price and availability, it still remains to be seen whether and to what extent this new class will 'take off' on the recreational drug market.

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Tables

Table 1: Comparison of different nomenclature for the discussed substances. Table based on the Public Health Alert Report, prepared by NPS Discovery (CFSRE) and Cayman Chemical, and released on August 31, 2021[18].

Initial naming	Synonyms	IUPAC naming	New systematic naming
MDA-19	MDA19 MDA 19	(Z)-*N*-(1-hexyl-2-oxoindolin-3-ylidene)benzohydrazide	BZO-HEXOXIZID
Pentyl MDA-19	5C-MDA-19 MDA-19 pentyl analog	(Z)-*N*-(1-pentyl-2-oxoindolin-3-ylidene)benzohydrazide	BZO-POXIZID
5F-MDA-19	MDA-19 5-fluoropentyl analog	(Z)-*N*-(1-(5-fluoropentyl-2-oxoindolin-3-ylidene) benzohydrazide	5F-BZO-POXIZID
4en-pentyl MDA-19		(Z)-N'-(2-oxo-1-(pent-4-en-1-yl)indolin-3-ylidene) benzohydrazide	BZO-4en-POXIZID[62]
CHM-MDA-19	Cyclohexylmethyl MDA-19	(Z)-*N*-(1-(cyclohexylmethyl)-2-oxoindolin-3-ylidene) benzohydrazide	BZO-CHMOXIZID

Table 2: Potency (EC_{50}) and efficacy (E_{max} relative to CP55,940) values and assessment of cannabinoid receptor selectivity of BZO-HEXOXIZID and analogs at either the CB_1 of CB_2 receptor.

Compound	CB_1		CB_2		Ratio of potencies CB_1/CB_2	Receptor selectivity[a] (CB_1/CB_2)
	EC_{50} (nM) (95% CI)	E_{max} (%) (95% CI)	EC_{50} (nM) (95% CI)	E_{max} (%) (95% CI)		
BZO-HEXOXIZID	**721** (428 – 1192)	**165** (149 – 180)	**25.9** (10.0 – 67.5)	**35.0** (31.0 – 39.1)	27.8	-0.62
BZO-POXIZID	**244** (142-420)	**686** (609 – 768)	**12.2** (3.95 – 40.1)	**59.8** (51.8 – 68.2)	20.0	-0.09
5F-BZO-POXIZID	**226** (136 – 378)	**731** (657– 810)	**4.11** (0.86 – 18.0)	**51.7** (40.9 – 63.5)	55.0	-0.44
BZO-4en-POXIZID	**532** (227 – 1192)	**399** (328 – 480)	**12.6** (2.53 – 63.1)	**54.1** (43.3 – 65.7)	42.2	-0.61
Seized powder BZO-4en-POXIZID	***521*** *(300 – 882)*	***318*** *(280 – 359)*	***14.5*** *(2.20 – 97.7)*	***54.1*** *(41.5 – 67.9)*	*35.9*	*-0.64*
BZO-CHMOXIZID	**84.6** (23 – 275)	**716** (566 – 876)	**2.21** (0.72 – 7.03)	**69.2** (59.7 – 79.2)	38.3	-0.42
JWH-018	**23.9** (11.3 – 52.9)	**340** (306 – 376)	**6.78** (2.93 – 14.9)	**74.0** (66.7 – 81.4)	3.53	-0.27
CP55,940	**0.69** (0.25 – 1.74)	**99.7** (87.5 – 112)	**0.49** (0.16 - 1.37)	**100** (87.4 - 113)	1.41	0

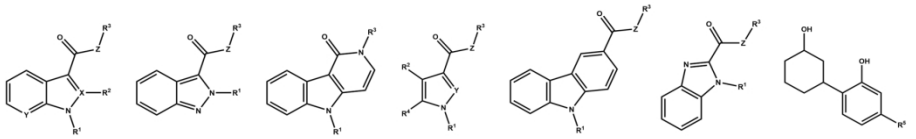
[a]Receptor selectivity calculated in a way similar to bias calculation, using relative intrinsic activities.

Figure captions

Figure 1: Overview of the 7 general SCRA scaffolds covered by the generic control measure in China, in effect as of July 1st, 2021. Figures based on the official announcement document, released by the Office of China National Narcotics Control Commission on May 12th, 2021[17].

Figure 2: Chemical structures of the OXIZIDs evaluated in this report, together with the reference compound CP55,940 and the prototypic SCRA JWH-018. Structures were made with the ChemDraw 19 Professional software.

Figure 3: Activation profiles obtained for BZO-HEXOXIZID and analogs, JWH-018 and reference compound CP55,940 at the CB_1 receptor (Panel A) and the CB_2 receptor (Panel B). Panel C and D depict activation profiles at the CB_1 receptor (C) and CB_2 receptor (D) of the seized BZO-4en-POXIZID powder, compared to the standard of the BZO-4en-POXIZID, JWH-018 and the reference compound CP55,940. Each datapoint represents the mean ± standard error of the mean (SEM). All data was normalized to the maximal response of CP55,940, arbitrarily set at 100%.



- R^1 represents C_3-C_8 alkyl group whether or not substituted; or heterocyclic group containing 1-3 heteroatoms whether or not substituted; or methyl or ethyl substituted by a heterocyclic group containing 1-3 heteroatoms whether or not substituted.
- R^2 represents a hydrogen atom or methyl group or no atom
- R^3 represents a C_6-C_{10} aryl group whether or not substituted; or a C_3-C_{10} alkyl group whether or not substituted; or a heterocyclic group containing 1-3 heteroatoms whether or not substituted; or a methyl or ethyl group substituted by a heterocyclic group containing 1-3 heteroatoms whether or not substituted
- R^4 represents a hydrogen atom; phenyl group whether or not substituted; benzyl group whether or not substituted
- R^5 represents a C_3-C_{10} alkyl group whether or not substituted
- X represents N or C
- Y represents N or CH
- Z represents O or NH or no atom

Figure 1: Overview of the 7 general SCRA scaffolds covered by the generic control measure in China, in effect as of July 1st, 2021. Figures based on the official announcement document, released by the Office of China National Narcotics Control Commission on May 12th, 2021[15].

338x140mm (150 x 150 DPI)

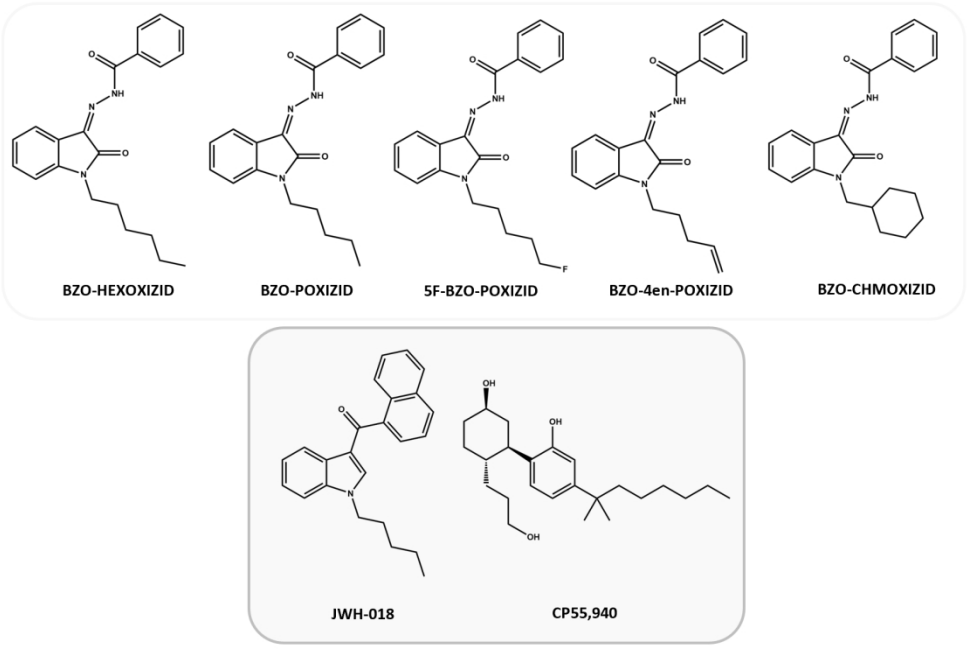


Figure 2: Chemical structures of the OXIZIDs evaluated in this report, together with the reference compound CP55,940 and the prototypic SCRA JWH-018. Structures were made with the ChemDraw 19 Professional software.

144x96mm (330 x 330 DPI)

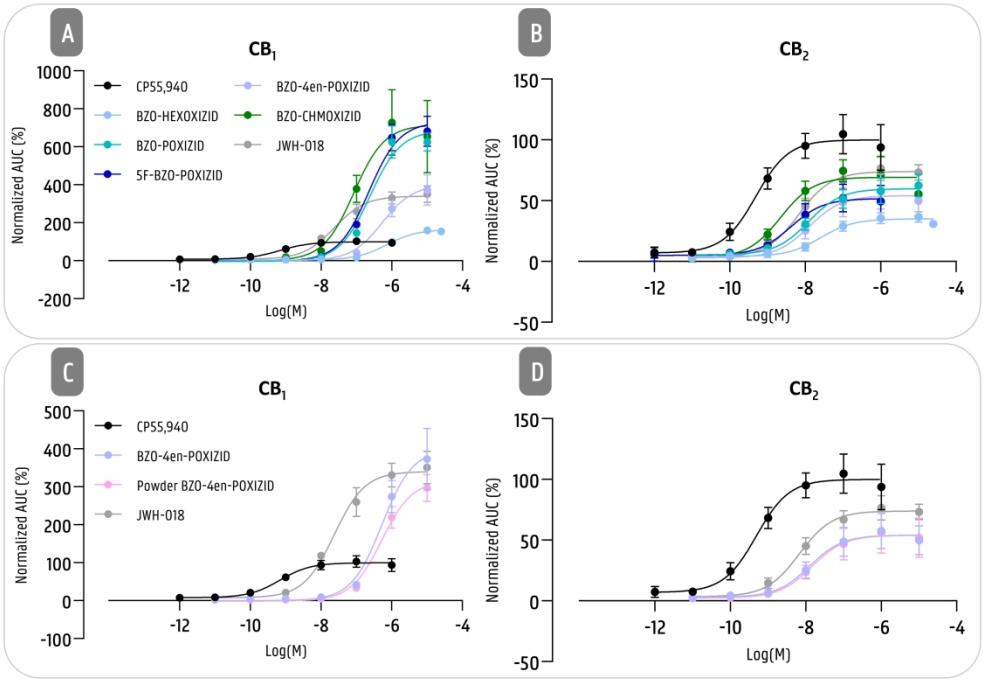


Figure 3: Activation profiles obtained for BZO-HEXOXIZID and analogs, JWH-018 and reference compound CP55,940 at the CB_1 receptor (Panel A) and the CB_2 receptor (Panel B). Panel C and D depict activation profiles at the CB_1 receptor (C) and CB_2 receptor (D) of the seized BZO-4en-POXIZID powder, compared to the standard of the BZO-4en-POXIZID, JWH-018 and the reference compound CP55,940. Each datapoint represents the mean ± standard error of the mean (SEM). All data was normalized to the maximal response of CP55,940, arbitrarily set at 100%.

298x207mm (330 x 330 DPI)