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Kinetic multiplex assay to assess biased signaling of clinical GPCR agonists

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HU910 (PubChem CID: 67207173)
JWH133 (PubChem CID: 6918505)
CP55,940 (PubChem CID: 104895)
Anandamide (PubChem CID: 5281969)
2-AG (PubChem CID: 5282280)
Dronabinol (PubChem CID: 16078)
Nabilone (PubChem CID: 5284592)
Cannabidiol (PubChem CID: 644019)
Lenabasum (PubChem CID: 3083542)
Olorinab (PubChem CID: 60164925)
CMX-020 (PubChem CID: 68316721)
Cannabinol (PubChem CID: 2543)
KN387271 (PubChem CID: 9845561)
GW842166X (PubChem CID: 10253143)
S-777469 (PubChem CID: 865880)
LY-2828360 (PubChem CID: 46833780)
PRS-211375 (PubChem CID: 10174045)
ART-27.13 (PubChem CID: 11675994)
EHP-101 (PubChem CID: 118465221)
Tedralinab (PubChem CID: 67029278)
TAK937 (PubChem CID: 49869061)

ABSTRACT

Studying biased signaling of G protein-coupled receptors (GPCRs) holds promise for the identification of ligands with a better therapeutic window. However, proper examination of biased signaling remains challenging by risking introduction of system or observation bias. Therefore, we developed a novel multiplex assay that simultaneously and kinetically detects cAMP production and β-arrestin-2 recruitment in the same well. To investigate the applicability of the kinetic multiplex assay, we profiled seventeen clinically tested agonists for the cannabinoid CB₂ receptor (CB₂R), a promising GPCR for treating tissue injury and inflammation. Agonist-mediated CB₂R activation and signaling was time sensitive, dependent on the agonist. Similar potency and efficacy parameters were obtained from semi-kinetic and kinetic analysis, while the latter provided additional signaling rate constants for signaling onset (k_1) and decline (k_2). Fast CB₂R engagement (k_{on}) of agonists resulted in increased affinity and potency. Slow dissociation of agonists extended the interaction between CB₂R and β-arrestin-2. Moreover, superagonists Tedalinab, Olorinab, PRS-211375 and ART-27.13 were characterized by fast k_1 values. No significant biased signaling was observed for the investigated CB₂R agonists. To validate the assay, the reported biased compound JWH145 was tested in a multiplex assay developed for CB₁R and confirmed that our new method is able to detect bias. Altogether, this study accentuates the potential of multiplexing functional responses and performing kinetic analyses to provide an extensive preclinical profile for agonists that may better predict their *in vivo* pharmacological effects. Ultimately, providing full kinetic context for binding and signaling of GPCR agonists could advance drug discovery efforts.

Abbreviations: Δ⁹-THC, Δ⁹-tetrahydrocannabinol; 2-AG, 2-arachidonoylglycerol; AEA, anandamide; AUC, area under the curve; BCA, bicinchoninic acid; CB₁R, cannabinoid CB₁ receptor; CB₂R, cannabinoid CB₂ receptor; CBD, cannabidiol; CBN, cannabinol; CHO, Chinese Hamster Ovary; DTT, DL-dithiothreitol; ECB, endocannabinoid; ET, engagement time; FCS, fetal calf serum; FSK, Forskolin; GPCR, G protein-coupled receptors; HEK293T, human embryonic kidney 293 T; IR, initial rate; NSB, nonspecific binding; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; Rt, room temperature; RT, residence time; SSR, steady-state response; TB, total binding.

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1. Introduction

Biased signaling, also known as ligand bias, biased agonism or functional selectivity, describes the ability of a ligand to preferentially activate one pathway over another by stabilizing distinct conformations of the receptor [1–4]. Bias on G protein-coupled receptors (GPCRs) is most frequently observed between G proteins and β -arrestins but may also occur amongst the G protein or β -arrestin family subtypes [5,6]. Consequently, activation of distinct pathways by an agonist may lead to different biological effects. Therefore, studying biased signaling may provide the potential to increase the therapeutic window if a specific signaling pathway is associated with efficacy, while another with side effects [1–4,7]. Recently, this was exemplified by the FDA approval of the first GPCR biased agonist, oliceridine, for the μ -opioid receptor to treat moderate to severe acute pain in adults [8]. G protein signaling after μ -opioid receptor activation improved antinociception, while β -arrestin recruitment was related to adverse effects such as respiratory repression [9].

The *in vitro* study of biased signaling, however, is challenging and influenced by many system-dependent and experimental factors [1,10,11]. Different receptor, transducer or effector levels, or stoichiometry between these components in various cellular backgrounds may introduce system bias [1,12]. Furthermore, interpretation of bias is dependent on the choice of reference ligand, such as a tool compound to report 'ligand benchmark-bias' or the endogenous ligand for quantification of 'ligand physiology-bias'. A tool compound with a balanced profile across studied pathways allows for the identification of 'ligand pathway-bias' [1]. Moreover, most commonly used functional assays are employed as endpoint assays, i.e., measured at one specific time point. However, agonist-mediated receptor activation may change over time and could be influenced by the dynamics and kinetics of the ligand-receptor interaction [1,10]. The importance of kinetic context for biased signaling was first highlighted in a study on the dopamine D₂ receptor, which demonstrated that the direction of ligand bias changed over time and conclusions on bias were therefore highly dependent on the selected time point [13]. In addition, biased agonists displayed a longer residence time (RT) at the receptor compared to the endogenous agonist [13]. Functional parameters have previously been correlated to the target-binding kinetics of agonists at several GPCRs [14–16]. Important developments in the field such as the TRUPATH platform, which allows for screening of biased G protein signaling in separate assays, are made to include the kinetic context of signaling [17,18]. Nevertheless, the majority of studies that report on biased signaling are performed with separate cellular assays for the different signaling pathways, use endpoint assays and/or do not quantify the kinetic signaling traces with fit-for-purpose mathematical analyses. Thus, a novel design of *in vitro* assays and methods of data analysis to report functional parameters in conjunction with kinetic parameters, such as binding kinetics and signaling constants, is warranted to study the *in vitro* pharmacology profile of candidate drugs [19,20].

Targeting of cannabinoid CB₂ receptor (CB₂R) has demonstrated great potential in preclinical studies for the treatment of autoimmune conditions, pain and diseases with an inflammatory component. This receptor is predominantly expressed on immune cells and plays a key role in the regulation of several inflammatory processes via activation by the endocannabinoids (eCBs) anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [21–25]. To date, the focus has been on activation of CB₂R to enhance immunosuppressive effects [25,26]. A recent review by Brennecke *et al.* described at least twenty CB₂R agonists that have progressed to clinical development [25]. Of these, three are currently on the market that are described to non-selectively activate CB₂R: Dronabinol, a synthetic Δ^9 -tetrahydrocannabinol (Δ^9 -THC); Nabilone, a synthetic Δ^9 -THC analogue and cannabidiol (CBD) (Fig. 1, Table S1) [21,25]. Additionally, combinations of THC and CBD have been approved for the treatment of multiple sclerosis-associated spasticity [27]. Of note, we recently demonstrated that CBD does not bind or activate CB₂R, and the

therapeutic effect might be due to polypharmacology at other proteins [28,29]. Five CB₂R agonists are currently still in clinical trials, whereas the development of the remaining agonists has been discontinued due to a variety of reasons [25]. Several issues have been hypothesized to explain the poor preclinical to clinical translation, including, but not limited to, a lack of translational animal models or differential signaling bias at the receptor level [25,30].

In various studies, biased signaling of diverse agonists and inverse agonists at cannabinoid receptors has been investigated [31–38]. For CB₁R, biased ligands affecting cAMP production and β -arrestin-2 recruitment have been identified including synthetic ligand JWH145, which exhibited full agonism in inhibiting cAMP production while showing no activity in β -arrestin-2 recruitment [33]. A large consensus study on CB₂R combined the results from several independent academic and industry laboratories for a well-defined set of commonly used CBR ligands [35]. All ligands were screened on activation in five separate *in vitro* endpoint assays, i.e., GTP γ S binding, cAMP production, β -arrestin-2 recruitment, pERK stimulation and GIRK activation. The most prominent bias at CB₂R was found between inhibition of cAMP production and β -arrestin-2 recruitment, although bias factors remained low compared to other GPCRs. Furthermore, agonists HU308, HU910 and JWH133 were most balanced between all pathways and were thus recommended by the authors for use in further bias studies. To the best of our knowledge, bias at CB₂R has not been studied with a focus on the kinetic context and in general little is known about the binding kinetics of CB₂R agonists.

Here, we aimed to develop a novel multiplex assay that simultaneously detects cAMP production and β -arrestin-2 recruitment after receptor activation in a time-dependent manner in a single well. Seventeen CB₂R agonists that were examined in clinical trials were screened to investigate the applicability of this novel multiplex assay (Fig. 1, Table S1). These agonists encompassed various chemotypes, clinical development phases and indications (Fig. 1, Table S1). Furthermore, we included four widely used preclinical agonists and the endocannabinoids as benchmarks. Affinity and binding kinetics of the agonists were determined to ensure interaction with CB₂R and to obtain a more complete preclinical profile of the agonists. We explored the time-dependency of receptor activation by use of various methods of data analysis for the multiplex assay, i.e., endpoint, semi-kinetic and kinetic. In our novel multiplex system, we did not detect statistically significant bias for any of the CB₂R agonists relative to HU308. However, small differences were observed in trends for bias signaling, which were in some cases dependent on the method of analysis. For proof-of-concept we applied our multiplex system to CB₁R to qualitatively show our multiplex system is able to detect biased signaling. Altogether, this study highlights and provides the potential of multiplexing functional readouts and including a kinetic view to CB₂R drug discovery programs, which can be extended to other GPCRs for an improved screening of biased signaling.

2. Materials and methods

2.1. Chemical and reagents

RO6957022 and [³H]RO6957022 (specific activity 82.83 Ci mmol⁻¹) were synthesized in-house as previously published [39]. [³H]CP55,940 (specific activity 108.5 Ci mmol⁻¹ #NET1051250UC), [³⁵S]GTP γ S (specific activity 1250 Ci mmol⁻¹ #NEG030H250UC), and GF/C filter plates (#6055690) were purchased from Revvity (Waltham, MA, USA). Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL, USA). CP55,940 (#C1112), AM630 (#SML0327), and DL-dithiothreitol (DTT, #646563) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Tedalinab (#T013730) and forskolin (FSK, #F701800) were from Toronto Research Chemicals. Δ^9 -THC (#12068), LY-2828360 (#26791) and JWH145 (#10825) from Cayman Chemical

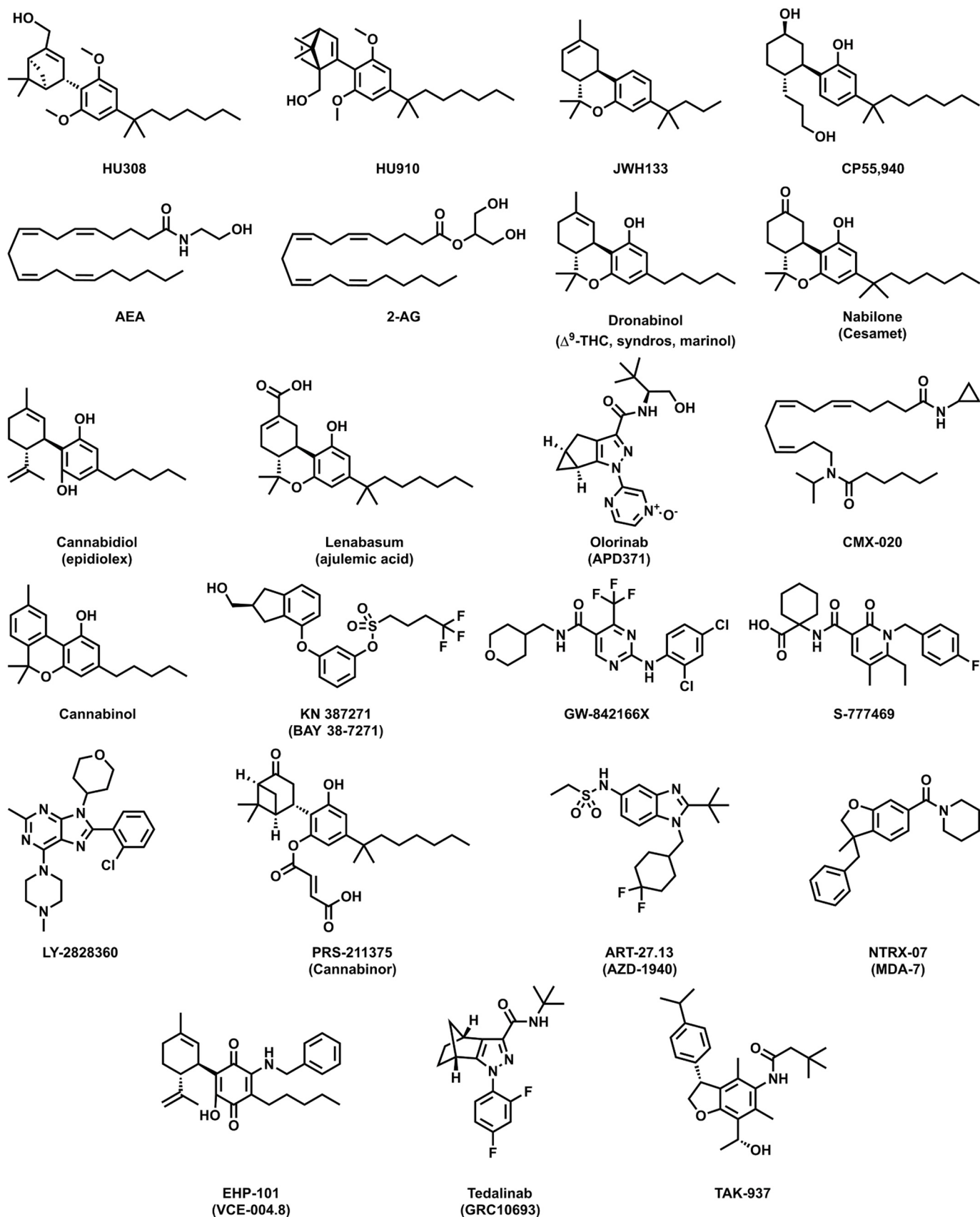


Fig. 1. Chemical structures of cannabinoid CB₂ receptor agonists used in this study. Benchmark agonists (HU308, HU910, JWH133 and CP55,940) and endocannabinoids (AEA, 2-AG). Clinical agonists ordered on highest phase of clinical development reached (in January 2024): launched (Dronabinol, Nabilone, Cannabidiol), phase 3 (Lenabasum), phase 2 (Olorinab, CMX-020, Cannabinol, KN 387271, GW-842166X, S-777469, LY-282360, PRS-211375), phase 1 (ART-27.13, NTRX-07, EHP-101, Tedalinab, TAK-937) with alternative names in between brackets.

(Ann Arbor, MI, USA). Anandamide (AEA, #1339), 2-Arachidonylglycerol (2-AG, #1298), JWH133 (#1343), cannabidiol (CBD, #1570), cannabinol (CBN, #3130) and phenylmethylsulfonyl fluoride (PMSF, #4486) were purchased from Tocris Bioscience (Bristol, UK). GDP (#J61646) was from Thermo Fisher Scientific (Waltham, MA, USA), EHP-101 (#HY-128872) from MedChemExpress (Monmouth Junction, NJ, USA) and HU308 (#H800010) was obtained from LKT Laboratories (St. Paul, MN, USA). Nabilone, Lenabasum, Olorinab, CMX-020, KN 387271, GW-842166X, S-777469, PRS-211375, ART-27.13, NTRX-07 and TAK-937 were provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland) and HU910 was a kind gift from Pal Pacher (NIH, MD, USA). FuGENE® 6 Transfection Reagent (#E2692), Nano-Glo® Viva-zine™ Substrate (#N2581), GloSensor™ cAMP reagent (#E1291) and pGloSensor™-22F cAMP Plasmid (#E2301) were from Promega (Madison, WI, USA) and 96-well solid white flat bottom microplates (#3917) were obtained from Corning (Corning, NY, USA). All buffers and solutions were prepared using Millipore water (deionized using a MilliQ A10 Biocel™ with a 0.22 µm filter) and analytical grade reagents and solvents. Buffers were prepared at room temperature (rt) and stored at 4 °C, unless stated otherwise.

2.2. Physicochemical properties and pharmacokinetic properties determination

2.2.1. Lipophilicity

For the determination of the octanol/water distribution coefficient (LogD), the Carrier-Mediated Distribution System (CAMDIS)-assay was used as described previously [40].

2.2.2. Kinetic aqueous solubility (Lysa)

The compounds' solubility was assessed in a phosphate buffer with a pH of 6.5, originating from a 10 mM stock solution in DMSO that had been evaporated. For each compound, two samples were desiccated and reconstituted in the pH 6.5 phosphate buffer. Post-dissolution, the solutions underwent filtration and were subsequently diluted to three distinct concentrations. RapidFire mass spectrometry analysis was conducted on these dilutions. Quantification of each compound was achieved using a calibration curve comprising six points, which was established using the initial DMSO solution.

2.2.3. Lipid membrane binding assay (Limba LogDbrain)

The Lipid Membrane Binding Assay (LIMBA LogDbrain) was conducted consistent with previously published methods, allowing for high to medium throughput analysis [41].

2.2.4. Passive membrane permeability assay (PAMPA)

The PAMPA assay was carried out as a high-throughput experiment as previously described [41].

2.2.5. Human plasma protein binding (free fraction %)

Human plasma with EDTA as an anticoagulant was sourced from BioreclamationIVT (New York, USA) [42,43]. To determine the free fraction of agonist, a 96-well equilibrium dialysis device with a Teflon build and a 150 µL half-cell volume was used. This device featured a membrane with a molecular weight cut-off between 12 and 14 kDa, chosen to reduce non-specific binding. Both the test compound and a known reference, diazepam, were assessed in groups ranging from 2 to 5 wells, starting at a concentration of 1000 nM. Each well was filled with equal amounts of a plasma sample containing the test compound or diazepam and a blank dialysis buffer, specifically Soerensen's buffer. The pH of the plasma and buffer was adjusted to 7.4 on the day of the experiment. The dialysis unit was then incubated at 37 °C with 5 % CO₂ for 5 h, which is generally sufficient for equilibrium to be reached for most small molecules under 600 Da. Following incubation, the samples were prepared for LC-MS/MS analysis. Each protein binding assessment was conducted in triplicate to ensure accuracy. The integrity of the

dialysis membrane was verified by measuring the unbound fraction of diazepam in each well. At equilibrium, the concentration of the unbound drug in the plasma should equal that in the buffer, allowing for the calculation of the unbound fraction as the buffer concentration post-dialysis divided by the plasma concentration post-dialysis, multiplied by 100. Additionally, the recovery of the device was evaluated by comparing the concentration of the compound in the plasma before and after dialysis, with acceptable recovery rates ranging from 80 % to 120 % for the data to be considered valid.

2.2.6. P-glycoprotein-mediated efflux ratio

The generation of human P-gp efflux ratio values was conducted as previously reported [41].

2.3. Cell culture and membrane preparation

For functional assays, human embryonic kidney 293 T cells (HEK293T, ATCC #CRL-3216) and HEK293T cells stably expressing full-length hCB₂R-SmBiT and LgBiT-β-arrestin-2 (HEK293T CB₂R-SmBiT LgBiT-β-arrestin-2) or hCB₁R-LgBiT and SmBiT-β-arrestin-2 constructs (HEK293T CB₁R-LgBiT SmBiT-β-arrestin-2-366 truncated) were grown in monolayers. Stably expressing cell lines with CBRs were a kind gift by Christophe Stove, Ghent University, Belgium [44,45]. All cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % FCS, 2 mM Glutamax, 100 IU/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere at 37 °C and 5 % CO₂. For binding assays, Chinese Hamster Ovary (CHO) cells stably expressing hCB₂R or hCB₁R (CHOK1_hCB₂bgal, #93-0706C2 and CHOK1_hCB₁bgal, #93-0959C2, PathHunter EA Parental cell line, female, DiscoverX) were cultured in Ham's F12 Nutrient Mixture supplemented with 10 % (v/v) fetal calf serum (FCS), 2 mM Glutamax, 100 IU/mL penicillin, 100 µg/mL streptomycin, 300 µg/mL hygromycin and 800 µg/mL G418 in a humidified atmosphere at 37 °C and 5 % CO₂. All cells were subcultured twice weekly when reaching 80–90 % confluence on 10 or 15 cm ø plates by trypsinization and used for further experiments within 20 passages.

2.4. Membrane preparation

HEK293T CB₂R-SmBiT LgBiT-β-arrestin-2, CHOK1_hCB₂bgal and CHOK1_hCB₁bgal cells were harvested when reaching 80–90 % confluence in 15 cm ø plates after one week subculture at a 1:15, 1:6 or 1:6 ratio, respectively. The cells were detached by scraping into 5 mL phosphate-buffered saline (PBS) and subsequently centrifuged at 2000 × g for 5 min. Pellets were resuspended in ice-cold Tris buffer (50 mM Tris-HCl, pH 7.4) and homogenized with an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany) using the thick rod on setting 1 for 15 seconds (s). Cytosolic and membrane fractions were separated using an Optima LE-80 K ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) at 100,000 × g for 20 min at 4 °C. The membrane fractions were subjected to another round of homogenization (thin rod on setting 1 for 15 s) and centrifugation. The final pellets were resuspended and homogenized in ice-cold Tris buffer (thin rod on setting 1 for 15 s) and subsequently aliquoted and stored in 100 µL aliquots at –80 °C. Membrane protein concentrations were determined using a BCA protein determination assay, as described by the manufacturer (Pierce BCA protein assay kit) [46].

2.5. [³H]RO6957022 binding assays

[³H]RO6957022 displacement and competition association assays have previously been described with the main difference that the incubation temperature was changed to 10 °C for improved separation of kinetic differences without affecting the equilibrium of radioligand binding [39]. In short, CHOK1_hCB₂bgal or HEK293T CB₂R-SmBiT LgBiT-β-arrestin-2 membranes were thawed and subsequently

homogenized using the Ultra Turrax homogenizer. For experiments with endocannabinoids and CMX-020, membranes were preincubated for 30 min with 50 μ M PMSF. The reactions were carried out in 100 μ L assay buffer (50 mM Tris-HCl (pH 7.4), 0.1 % (w/v) bovine serum albumin (BSA)) containing 1 μ g (CHOK1_hCB₂bgal) or 10 μ g (HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2) of membrane protein and 1.5 nM [³H] RO6957022. Incubations were performed at 10 °C for 2 h. Therefore, assay buffer, (radio)ligands and membranes were precooled to 10 °C for 30 min prior to the experiment. Nonspecific binding (NSB) was determined using 10 μ M AM630 and vehicle (i.e., acetonitrile for endocannabinoids and CMX-020, and DMSO for all other compounds) concentrations were constant and kept < 1 % in all samples. Total radioligand binding (TB) did not exceed 10 % of the amount added to prevent ligand depletion. For all assays, incubations were terminated by rapid vacuum filtration with ice-cold 50 mM Tris-HCl (pH 7.4), 0.1 % (w/v) BSA buffer through Whatman GF/C filters using a Filtermate 96-well harvester (Revvity, Waltham, MA, USA). Filters were dried for at least 30 min at 55 °C and subsequently 25 μ L MicroScint scintillation cocktail was added per well. Filter-bound radioactivity was measured by scintillation spectrometry using a Microbeta² 2450 counter (Revvity, Waltham, MA, USA).

2.5.1. Displacement assays

Binding affinity of all clinical agonists for CB₂R was determined in displacement assays using radioligand and six increasing concentrations of competing compound (ranging from 0.01 nM to 10 μ M) on CHOK1_hCB₂bgal membranes. Homologous displacement assays were performed on HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 membranes with three concentrations of [³H]RO6957022 of ~0.5 nM, ~1.5 nM and ~5.0 nM in the presence of competing RO6957022 (ranging from 0.01 nM to 1 μ M) in assay buffer. The reaction mixture was incubated for 2 h at 10 °C, after which incubations were terminated and receptor-bound radioactivity was determined as described in [Section 2.5 \[³H\] RO6957022 binding assays](#).

2.5.2. Competition association assays

Binding kinetics were assessed in competition association experiments using radioligand and competing compound at its IC₅₀ concentration obtained from displacement assays. Competition was initiated by addition of CHOK1_hCB₂bgal membrane homogenates at different time points for 2 h, after which incubations were terminated and receptor-bound radioactivity was determined as described in [Section 2.5 \[³H\] RO6957022 binding assays](#).

2.6. [³H]CP55,940 displacement assays

Binding affinity for CB₁R was determined in [³H]CP55,940 displacement assays. CHOK1_hCB₁bgal membranes were homogenized and diluted to 2.5 μ g protein per well in assay buffer (50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.1 % (w/v) BSA). Membranes were incubated with six increasing concentrations (ranging from 0.01 nM to 10 μ M) of competing compound in the presence of 1.5 nM [³H]CP55,940. Incubations were for 2 h at 25 °C. NSB was determined using 10 μ M SR141716A and vehicle (i.e., acetonitrile for endocannabinoids and CMX-020, and DMSO for all other compounds) concentrations were constant and kept < 1 % in all samples. TB did not exceed 10 % of the amount added to prevent ligand depletion. Incubations were terminated as described in [Section 2.5 \[³H\] RO6957022 binding assays](#) except using ice-cold wash buffer containing 50 mM Tris-HCl, 5 mM MgCl₂ and 0.1 % BSA.

2.7. Multiplexed cAMP production and β -arrestin-2 recruitment assay

Inhibition of cAMP production and β -arrestin-2 recruitment after CB₂R or CB₁R activation were measured using the GloSensor™ and NanoBiT® technologies, respectively, in a multiplexed manner. To this

end, HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 or HEK293T CB₁R-LgBiT SmBiT- β -arrestin-2-366 truncated cells were seeded as 2×10^6 cells on 10 cm ϕ plates to reach 60 % confluence on the day of transfection. The next day, cells were transfected with 10 μ g pGloSensor™-22F cAMP plasmid using FuGENE® 6 transfection in a 3:1 reagent:DNA ratio [47]. In short, a mix of 10 μ g GloSensor plasmid and 30 μ L FuGENE® 6 was incubated in 850 μ L Opti-MEM™ at rt for 15 min. The mixture was added to the cells without replacing the medium. Cells were left to grow overnight at 37 °C with 5 % CO₂ to reach ~90 % confluence. The next day, transfected cells were seeded at a density of 50,000 cells/well for HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 and 100,000 cells/well for CB₁R-LgBiT SmBiT- β -arrestin-2-366 truncated in poly-D-lysine treated solid white, flat bottom 96-well plates in culture medium and incubated for another 22–24 h at 37 °C with 5 % CO₂. On the assay day, the culture medium was replaced by 30 μ L assay medium (CO₂ independent medium (Gibco cat. nr. 18045088) + 10 % (v/v) FCS) and 50 μ L equilibration reagent in assay medium. The equilibration reagent for the GloSensor™ only contained 4 % (v/v) GloSensor™ cAMP reagent, NanoBiT® reagent contained 2 % (v/v) Vivazine™ substrate and the “multiplex reagent” contained a combination of the two reagents, i.e., 2 % (v/v) Vivazine™ substrate and 4 % (v/v) GloSensor™ cAMP reagent. The plate was incubated at 25 °C for 2 h in the dark after which baseline cAMP levels and β -arrestin recruitment were measured for 7.5 min in the plate reader using the protocol as described below.

To induce cAMP production, cells were prestimulated with 1 μ M forskolin (FSK) for HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 cells and 0.1 μ M for CB₁R-LgBiT SmBiT- β -arrestin-2-366 truncated cells, i.e., EC₉₀ value for cAMP production, until reaching a stable plateau of cAMP production. Specifically, induction of cAMP production was initiated by addition of 10 μ L FSK mixture and luminescence was monitored for 52.5 min in the plate reader. For experiments with endocannabinoids or CMX-020, the FSK prestimulation mixture additionally contained 50 μ M phenylmethylsulfonyl fluoride (PMSF). Immediately after, cells were stimulated with 10 μ L increasing concentrations of compound of interest (ranging from 0.01 nM to 10 μ M). Compounds were added using a MINI 96 portable electronic pipette to ensure no time delay (INTEGRA Biosciences, Tokyo, Japan). The maximal response was determined by 1 μ M CP55,940 and vehicle (i.e., acetonitrile for endocannabinoids and CMX-020, and DMSO for all other compounds) concentrations were constant and kept < 1 % in all samples. Luminescence was measured for another 90 min in the plate reader resulting in a total read time of 150 min.

The simultaneous luminescent signals were measured in a Wallac EnVision 2104 Multilabel reader (Revvity, Waltham, MA, USA) at rt. To this end, the individual signals were detected by two emission filters in a dual luminescent manner. Specifically, the Cy3 595 filter (595/60 nm, barcode 229) was used to detect the cAMP responses and emission filter NanoBRET Blue (460/80 nm, barcode 703) was used to detect β -arrestin-2 recruitment to CB₂R. Each wavelength was measured for 250 ms/well and the total interval between plate repeats was 90 s.

To investigate cAMP responses independent of CB₂R, the same protocol was applied to parental HEK293T cells, but only using the GloSensor™ equilibration reagent containing 4 % (v/v) GloSensor™ cAMP reagent.

2.8. In silico docking

Each ligand was docked with Glide (Schrödinger Suite 2023–4, Schrödinger, LLC, New York, NY) to the binding cavities of all publicly available CB₂R x-ray and cryo-EM structures (6KPC, 6KPF, 6PT0, 8GUQ, 8GUT, 8GUS, 8GUR). From each pair of binding cavity and docked ligand the combination with the highest docking score was used.

2.9. Data analysis and statistics

All experimental data were analyzed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). All values obtained are

means $\pm$ standard error of the mean (SEM) of at least three independent experiments performed in duplicate, unless stated otherwise.

2.9.1. Displacement assays

[³H]RO6957022 (CB₂R) and [³H]CP55,940 (CB₁R) assays were baseline-corrected with NSB and normalized to this value (0 %) and TB (100 %). The equilibrium dissociation constant (K_D) of [³H]RO6957022 and receptor expression level (B_{max}) were calculated from homologous displacements by non-linear regression analysis, using the “one-site homologous” model. The half-maximal inhibitory concentrations (pIC_{50}) of the compounds in [³H]RO6957022 and [³H]CP55,940 displacement assays were obtained by non-linear regression analysis of the displacement curves and further converted into inhibitory constant pK_i using the Cheng-Prusoff equation [48] with the experimentally determined K_D values 0.78 nM and 0.84 nM, respectively (data not shown). CB₂R selectivity of the compounds was determined when two pK_i values were obtained using Eq. 1:

$$\text{Selectivity} = 10^{(pK_i \text{ CB}_2\text{R} - pK_i \text{ CB}_1\text{R})} \quad (1)$$

2.9.2. Competition association assays

From [³H]RO6957022 competition association assays, the k_{on} and k_{off} values of compounds of interest were determined by non-linear regression analysis, using the “kinetics of competitive binding” model as described by Motulsky and Mahan [49]:

$$K_a = k_1 [L] \bullet 10^{-9} + k_2$$

$$K_b = k_3 [I] \bullet 10^{-9} + k_4$$

$$S = \sqrt{(K_a - K_b)^2 + 4 \bullet k_1 \bullet k_3 \bullet [L] \bullet [I] \bullet 10^{-18}}$$

$$K_f = 0.5(K_a + K_b + S)$$

$$K_s = 0.5(K_a + K_b - S)$$

$$Q = \frac{B_{max} \bullet k_1 \bullet [L] \bullet 10^{-9}}{K_f - K_s}$$

$$[Y] = Q \left(\frac{k_4 \bullet (K_f - K_s)}{K_f \bullet K_s} + \frac{k_4 - K_f}{K_f} \bullet e^{(-K_f \bullet X)} - \frac{k_4 - K_s}{K_s} \bullet e^{(-K_s \bullet X)} \right)$$

Where [L] is the radioligand concentration per experiment (~ 1.5 nM), I is the IC₅₀ concentration of compound (nM), X is the time (s), and Y is the specific binding of the radioligand (dpm). K_a and K_b are the observed association rate constants (k_{obs}) of the radioligand and the compound of interest, respectively. The parameters K_a , K_b , S, Q, K_s and K_f are composite parameters used to simplify the entry of the equation into curve-fitting programs. These parameters are not reported or used to interpret the data. k_1 and k_3 are the association rate constants (k_{on} in $M^{-1}s^{-1}$) of [³H]RO6957022 (determined per experiment) and the compound of interest, respectively. Similarly, k_2 and k_4 are the dissociation rate constants (k_{off} in s^{-1}) of [³H]RO6957022 (experimentally determined at $4.3 \times 10^{-4} s^{-1}$, data not shown) and the compound of interest, respectively. The engagement time (ET in seconds) of the compounds of interest was determined at 1 μM of compound using Eq. 2 [50]:

$$ET = \frac{1}{k_{on} \bullet 10^{-6}} \quad (2)$$

The residence time (RT in min) was calculated using Eq. 3 [14]:

$$RT = \frac{1}{k_{off} \bullet 60} \quad (3)$$

The association and dissociation rate constants were used to calculate the kinetic K_D using Eq. 4:

$$K_D = \frac{k_{off}}{k_{on}} \quad (4)$$

2.9.3. Inhibition of forskolin-induced cAMP production (multiplex signal 1)

Functional forskolin-induced cAMP data from the multiplex assay was analyzed based in endpoint, semi-kinetic and kinetic approaches. Forskolin-induced cAMP responses after 7.5, 15, 30 and 60 min of agonist addition were baseline-corrected with the vehicle response and normalized to 1 μM CP55,940. Dose-response curves were generated and pEC_{50} and E_{max} values were determined using non-linear regression curve fitting ‘log(agonist) vs. response’ (three parameters).

Forskolin-induced cAMP time traces from the multiplex assay was corrected for well-to-well variation by subtracting the mean of the baseline measurement and subsequently subtracting the mean of the final five FSK measurements. Next, vehicle response was subtracted, and the data was inverted to represent inhibition of cAMP production. The agonist-induced inhibition of cAMP production was quantified by taking the net area under the curve (AUC) from the final five FSK measurements until the end of agonist measurement (54–150 min). Dose-response curves were generated and pEC_{50} and E_{max} values were determined using non-linear regression curve fitting ‘log(agonist) vs. response’ (three parameters) after normalization to 1 μM CP55,940.

The agonist-induced inhibition of cAMP production was further kinetically analyzed by applying the ‘Baseline then rise-and-fall to baseline time course’ equation according to Hoare *et al.* [20]. This Eq. (5) was provided as a plug-in, which was downloaded into GraphPad Prism [51]:

$$Y = IF \left(X \right. \\ \left. < X_0, \text{ Baseline, Baseline} + \left(\frac{IR}{k_1 - k_2} (e^{-k_2(X-X_0)} - e^{-k_1(X-X_0)}) \right) \right) \quad (5)$$

Where the IF statement is a logic gate term used within Graphpad Prism, in this case to define the start of the rise phase of the curve (X_0), ‘IR’ is a fitting constant (cps min^{-1}), which is equal to the initial rate of signaling and represented by the initial linear phase of signal generation after receptor activation, X is the time (min), X_0 is the time at which the signal starts, and Y is the luminescent signal (cps). k_1 and k_2 are the observed signaling rate constants for signal onset and decline, respectively (min^{-1}), where k_1 is constrained to be greater than k_2 . When time traces did not fall back to baseline in the assay time, as was the case for lower agonist concentrations, the ‘baseline then rise to steady state time course’ was fitted according to Eq. 6:

$$Y = IF \left(X < X_0, \text{ Baseline, SSR} \bullet \left(1 - e^{-k(X-X_0)} \right) + \text{Baseline} \right) \quad (6)$$

Where the IF statement is a logic gate term used within Graphpad Prism, in this case to define the start of the rise phase of the curve (X_0), SSR is the steady-state response representing the maximal activation as time reaches infinity (cps), X is the time (min), X_0 is the time at which the signal starts, and Y is the luminescent signal (cps). k is the observed signaling rate constant (min^{-1}).

Dose-response curves were generated from the Initial Rate data and pIR_{50} and IR_{max} values were determined using non-linear regression curve fitting ‘log(agonist) vs. response’ (three parameters) after normalization to 1 μM CP55,940. More detailed information on the use of these models and a step-by-step procedure can be found in [Supplementary methods S1.2.1](#).

2.9.4. β -arrestin-2 recruitment (multiplex signal 2)

Functional β -arrestin-2 recruitment data from the multiplex assay was analyzed based on an endpoint, semi-kinetic and kinetic approach. Recruitment of β -arrestin-2 after 7.5, 15, 30 and 60 min of agonist addition were baseline-corrected with the vehicle response and

normalized to 1 μM CP55,940. Dose-response curves were generated and pEC_{50} and E_{max} values were determined using non-linear regression curve fitting 'log(agonist) vs. response' (three parameters).

Functional β -arrestin-2 recruitment time traces from the multiplex assay was corrected for well-to-well variation by subtracting the mean of the baseline (final five FSK measurements count as baseline) and subsequently vehicle response was subtracted. The β -arrestin-2 recruitment to CB_2R was quantified by taking the net area under the curve (AUC) from the baseline measurements until the end of agonist measurement (54–150 min). Dose-response curves were generated and pEC_{50} and E_{max} values were determined using non-linear regression curve fitting 'log(agonist) vs. response' (three parameters) after normalization to 1 μM CP55,940.

The β -arrestin-2 recruitment was further kinetically analyzed by applying the 'Baseline then rise-and-fall to steady state time course' equation according to Hoare *et al.* [20]. This Eq. (7) was provided as a plug-in, which was downloaded into GraphPad Prism [51]:

$$Y = \text{IF}(X)$$

$$< X_0, \text{Baseline}, \text{Baseline} + \text{SSR}(1 - D) \bullet e^{-k_1(X-X_0)} + (D-1)e^{-k_2(X-X_0)} \quad (7)$$

Where the IF statement is a logic gate term used within Graphpad Prism, in this case to define the start of the rise phase of the curve (X_0), SSR is the steady-state response representing the maximal activation as time reaches infinity (cps), D is a unitless fitting constant, X is the time (min), X_0 is the time at which the signal starts, and Y is the luminescent signal (cps). k_1 and k_2 are the observed signaling rate constants (min^{-1}) for signal onset and decline, respectively, where k_1 is constrained to be greater than k_2 . Initial rate can be calculated using Eq. 8:

$$\text{IR} = \text{SSR}(D \bullet k_1 - (D-1)k_2) \quad (8)$$

When time traces did not fall back to baseline in the assay time, as was the case for lower agonist concentrations, the 'baseline then rise to steady state time course' was fitted as described for 2.9.3 Inhibition of forskolin-induced cAMP production.

Dose-response curves were generated from the Initial Rate data and pIR_{50} and IR_{max} values were determined using non-linear regression curve fitting 'log(agonist) vs. response' (three parameters) after normalization to 1 μM CP55,940. More detailed information on the use of these models and a step-by-step procedure can be found in Supplementary methods S1.2.2.

2.9.5. Bias calculation

Dose-response curves from the endpoint analysis (early and late time point), semi-kinetic AUC and kinetic initial rate analysis for all compounds and for each functional readout were analyzed using the Black and Leff operational model to calculate the transduction coefficient Log (τ/K_A) or LogR values as previously described [52,53]. In the model, the slope (n) was set to 1, basal to 0 as the data was baseline corrected and E_{max} was set to the maximal activation in the system (Tetalinab). For each functional readout, the LogR value of the agonists was compared to most balanced agonist (HU308 for CB_2R and CP55,940 for CB_1R [54]) generating transduction ratios ΔLogR using Eq. 9:

$$\Delta\text{LogR}_{\text{agonist}} = \text{LogR}_{\text{agonist}} - \text{LogR}_{\text{HU308}} \quad (\text{Eq. 9})$$

The relative bias of each agonist between the cAMP and β -arrestin-2 pathway, represented as $\Delta\Delta\text{LogR}$, was calculated using Eq. 10:

$$\Delta\Delta\text{LogR}_{\text{agonist}} = \Delta\text{LogR}_{\text{agonist, cAMP}} - \Delta\text{LogR}_{\text{agonist, } \beta\text{-arrestin}} \quad (10)$$

Ultimately, the bias factor was calculated as the inverse logarithm of the $\Delta\Delta\text{LogR}$ values using Eq. 11:

$$\text{Bias factor} = 10^{\Delta\Delta\text{LogR}_{\text{agonist}}} \quad (11)$$

Pooled SEM values were calculated for $\Delta\text{LogR}_{\text{agonist}}$ and $\Delta\Delta$

$\text{LogR}_{\text{agonist}}$ using Eqs. 12 and 13 respectively:

$$\text{Pooled SEM} = \sqrt{(\text{SEM}_{\text{agonistX}})^2 + (\text{SEM}_{\text{HU308}})^2} \quad (12)$$

$$\text{Pooled SEM} = \sqrt{(\text{SEM}_{\text{agonist cAMP}})^2 + (\text{SEM}_{\text{agonist } \beta\text{-arrestin2}})^2} \quad (13)$$

Statistical analysis was performed on $\Delta\Delta\text{LogR}$ values to test for significance of ligand bias relative to balanced agonist HU308. To this end, a one-way ANOVA with Dunnett's multiple comparisons test was performed. $p < 0.05$ was considered statistically significant.

2.9.6. Constitutive receptor activity

Statistical analysis for constitutive receptor activity was performed using a two-way ANOVA with Tukey's multiple comparison test to compare constitutive receptor activity of 1 μM inverse agonist AM630 relative to its vehicle condition. P values were indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Detection of binding affinity of agonists at CB_2R and selectivity over CB_1R

Prior to the development of the novel multiplexed signaling assay, all agonists were screened for their binding affinity at CB_2R in [^3H] RO6957022 binding assays to ensure interaction with CB_2R (Table 1). Inverse agonist radioligand [^3H]RO6957022 was used due to its monophasic nature opposed to agonist [^3H]CP55,940. [^3H]CP55,940 shows a biphasic binding kinetic profile, thus preventing kinetic binding parameter determination from the (monophasic) Motulsky & Mahan model [39,49].

From [^3H]RO6957022 displacement assays, it was observed that most agonists displayed affinity for CB_2R , which ranged from a moderate pK_i value of 6.31 ± 0.07 for AEA to high affinities below 10 nM for CP55,940, Dronabinol, ART-27.13, Tetalinab and TAK-937 (Table 1). In contrast, cannabidiol (CBD), GW-842166X and EHP-101 only showed limited displacement of [^3H]RO6957022, indicative of low affinity for CB_2R .

To get insight in selectivity over CB_1R , binding affinities of all agonists were determined in [^3H]CP55,940 displacement assays at this receptor (Table 1). Agonists CBD, GW-842166X and EHP-101 were devoid of CB_1R affinity, as was the case for CB_2R . As expected, no pronounced selectivity for either CBR was found for the endocannabinoids or CMX-020, a 2-AG derivative. Similarly, no selectivity was observed for CP55,940, Dronabinol, Nabilone, Lenabasum, Cannabinol, KN 387271 and TAK-937. More than 15-fold CB_2R selectivity was detected for HU910, LY-2828360, PRS-211375 and ART-27.13. Furthermore, exclusive CB_2R affinity (>100 fold) was observed for HU308, JWH133, Olorinab, S-777469, NTRX-07 and Tetalinab.

3.2. Development and validation of a kinetic multiplex assay for CB_2R

To investigate biased signaling in one cellular system and in a kinetic context, we developed a novel multiplex assay. The new signaling assay incorporated two luminescent technologies for detection of inhibition of cAMP production, via $\text{G}\alpha_i$ activation, and β -arrestin-2 recruitment after CB_2R activation to improve the detection of potential biased signaling of clinically relevant agonists. The inhibition of cAMP production was measured via the GloSensor technology, which was visualized by addition of GloSensor cAMP reagent. The NanoBiT technology was used to detect β -arrestin-2 recruitment to CB_2R for which Vivazine was used as substrate (Table S3). The two technologies were combined in a multiplex assay to simultaneously measure both signaling events under the same assay conditions and in the same cellular background, and as such improve the validity of any observed signaling bias.

Table 1

Binding affinity and kinetic binding parameters of benchmark agonists, endocannabinoids (eCB) and clinical agonists on CB₂R and selectivity over CB₁R.

	Agonist	CB ₂ R				CB ₁ R			
		pK _i (K _i in nM) or displacement at 10 μM (%) ^a	k _{on} (M ⁻¹ s ⁻¹) ^b	ET (s) ^c	k _{off} (s ⁻¹) ^d	RT (min) ^e	K _D (nM) ^f	pK _i (K _i in nM) or displacement at 10 μM (%) ^g	CB ₂ R selectivity ^h
Benchmark	HU308 [†]	7.03 ± 0.10 (92.4)	(7.0 ± 2.3) × 10 ³	143.5 ± 47.9	(2.3 ± 0.4) × 10 ⁻⁴	71.0 ± 11.3	33.7	21 ± 9	N.A.
	HU910	7.18 ± 0.09 (65.7)	(1.4 ± 0.2) × 10 ⁴	72.4 ± 11.9	(4.5 ± 1.0) × 10 ⁻⁴	37.0 ± 7.9	32.6	5.62 ± 0.13 (2384)	36.3
	JWH133	7.40 ± 0.07 (39.7)	(7.6 ± 3.6) × 10 ⁴	13.1 ± 6.2	(2.5 ± 1.1) × 10 ⁻³	6.7 ± 3.0	32.6	48 ± 2	N.A.
	CP55,940 [†]	8.85 ± 0.08 (1.4)	(1.8 ± 0.4) × 10 ⁶	0.6 ± 0.1	(5.2 ± 0.9) × 10 ⁻⁴	32.3 ± 5.5	0.3	8.91 ± 0.16 (1.2)	0.9
eCB	AEA [†]	6.31 ± 0.07 (484.5)	(6.6 ± 0.5) × 10 ³	152.3 ± 10.4	(2.4 ± 0.1) × 10 ⁻³	6.8 ± 0.4	371.7	6.60 ± 0.07 (254.1)	0.5
	2-AG [†]	7.01 ± 0.06 (97.3)	(5.3 ± 1.0) × 10 ⁴	18.8 ± 3.6	(2.3 ± 0.3) × 10 ⁻³	7.4 ± 1.0	42.7	6.60 ± 0.15 (251.8)	2.6
Clinical	Dronabinol	8.07 ± 0.26 (8.6)	(3.5 ± 1.5) × 10 ⁵	2.9 ± 1.2	(7.8 ± 3.7) × 10 ⁻³	2.1 ± 1.0	22.5	7.70 ± 0.09 (20.0)	2.3
	Nabilone	7.85 ± 0.07 (14.1)	(1.0 ± 0.2) × 10 ⁵	9.9 ± 1.6	(6.3 ± 0.3) × 10 ⁻⁴	26.6 ± 1.3	6.2	8.17 ± 0.06 (6.8)	0.5
	Cannabidiol	53 ± 2	N.A.	N.A.	N.A.	N.A.	N.A.	10 ± 7	N.A.
	Lenabasum	6.51 ± 0.11 (310.0)	(1.8 ± 0.9) × 10 ⁴	55.8 ± 29.1	(2.6 ± 0.4) × 10 ⁻³	6.4 ± 1.0	144.9	6.49 ± 0.08 (321.9)	1.0
	Olorinab [†]	7.45 ± 0.09 (35.3)	(2.5 ± 0.4) × 10 ⁴	40.1 ± 5.9	(3.7 ± 0.6) × 10 ⁻⁴	44.9 ± 6.6	14.9	-15 ± 8	N.A.
	CMX-020	6.37 ± 0.15 (428.9)	N.D.	N.D.	N.D.	N.D.	N.D.	7.05 ± 0.01 (88.2)	0.2
	Cannabinol	7.44 ± 0.16 (36.3)	N.D.	N.D.	N.D.	N.D.	N.D.	6.91 ± 0.07 (122.3)	3.4
	KN 387271	7.40 ± 0.08 (39.6)	N.D.	N.D.	N.D.	N.D.	N.D.	7.84 ± 0.05 (14.5)	0.4
	GW-842166X	27 ± 1	N.A.	N.A.	N.A.	N.A.	N.A.	2 ± 1	N.A.
	S-777469	6.37 ± 0.02 (423.3)	(1.0 ± 0.1) × 10 ⁴	96.2 ± 10.5	(2.5 ± 0.7) × 10 ⁻³	6.7 ± 2.0	239.1	-11 ± 3	N.A.
	LY-2828360	7.43 ± 0.14 (36.9)	(5.5 ± 0.7) × 10 ⁴	18.1 ± 2.3	(1.8 ± 0.5) × 10 ⁻³	9.2 ± 2.3	32.9	5.69 ± 0.06 (2040)	55.3
	PRS-211375	7.45 ± 0.11 (35.4)	(2.7 ± 0.7) × 10 ⁴	36.8 ± 10.1	(5.3 ± 0.6) × 10 ⁻⁴	31.6 ± 3.8	19.4	6.13 ± 0.06 (743.6)	21.0
	ART-27.13	8.93 ± 0.05 (1.2)	(4.1 ± 0.3) × 10 ⁵	2.4 ± 0.2	(2.9 ± 0.4) × 10 ⁻⁴	57.4 ± 8.4	0.7	7.71 ± 0.01 (19.7)	16.7
	NTRX-07	6.47 ± 0.09 (337.5)	(4.0 ± 1.1) × 10 ⁴	25.3 ± 6.9	(6.5 ± 0.9) × 10 ⁻³	2.6 ± 0.4	163.6	31 ± 3	N.A.
	EHP-101	24 ± 22	N.A.	N.A.	N.A.	N.A.	N.A.	-25 ± 11	N.A.
	Tedalinab	8.23 ± 0.08 (5.9)	(8.3 ± 1.5) × 10 ⁵	1.2 ± 0.2	(2.4 ± 0.4) × 10 ⁻³	7.0 ± 1.0	2.9	6.05 ± 0.05 (885.1)	N.A.
	TAK-937	8.89 ± 0.05 (1.3)	(6.1 ± 1.6) × 10 ⁵	1.6 ± 0.4	(1.8 ± 0.3) × 10 ⁻⁴	93.2 ± 13.9	0.3	8.12 ± 0.11 (7.6)	5.8

^a, ^b, ^d Binding parameters of agonists on CB₂R were determined in [³H]RO6957022 displacement (affinity pK_i) and competition association (association, k_{on}, and dissociation, k_{off}, rate constants) assays on CHOK1_hCB₂gal membranes at 10 °C. ^c Engagement time (ET) of the agonist to the receptor was determined for 1 μM agonist by employing Eq. 2 and is expressed in seconds (s), whereas k_{on} is expressed in M⁻¹ s⁻¹. ^e Residence time (RT) was determined following Eq. 3 and is expressed in min, whereas k_{off} is expressed in s⁻¹. ^f Kinetic K_D values were determined using Eq. 4. ^g Binding affinity (pK_i) of agonists on CB₁R was determined in [³H]CP55,940 displacement assays on CHOK1_hCB₁gal membranes at 25 °C. Negative values imply big error margins due to negligible binding. ^h CB₂R selectivity was calculated using Eq. 1. In the absence of CB₁R affinity N.A. is stated. Values represent the mean ± SEM of at least three independent experiments performed in duplicate. N.A. is not applicable, N.D. is not detectable. [†] CB₂R data previously published [50].

The multiplex assay was validated using full agonist CP55,940 as a reference (Fig. 2). The pGloSensor plasmid was transfected into HEK293T cells already stably expressing CB₂R-SmBiT and LgBiT-β-arrestin-2 (Table S3). After transfection, the cells were equilibrated with either cAMP reagent, Vivazine or a combination of cAMP reagent and Vivazine (i.e., termed “multiplex reagent”) for 2 h. Subsequently, cells were stimulated with 1 μM FSK to increase cAMP levels prior to inhibition due to CB₂R activation, which did not induce any detectable β-arrestin-2 recruitment (Fig. S1). After 1 h of FSK treatment, cells were stimulated with increasing concentrations of CP55,940 and the responses for the three different reagents were monitored on the cAMP channel (595 nm filter) and β-arrestin channel (460 nm filter). Experiments were carried out in the absence of phosphodiesterase (PDE) inhibitors, which are often used in endpoint assays to avoid cAMP breakdown. The presence of PDE inhibitors changed the shape of kinetic signaling graphs, thereby potentially eliminating important kinetic information (data not shown). Specific luminescent signals were only detected on the cAMP channel, but not the β-arrestin channel, for cells

that were pre-equilibrated with cAMP reagent (Fig. 2a,b). *Vice versa*, in the presence of Vivazine, luminescent signals were only evident on the β-arrestin channel and not the cAMP channel (Fig. 2c,d). Pre-equilibration with the multiplex reagent resulted in the detection of CB₂R activation on both channels, and the traces were similar to the individually observed cAMP and β-arrestin responses (Fig. 2a-f). To quantify the results, the area under the curve (AUC) was determined from all time traces and presented in dose-response curves (Fig. 2g,h). This confirmed the lack of agonist-mediated responses measured on the cAMP channel for Vivazine only, whereas the pEC₅₀ values of CP55,940 obtained with cAMP reagent or multiplex reagent were similar, 8.86 ± 0.01 and 8.55 ± 0.03, respectively (Table S4). Similarly, CP55,940-induced β-arrestin-2 recruitment was only measured in the presence of Vivazine or multiplex reagent, which resulted in similar pEC₅₀ values, i.e., 8.20 ± 0.08 and 8.16 ± 0.04, respectively. As this protocol clearly distinguished between the responses obtained for inhibition of cAMP production and β-arrestin-2 recruitment of full agonist CP55,940, we continued screening the remaining benchmark agonists, endocannabinoids and

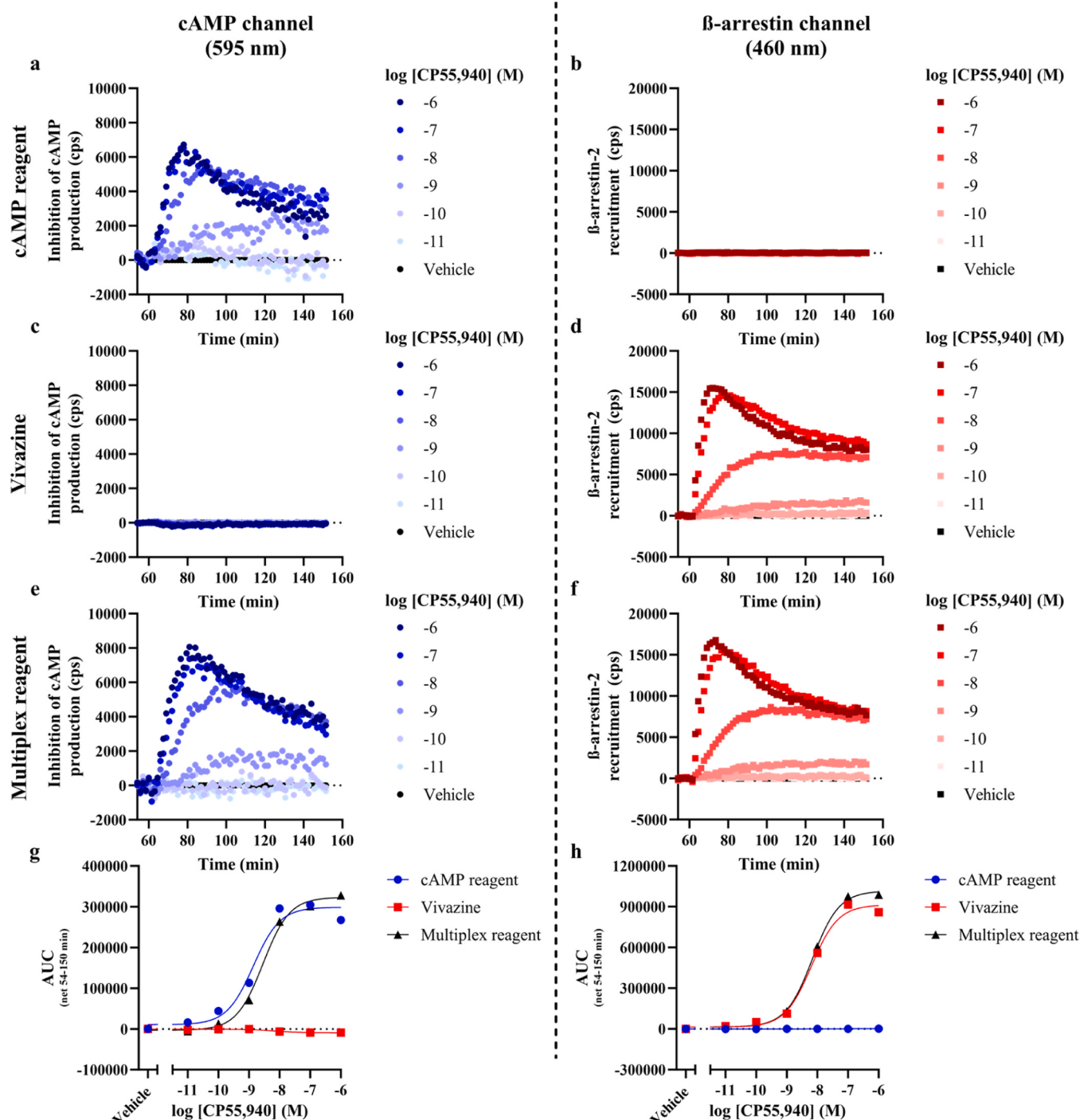


Fig. 2. Validation and characterization of the multiplexed cAMP production and β -arrestin-2 recruitment assay on HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 cells with full agonist CP55,940. Representative forskolin- and vehicle-corrected time traces of CP55,940 stimulation on HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 cells pre-equilibrated with (a, b) only cAMP reagent, (c, d) only Vivazine, or (e, f) multiplex reagent (cAMP reagent and Vivazine) for 2 h at 25 °C. The signals were simultaneously recorded at the (a, c, e) cAMP channel (595 nm) and (b, d, f) β -arrestin channel (460 nm). Area Under the Curve (AUC) was determined from the time traces after detection on the (g) cAMP channel or (h) β -arrestin channel. Data are shown as mean from a (a-f) representative experiment or (g-h) mean of two independent experiments all performed in duplicate.

clinical agonists with the FSK-induced multiplex assay.

3.3. Characterization of time traces by benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay

All benchmark agonists, endocannabinoids and clinical agonists were screened in the multiplex assay and the signaling time traces at

different concentrations of agonist were recorded in the two specific readouts (Figs. 3, S2-S5, Tables 2 and 3). Typically, at high concentrations of agonist the inhibition of cAMP production increased for the first 20 min after which the signals decreased with a tendency to return to baseline levels, although a complete return was not observed within the selected assay time (Figs. 3, S2, S3). At lower concentrations of agonist, the inhibition of cAMP production increased, although at a lower rate

Table 2
Inhibition of forskolin-induced cAMP production by benchmark agonists, endocannabinoids (eCB) and clinical agonists determined in the multiplex assay on CB₂R.

	Agonist	pEC ₅₀	E _{max} or receptor activation at 1 μM (%)	pIR ₅₀	IR _{max} or receptor activation at 10 μM (%)	k ₁ (min ⁻¹)	k ₂ (min ⁻¹)
Benchmark	HU308	6.57 ± 0.03	95 ± 3	6.03 ± 0.11	90 ± 4	0.26 ± 0.08	0.012 ± 0.002
	HU910	6.93 ± 0.08	102 ± 5	6.44 ± 0.17	89 ± 9	0.24 ± 0.08	0.012 ± 0.005
	JWH133	6.96 ± 0.07	88 ± 5	6.46 ± 0.12	79 ± 10	0.15 ± 0.01	0.026 ± 0.005
	CP55,940	9.15 ± 0.16	103 ± 5	7.90 ± 0.22	102 ± 1	0.24 ± 0.07 [§]	0.015 ± 0.002 [§]
eCB	AEA	6.41 ± 0.12	73 ± 12	5.73 ± 0.21	65 ± 5	0.15 ± 0.05 [§]	N.A.
	2-AG	6.15 ± 0.17	92 ± 16	6.16 ± 0.26	94 ± 7	0.26 ± 0.04	0.011 ± 0.001
Clinical	Dronabinol	5.66 ± 0.06	79 ± 19	6.65 ± 0.20	24 ± 1	0.08 ± 0.03	N.A.
	Nabilone	7.80 ± 0.17	99 ± 13	6.85 ± 0.19	81 ± 12	0.23 ± 0.02	0.004 ± 0.001
	Cannabidiol	N.D.	-8 ± 2 [§]	N.D.	4 ± 0 [#]	N.D.	N.D.
	Lenabasum	6.50 ± 0.07	132 ± 19	6.13 ± 0.15	93 ± 11	0.14 ± 0.01 [§]	0.007 ± 0.001 [§]
	Olorinab	7.88 ± 0.08	61 ± 14	6.92 ± 0.14	79 ± 10	0.14 ± 0.04 [§]	0.042 ± 0.015 [§]
	CMX-020	6.47 ± 0.13	109 ± 7	6.70 ± 0.58	53 ± 11	0.14 ± 0.03 [§]	N.A.
	Cannabinol	5.70 ± 0.05	81 ± 17	6.53 ± 0.02	10 ± 1	0.04 ± 0.01	N.A.
	KN 387271	7.96 ± 0.20	78 ± 11	7.29 ± 0.08	109 ± 29	0.30 ± 0.11 [§]	0.009 ± 0.002 [§]
	GW-842166X	N.D.	37 ± 3 [§]	N.D.	65 ± 13 [#]	0.19 ± 0.01 [§]	N.D.
	S-777469	7.04 ± 0.12	86 ± 12	6.71 ± 0.20	88 ± 10	0.22 ± 0.02	0.011 ± 0.002
	LY-2828360	6.89 ± 0.25	26 ± 5	6.33 ± 0.18	43 ± 7	0.24 ± 0.05	0.014 ± 0.003
	PRS-211375	7.18 ± 0.33	94 ± 11	6.41 ± 0.17	105 ± 4	0.24 ± 0.01	0.005 ± 0.002
	ART-27.13	9.15 ± 0.27	96 ± 11	7.93 ± 0.33	113 ± 23	0.24 ± 0.01 [§]	0.012 ± 0.000 [§]
	NTRX-07	6.70 ± 0.17	110 ± 27	6.59 ± 0.26	79 ± 3	0.20 ± 0.03 [§]	0.006 ± 0.002 [§]
	EHP-101	N.D.	19 ± 2 [§]	N.D.	5 ± 1 [#]	N.D.	N.D.
	Tedalinab	9.38 ± 0.25	97 ± 6	8.53 ± 0.14	114 ± 16	0.26 ± 0.03 [§]	0.018 ± 0.002 [§]
	TAK-937	8.56 ± 0.11	81 ± 3	7.07 ± 0.16	85 ± 3	0.20 ± 0.01 [§]	0.008 ± 0.001 [§]

Potency (pEC₅₀, pIR₅₀) and efficacy (E_{max}, IR_{max}) values were determined from AUC and initial rate dose-response curves, respectively, derived from the cAMP time traces in the multiplex assay. In the absence of a DRC, maximal receptor activation (%) was determined at 10 ([#]) or 1 μM ([§]) of agonist. Signaling rate constants k₁ and k₂ were determined at 10 μM or 1 μM ([§]) of agonist. Data are mean ± SEM from at least three independent experiments performed in duplicate. N.A. is not applicable, N.D. is not detectable.

than with high agonist concentrations, to reach a steady plateau within our assay time.

Strikingly, for several clinical agonists (AEA, Dronabinol, CBD, Lenabasum, CMX-020, CBN, KN 387271, GW-842166X, NTRX-07 and EHP-101), the inhibition of cAMP production by 10 μM of agonist did not return to basal levels, whereas by 1 μM agonist it did (Figs. S2 and S3). Of note, GW-842166X, CBD, EHP-101 had low or no detectable affinity for CB₂R (Table 1) but they modified cAMP levels at high compound concentrations. Intriguingly, 10 μM Olorinab induced a small inhibition of cAMP levels, which quickly returned to forskolin baseline levels and even further increased cAMP levels (Fig. S3a). To this end, the cAMP production of saturating concentrations (>100x K_i value) or 10 μM of all agonists was investigated in parental HEK293T cells devoid of CB₂R expression (Fig. S6). For multiple agonists (Dronabinol, CBD, Lenabasum, Olorinab, CMX-020, CBN, KN 387271, GW-842166X, LY-2828360, ART-27.13, NTRX-07 and TAK-937), we observed CB₂R-independent cAMP responses. This can be a result from off-target effects, i. e. unrelated to CB₂R. Consequently, for these agonists 10 μM was removed from the dose-response curves in the semi-kinetic and kinetic analysis, i.e., the methods of analysis using the full time traces.

In almost all cases, a high concentration of agonist induced a quick increase in β-arrestin-2 recruitment, which fell back to a steady state level above the baseline (Figs. 3, S4, S5). On the other hand, lower concentrations of agonist caused recruitment of β-arrestin-2, which increased towards a steady plateau above baseline within the timeframe of the assay. No increase in luminescence, as a consequence of β-arrestin-2 recruitment to CB₂R, was observed for any concentration of CBD or EHP-101, while a small increase in luminescence was observed by 10 and 1 μM GW-842166X (Figs. S4i, S5k, S5e).

An advantage of the multiplex assay is the possibility of observing the difference in initiation of the two signaling events over time, which was visualized for agonists CP55,940, HU308, 2-AG, Tedalinab, HU910, and PRS-211375at a saturating concentration (Figs. 3, S2–S5). In all cases, the peak response of β-arrestin-2 recruitment was reached earlier than the peak response of the inhibition of cAMP production. The peak response for both β-arrestin-2 recruitment and inhibition of cAMP production for PRS-211375 was markedly higher than the peak responses of CP55,940-mediated activation (Fig. 3a,f). Similarly, the peak response for β-arrestin-2 recruitment by Tedalinab was higher than by CP55,940, while the cAMP peak responses were similar (Fig. 3a,d). Moreover,

Table 3 β -arrestin-2 recruitment by benchmark agonists, endocannabinoids (eCB) and clinical agonists determined in the multiplex assay on CB₂R.

	Agonist	pEC ₅₀	E _{max} or receptor activation at 10 μ M (%)	pIR ₅₀	IR _{max} or receptor activation at 10 μ M (%)	k ₁ (min ⁻¹)	k ₂ (min ⁻¹)
Benchmark	HU308	6.06 ± 0.01	83 ± 5	5.36 ± 0.14	80 ± 10	0.24 ± 0.01	0.025 ± 0.005
	HU910	6.40 ± 0.03	92 ± 5	5.90 ± 0.03	61 ± 5	0.20 ± 0.01	0.025 ± 0.005
	JWH133	6.33 ± 0.05	98 ± 3	5.78 ± 0.10	64 ± 1	0.18 ± 0.01	0.033 ± 0.009
	CP55,940	8.54 ± 0.04	102 ± 0	7.33 ± 0.09	104 ± 1	0.26 ± 0.01 [§]	0.046 ± 0.005 [§]
eCB	AEA	5.94 ± 0.03	41 ± 1	5.19 ± 0.02	24 ± 3	0.20 ± 0.00	0.006 ± 0.001
	2-AG	5.90 ± 0.14	84 ± 8	5.90 ± 0.14	90 ± 17	0.29 ± 0.01	0.036 ± 0.002
	Dronabinol	7.08 ± 0.01	10 ± 1	5.95 ± 0.17	4 ± 1	0.19 ± 0.02	0.002 ± 0.001
Clinical	Nabilone	7.45 ± 0.11	80 ± 2	6.26 ± 0.03	63 ± 8	0.21 ± 0.02	0.039 ± 0.000
	Cannabidiol	N.D.	-8 ± 1 [#]	N.D.	N.D.	N.D.	N.D.
	Lenabasum	6.34 ± 0.02	87 ± 2	5.47 ± 0.07	78 ± 10	0.24 ± 0.01	0.030 ± 0.001
	Olorinab	7.11 ± 0.04	114 ± 1	6.19 ± 0.09	157 ± 2	0.34 ± 0.02	0.048 ± 0.004
	CMX-020	6.95 ± 0.12	21 ± 2	6.01 ± 0.14	25 ± 6	0.63 ± 0.11	0.025 ± 0.004
	Cannabinol	N.D.	9 ± 1 [#]	N.D.	6 ± 1 [#]	0.49 ± 0.04	N.D.
	KN 387271	7.80 ± 0.08	52 ± 1	6.83 ± 0.11	62 ± 2	0.53 ± 0.05	0.016 ± 0.002
	GW-842166X	N.D.	39 ± 6 [#]	N.D.	19 ± 6 [#]	0.23 ± 0.03	N.D.
	S-777469	6.64 ± 0.07	80 ± 3	6.13 ± 0.05	78 ± 4	0.31 ± 0.01	0.029 ± 0.001
	LY-2828360	7.00 ± 0.09	16 ± 1	6.05 ± 0.12	18 ± 1	0.43 ± 0.03	0.013 ± 0.004
	PRS-211375	6.81 ± 0.12	108 ± 2	5.85 ± 0.11	133 ± 12	0.25 ± 0.01	0.045 ± 0.004
	ART-27.13	8.65 ± 0.20	112 ± 2	7.36 ± 0.07	148 ± 3	0.32 ± 0.01 [§]	0.051 ± 0.002 [§]
	NTRX-07	6.76 ± 0.12	52 ± 6	6.03 ± 0.07	65 ± 7	0.44 ± 0.02	0.030 ± 0.003
	EHP-101	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	Tedalinab	8.52 ± 0.08	137 ± 5	7.55 ± 0.07	234 ± 10	0.34 ± 0.01 [§]	0.063 ± 0.008 [§]
	TAK-937	8.28 ± 0.08	41 ± 2	6.90 ± 0.10	27 ± 3	0.27 ± 0.01	0.016 ± 0.003

Potency (pEC₅₀, pIR₅₀) and efficacy (E_{max}, IR_{max}) values were determined from AUC and initial rate dose-response curves, respectively, derived from the β -arrestin-2 recruitment time traces in the multiplex assay. [#]In the absence of a DRC, maximal receptor activation (%) was determined at 10 μ M of agonist. Signaling rate constants k₁ and k₂ were determined at 10 μ M or 1 μ M ([§]) of agonist. Data are mean ± SEM from at least three independent experiments performed in duplicate. N.A is not applicable, N.D. is not detectable.

agonist-induced differences between the steepness of the decline phase after the peak responses are observed. For instance, a steep decline in β -arrestin-2 recruitment is observed for CP55,940, 2-AG, Tedalinab and PRS-211375, while the decline is more moderate for HU308 and HU910. Altogether, the shape of the curves is a first indication towards agonist-mediated differences in functional responses. In particular, differences in maximal effect are observed, but the shape of the time traces also accentuate variations in the responses over time.

3.4. Endpoint quantification of CB₂R activation by benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay

To quantify the effects of the benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay, their potency and efficacy values were determined with three different methods of analysis. Initially, all data was analyzed at four different time points to investigate the time-dependency of activation. To this end, dose-response curves of cAMP production and β -arrestin-2 recruitment were created for 7.5, 15, 30 and 60 min after agonist addition (Fig. S7-S10). Note that, no dose-response curves could be generated for 90 min after agonist addition as cAMP production returned to vehicle conditions for all agonist

concentrations. No potency values could be determined for the inhibition of cAMP production or β -arrestin-2 recruitment by CBD, GW-842166X or EHP-101, which is in line with their lack of CB₂R affinity (Table 1, S5, S6). Potency and efficacy values were only quantified for the early (7.5 min) and late (60 min) time points and the differences between those were calculated (Fig. 4, Table S5, S6).

Potency and efficacy values of HU910, JWH133, AEA, 2-AG, Dronabinol, Lenabasum, CBN, KN 387271, S-777469 and Tedalinab on inhibition of cAMP production remained statistically unaffected over time (Figs. 4, S7b,c,e,f,g,j, S8c,d,f,l, Table S5). On the other hand, potency values for CP55,940, Nabilone, Olorinab, PRS-211375, ART-27.13 and TAK-937 were significantly increased over time, whereas there was no time-dependent effect on efficacy (Figs. 4, S7d,h, S8a,h,i,m, Table S5). Interestingly, NTRX-07's potency significantly decreased over time, while a seeming increase in efficacy was not statistically significant (Table S5). While no pEC₅₀ value could be determined for LY-2828360 after 7.5 min (pEC₅₀ <10 μ M), its potency increased over time to 7.45 ± 0.38 (Fig. S8g, Table S5). Alternatively, HU308's potency was not dependent on time, but its efficacy was significantly increased from partial (78 ± 4 %) to full agonism (106 ± 7 %, Figs. 4, S7a, Table S5).

The potency and efficacy of HU308 and 2-AG for β -arrestin-2

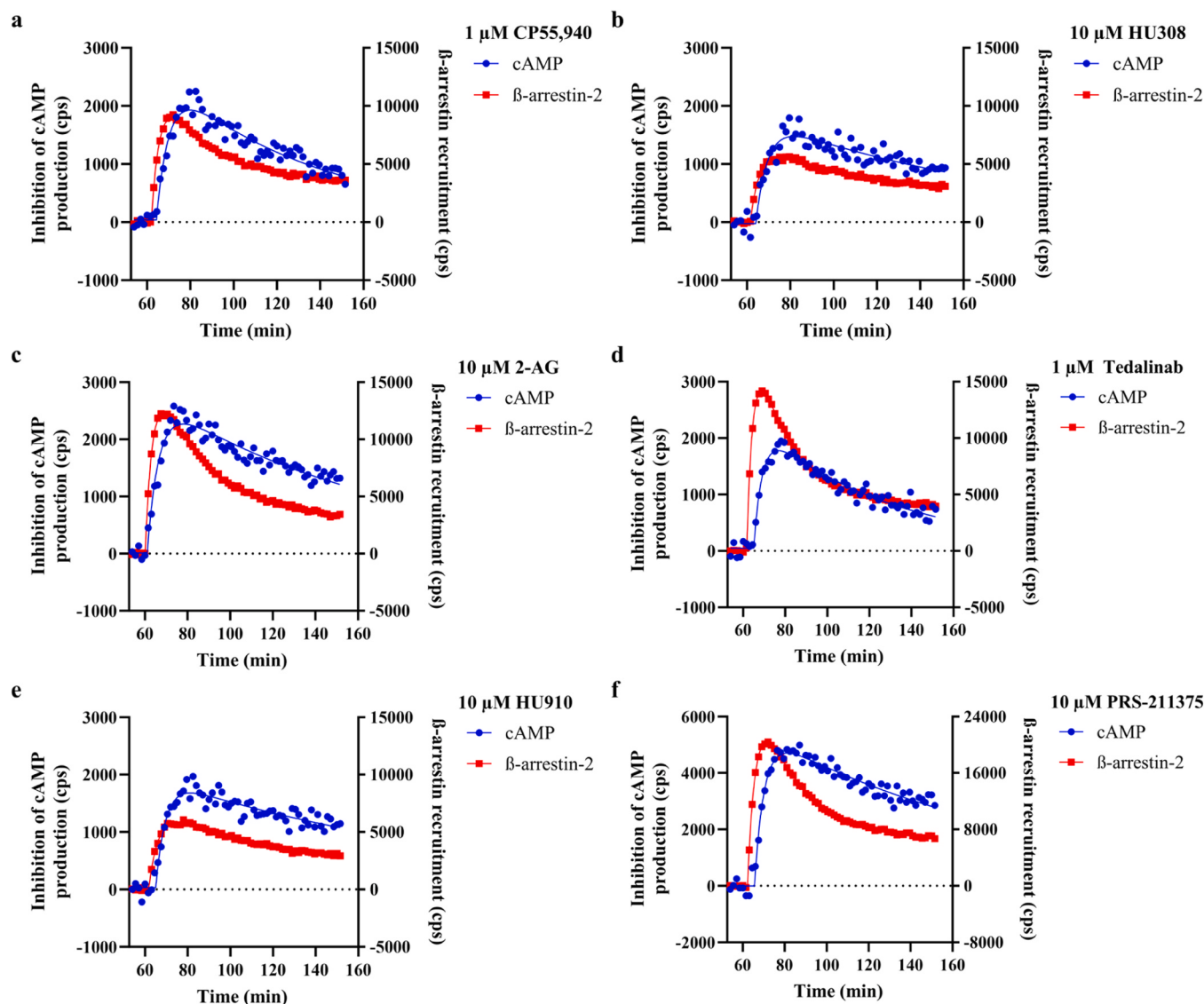


Fig. 3. Kinetic functional characterization of representative agonists CP55,940, HU308, 2-AG, Tedalinab, HU910 and PRS-211375 in the multiplex assay on HEK293T CB₂R-SmBiT LgBiT-β-arrestin-2 cells. Representative forskolin- and vehicle-corrected time traces for inhibition of cAMP production (left y-axis) and β-arrestin-2 recruitment (right y-axis) by (a) 1 μM CP55,940, (b) 10 μM HU308, (c) 10 μM 2-AG, (d) 1 μM Tedalinab (e) 10 μM HU910, or (f) 10 μM PRS-211375 in the multiplex assay on CB₂R. Data are shown as mean from a representative experiment all performed in duplicate. Representative dose-dependent time traces of all agonists can be found in Fig. 3.S2–3.S5.

recruitment were not dependent on time (Fig. 4, S9a,f, Table S6). Endocannabinoid AEA's potency was unaffected by longer incubation, but AEA's efficacy was significantly increased to $53 \pm 3\%$ after 60 min compared to $26 \pm 0\%$ at 7.5 min (Fig. 4, S9e, Table S6). The potency of the seventeen remaining agonists was significantly increased over time with varying effects on efficacy. Specifically, the efficacy was significantly increased for HU910, JWH133, CP55,940, Dronabinol, CMX-020, KN 387271, LY-2828360 and TAK-937, while the efficacy of Nabilone, Lenabasum, CBN, S-777469, PRS-211375 and NTRX-07 remained unaffected (Figs. 4, S9, S10, Table S6). On the other hand, the efficacy of Tedalinab was significantly decreased over time, and a similar trend was observed for Olorinab and ART-27.13 (Figs. 4, S10a,i,l, Table S4).

This method of analysis showed that activation by certain agonists is not time-dependent, whereas for other agonists potency and/or efficacy might be increased or decreased over time, and this may differ depending on functional assay. Furthermore, it suggests that interpretation of results is highly dependent on the selected time point for data analysis, i.e., endpoint analyses (and assays) can influence conclusions on ligand function and bias.

3.5. Semi-kinetic quantification of CB₂R activation by benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay

To incorporate the kinetic time traces, a second semi-kinetic method of analysis was pursued, which is commonly used to analyze time traces obtained from functional assays. To this end, the AUC of the full 90 min of activation was plotted against the agonist concentration and potency and efficacy values were determined for cAMP production and β-arrestin-2 recruitment. For agonists that showed CB₂R-independent cAMP production (Dronabinol, CBD, Lenabasum, Olorinab, CMX-020, CBN, KN 387271, GW-842166X, LY-2828360, ART-27.13, NTRX-07 and TAK-937), the curves were analyzed up to 1 μM in cAMP production (Fig. S6–S8). Furthermore, no potency values were determined for CBD, GW-842166X and EHP-101 due to their lack of cAMP production or β-arrestin-2 recruitment, which is in line with their lack of CB₂R affinity (Table 1–3).

Potency values determined for inhibition of cAMP production spanned a wide range. Dronabinol and CBN displayed the lowest potencies with pEC₅₀ values of 5.66 ± 0.06 and 5.70 ± 0.05 , respectively,

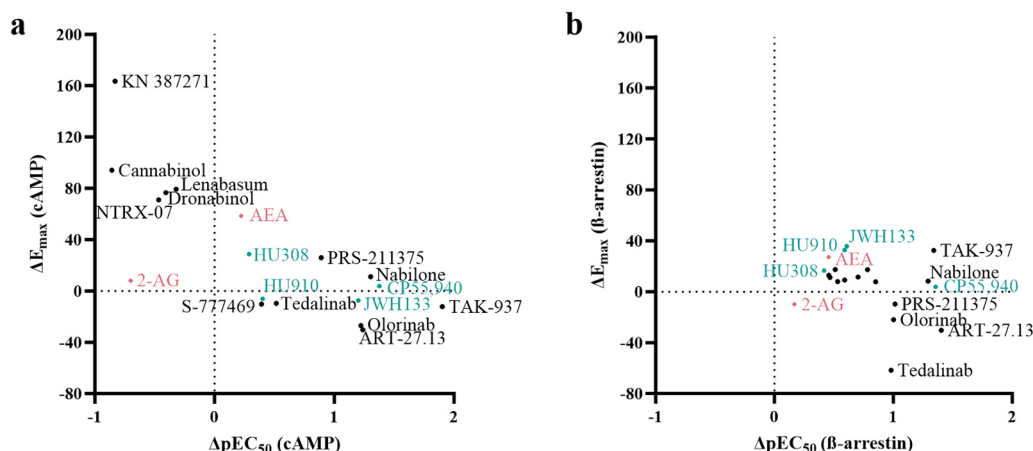


Fig. 4. Time-dependent effect on potency and efficacy of benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay on HEK293T CB₂R-SmBiT LgBiT- β -arrestin-2 cells. Differences in potency (ΔpEC_{50}) and efficacy ($\Delta E_{\max}$) from (a) cAMP production and (b) β -arrestin-2 recruitment assays by benchmark agonists, endocannabinoids and clinical agonists after 60 min of stimulation compared to 7.5 min. Benchmark agonists are turquoise hexagons (●), endocannabinoids coral diamonds (◆) and clinical agonists black circles (●). Data are shown as mean from at least three independent experiments performed in duplicate.

whereas agonists CP55,940, ART-27.13 and Tedalinab were full agonists and exhibited sub nanomolar potency with pEC_{50} values of 9.15 ± 0.16 , 9.15 ± 0.27 and 9.38 ± 0.25 , respectively (Table 2). HU308, HU910, 2-AG, Nabilone, Lenabasum, CMX-020, PRS-211375 and NTRX-07 behaved as full agonists with $E_{\max}$ values $> 90\%$, but with lower potency values compared to the before mentioned agonists, i.e., pEC_{50} values between 6.15 ± 0.17 and 7.80 ± 0.17 .

Secondly, the recruitment of β -arrestin-2 to CB₂R was determined in the multiplex assay (Table 3). Potency values, calculated from the AUC-derived dose-response curves, ranged from 5.90 ± 0.14 for 2-AG to 8.54 ± 0.04 for CP55,940. Various degrees of partial agonism were observed in the agonist set. HU910, JWH133 were full agonists with $E_{\max}$ values $> 90\%$, while Olorinab, PRS-211375, ART-27.13 and Tedalinab recruited β -arrestin-2 to CB₂R with a higher efficacy than reference full agonist CP55,940 with $E_{\max}$ values of $114 \pm 1\%$, $108 \pm 2\%$, $112 \pm 2\%$ and $137 \pm 5\%$, respectively, and could therefore be described as superagonists (exceeding $E_{\max}$ of CP55,940) in this readout. All remaining fourteen agonists displayed partial agonism in β -arrestin-2 recruitment with $E_{\max}$ values between 10% and 90% .

3.6. Kinetic quantification of CB₂R activation by benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay

To use the kinetic data to its fullest potential, kinetic signaling parameters were calculated using the kinetic mathematical model by Hoare *et al.*, i.e., currently the only equations available for convenient analysis of receptor activation and signal transduction time course data from cellular assays [20]. The initial rate (IR) was determined for each time trace and the kinetic potency (pIR_{50}) and kinetic efficacy ($IR_{\max}$) were obtained from the generated dose-response IR curve. This initial rate represents the rate of signal generation before the onset of regulatory mechanisms that turn down or turn off the signal [20]. Furthermore, the signaling rate constants for signal onset (k_1) and decline (k_2) were determined at a saturating concentration of agonist, i.e., $10 \mu M$ or $1 \mu M$ agonist (Table 2, 3), where k_1 represents the rate of onset of the response (the initial rise phase) and k_2 the rate of decline of the response (the later declining phase). From the kinetic trace fits, peak responses were obtained and also used to generate dose-response curves for parameter quantification. However, these results were identical to the AUC data and therefore not further analyzed (data not shown). No potency values were determined for CBD, GW-842166X and EHP-101 due to their lack of inhibition of cAMP production or β -arrestin-2 recruitment to CB₂R, which is in line with their lack of CB₂R affinity (Table 1-3).

Based on the shape of the agonist-induced inhibition of cAMP production time traces, a statistical comparison between two rise-and-fall equations was performed, i.e., the simpler 'rise-and-fall to baseline' which describes a decline of signaling, and more complex 'rise-and-fall to steady state', which describes a decline to levels above baseline. The first fit was statistically preferred for inhibition of cAMP production. The kinetic potencies ranged from pIR_{50} values of 5.73 ± 0.21 for AEA to 8.53 ± 0.14 for Tedalinab (Table 2). A large variety in kinetic efficacy was observed with full agonists HU308, CP55,940, 2-AG, Lenabasum, KN 387271, PRS-211375, ART-27.13 and Tedalinab displaying $IR_{\max}$ values over 90% . All other twelve agonists displayed partial agonism, which ranged from $10 \pm 1\%$ for CBN to $89 \pm 9\%$ for HU910. Moreover, signal onset and decline rate constants k_1 and k_2 for the agonist-mediated inhibition of cAMP production were calculated, where the rising phase of the curve is characterized by k_1 , and the fall phase by k_2 . These constants were determined at saturating concentrations of agonists, i.e., $10 \mu M$ for most agonists and $1 \mu M$ for potent agonists CP55,940, ART-27.13, Tedalinab and TAK-9337 (Table 2). Values for k_1 ranged from $0.04 \pm 0.01 \text{ min}^{-1}$ for slowly activating agonist CBN to $0.30 \pm 0.11 \text{ min}^{-1}$ for faster agonist KN 387271, a difference of maximally 7.5-fold, while k_2 values differed maximally 10-fold between fastest agonist Olorinab ($0.042 \pm 0.015 \text{ min}^{-1}$) and slowest detectable agonist Nabilone ($0.004 \pm 0.001 \text{ min}^{-1}$).

Based on the shape of the β -arrestin-2 recruitment traces, a statistical comparison between the two available rise-and-fall fits was performed, i.e., 'rise-and-fall to baseline' and 'rise-and-fall to steady state' equations, where in this case the latter was statistically preferred. The kinetic potencies ranged from pIR_{50} values of 5.19 ± 0.02 for AEA to 7.55 ± 0.07 for Tedalinab (Table 3). Superagonists Olorinab, PRS-211375, ART-27.13 and Tedalinab displayed high $IR_{\max}$ values of $157 \pm 2\%$, $133 \pm 12\%$, $148 \pm 3\%$ and $234 \pm 10\%$, respectively, while 2-AG behaved as a full agonist with a kinetic efficacy of $90 \pm 17\%$. All thirteen remaining agonists displayed partial agonism in β -arrestin-2 recruitment to CB₂R with $IR_{\max}$ values ranging from $4 \pm 1\%$ for Dronabinol to $80 \pm 10\%$ for HU308. The signal onset and decline rate constants k_1 and k_2 for the agonist-mediated β -arrestin recruitment were determined at saturating concentrations of agonist, i.e., $10 \mu M$ for most agonists and $1 \mu M$ for potent agonists (Table 3). The rising phase of the curve and corresponding parameter k_1 only differed less than 4-fold from slow agonist JWH133 ($0.18 \pm 0.01 \text{ min}^{-1}$) to faster agonist CMX-020 ($0.63 \pm 0.11 \text{ min}^{-1}$). The rate constant of the fall phase k_2 showed major differences with a maximal 32-fold difference between fastest agonist Tedalinab ($0.063 \pm 0.008 \text{ min}^{-1}$) and slowest detectable agonist Dronabinol ($0.002 \pm 0.001 \text{ min}^{-1}$).

The kinetic mathematical model allowed for a robust quantification of agonist-mediated inhibition of cAMP production and β -arrestin-2 recruitment. The two potency values from the semi-kinetic and kinetic analyses, i.e., pEC_{50} and pIR_{50} , respectively, correlated for both the inhibition of cAMP production (R^2 0.73, $p < 0.0001$) and β -arrestin-2 recruitment (R^2 0.90, $p < 0.0001$) (Fig. S11a,i). Furthermore, efficacy E_{max} and kinetic efficacy IR_{max} values were well correlated for both pathways (R^2 0.44, p 0.0006 and R^2 0.80, $p < 0.0001$) (Fig. S11b,j). The signal onset and decline rate constants, k_1 and k_2 , of the agonists were not predictive for the kinetic potencies for either inhibition of cAMP production or β -arrestin recruitment (Fig. S11c,d,k,l). However, k_1 values from the inhibition of cAMP production were not significantly correlated with differences in efficacy (ΔE_{max} values) from the time-dependent analysis, i.e., agonists with fast k_1 values tended to gain efficacy over time (Fig. S11g). For β -arrestin-2 recruitment signaling parameters, only a fast k_2 value positively correlated with ΔE_{max} values, whereas no statistically significant correlation was found between k_1 values and ΔE_{max} values (Fig. S11o,p). Interestingly, superagonists relative to CP55,940 in β -arrestin-2 recruitment were characterized by a decrease in efficacy over time, i.e., negative ΔE_{max} values, and all displayed fast k_1 values (Fig. S11o). Lastly, the interplay between inhibition of cAMP production and β -arrestin-2 recruitment was investigated in terms of the kinetic parameters. Kinetic potency values for all agonists in inhibition of cAMP production and β -arrestin-2 recruitment were significantly correlated ($R^2 = 0.93$, $p < 0.0001$) and only differed maximally 10-fold, indicating no overall pathway bias in the diverse agonist set (Fig. S11q). Similarly, kinetic efficacy (IR_{max}) values were significantly correlated ($R^2 = 0.53$, p 0.0002) (Fig. S11r). On the contrary, signal onset and decline rate constants k_1 and k_2 values were not correlated between the two readouts (k_1 : $p = 0.7044$, k_2 : $p = 0.4600$) (Fig. S11s,t).

Altogether, investigation of benchmark agonists, endocannabinoids and clinical agonists in the multiplex assay highlighted the diversity of signaling potencies and efficacies at CB₂R within this large set of agonists. Furthermore, it emphasized the possibility and importance of kinetic evaluation, including kinetic mathematical analysis, of receptor signaling. The kinetic mathematical equations allowed quantification of common pharmacological parameters, although with a kinetic context, i.e., kinetic potency (pIR_{50}) and kinetic efficacy (IR_{max}). Moreover, signal onset and decline rate constants k_1 and k_2 could be determined, which were not correlated between inhibition of cAMP production and β -arrestin-2 recruitment suggesting these parameters may play a role in biased signaling.

3.7. *In silico* docking to rationalize differences in binding affinities and signaling of CB₂R agonists

To structurally explore the (absence of) binding of the agonists in CB₂R, all agonists were docked to the binding pockets of all publicly available CB₂R x-ray and cryo-EM structures (6KPC, 6KPF, 6PTO, 8GUQ, 8GUT, 8GUS, 8GUR) [50,55,56]. From each pair of binding pocket and docked ligand the combination with the highest docking score was used for closer examination (Table S7, Fig. S12, S13). Although all ligands fit into the active state CB₂R binding pocket, there are marked differences with respect to the orientation and intermolecular interactions within the orthosteric pocket (Fig. S12, S13). Specifically, low docking scores were observed for CBD, GW-842166X and EHP-101 in the absence of favorable interactions with CB₂R (Table S7). This was in line with their lack of CB₂R affinity (Table 1).

The superagonists Tedalinab, ART-27.13, Olorinab and PRS-211375 displayed specific interactions in the CB₂R binding pockets that were not observed for any other agonist (Fig. S12, S13). Specifically, the six-membered terminal ring systems of Tedalinab, ART-27.13 and Olorinab make favorable interactions with the toggle switch region of CB₂R. Furthermore, the aromatic systems of Olorinab and Tedalinab undergo strong interactions with CB₂R including edge-to-face interactions with

surrounding aromatic protein moieties. Moreover, the 4,4-difluorocyclohexyl ring at the terminus of ART-27.13 allows for multiple favorable van der Waals interactions. The carboxylic acid moiety of PRS-211375 is in close proximity to K109, which introduces the possibility of a salt bridge formation. These specific interactions were not found in the remainder of the clinical agonists and thus might be responsible for triggering the observed superagonism in the β -arrestin-2 recruitment assay.

It is important to note that the *in silico* docking results cannot be used to interpret or predict biased signaling of the agonists. Particularly, since only active CB₂R structures in conjunction with the G protein are crystallized and to date, no CB₂R- β -arrestin-2 have been published.

3.8. Detection of binding kinetics of agonists at CB₂R

To further explore the kinetic context of CB₂R agonists, we determined the target binding kinetics in competition association assays of all agonists for which affinity could be determined (Fig. 5, Table 1). The association of [³H]RO6957022 to CB₂R was observed for 2 h in the absence (control) or presence of competing agonist (IC_{50} concentration). Agonists with similar residence times (RT) to the radioligand used, such as CP55,940, displayed an association curve with a similar shape to the control curve (Fig. 5a). Agonists with a longer RT compared to [³H]RO6957022, such as HU308 presented a characteristic overshoot (Fig. 5b). On the other hand, a slowly ascending curve was characteristic for agonists with a shorter RT compared to the radioligand, such as 2-AG and Tedalinab (Fig. 5c,d). Using the Motulsky-Mahan equation as described in Material and Methods, the kinetic binding parameters k_{on} and k_{off} were determined and subsequently these parameters were converted into engagement time (ET) for 1 μ M of agonist and RT, respectively [49]. ETs differed by 260-fold, with the fastest engagement to CB₂R for CP55,940 of below 1 s, and the slowest ETs for HU308 and AEA of 143.5 ± 47.9 s and 152.3 ± 10.4 s, respectively (Table 1). On the other hand, RTs only differed 44-fold. Dronabinol had the shortest interaction with CB₂R as represented by a RT of 2.1 ± 1.0 min, while TAK-937 displayed the longest RT of 93.2 ± 13.9 min (Table 1). Of note, due to the fast binding kinetics of CMX-020, CBN and KN 387271, their binding kinetics could not be quantified as these fell outside the temporal resolution of the assay.

To obtain a better understanding of the relationship between the affinity and kinetic binding parameters several correlation plots were constructed (Fig. S14). The equilibrium pK_i and kinetic pK_D values were significantly correlated, validating the competition association assay (R^2 0.93, $p < 0.0001$) (Fig. S14a). Furthermore, association rate constant k_{on} significantly correlated with affinity (R^2 0.78, $p < 0.0001$), while dissociation rate constant k_{off} did not correlate to affinity (Fig. S14b,c). This indicated that fast association is the driving factor for high affinity on CB₂R. The kinetic map, which presents the connection between k_{on} (x-axis), k_{off} (y-axis) and kinetic affinity K_D (diagonals lines), highlighted the diversity of combinations between k_{on} and k_{off} to reach similar affinities. Furthermore, it indicated no clear clustering of groups of agonists based on one of the binding parameters, underlining the chemical diversity of the agonists (Fig. S14d).

3.9. Exploration of the kinetic context of CB₂R agonists

To draw connections between molecular descriptors of CB₂R agonists, their binding and signaling, various correlation plots were constructed (Fig. S15). The investigated agonist set including benchmark agonists, endocannabinoids and clinical agonists displayed a high variety in chemotypes (Fig. 1). Moreover, the agonists were highly diverse in terms of lipophilicity (LogD, K_{ow} LogP), solubility, brain lipid binding, membrane permeation and protein binding (Table S2), providing a structurally and chemically diverse set of agonists to demonstrate the applicability of the new signaling assay. Nevertheless, none of the physicochemical parameters PSA, LogD or K_{ow} LogP of the agonists were

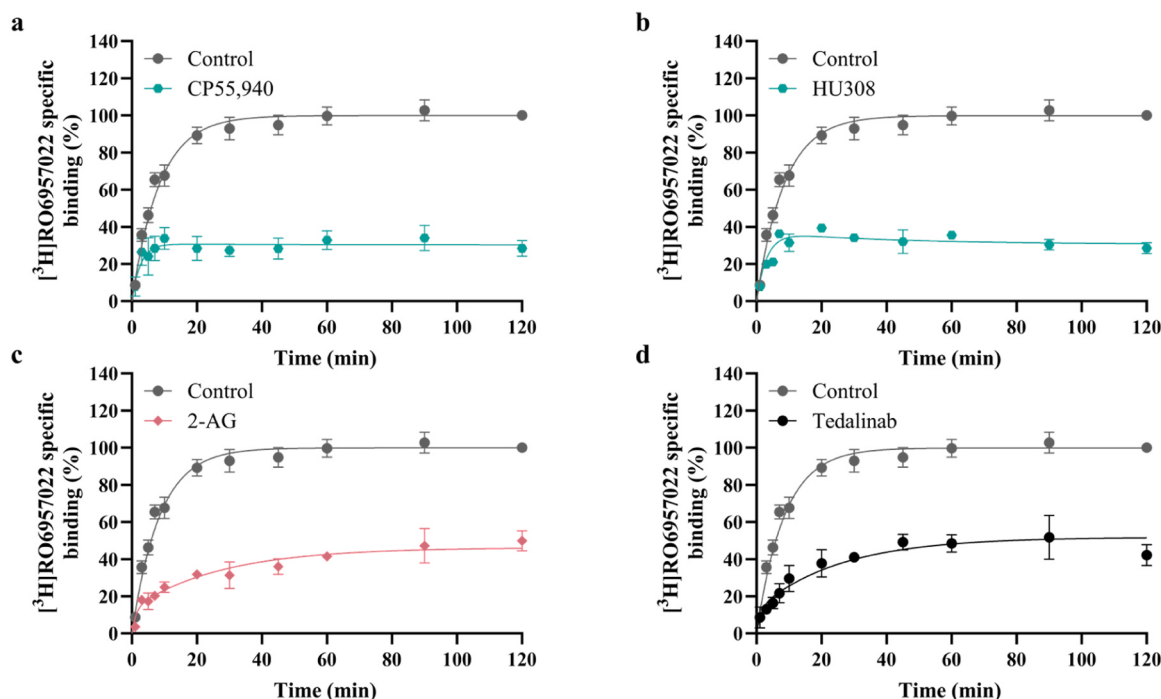


Fig. 5. Evaluation of CB₂R target binding kinetics of representative benchmark agonists CP55,940 and HU308, endocannabinoid 2-AG, and clinical agonist Tedalinab. Competition association assay with [³H]RO6957022 on CB₂R in the absence (control) or presence of an IC₅₀ concentration of representative competing agonists with (a) similar, (b) longer or (c, d) shorter receptor residence time (RT) compared to control, i.e., the radioligand used. Benchmark agonists are turquoise hexagons (●), 2-AG coral diamonds (◆) and clinical agonist Tedalinab black circles (●). Data are shown as mean ± SEM from three independent experiments performed in duplicate.

correlated with either affinity (pK_i) or association and dissociation rate constants k_{on} and k_{off} (Fig. S15). This suggested that the overall lipophilicity of the molecules, represented by different parameters, is not predictive for equilibrium or kinetic binding parameters at CB₂R.

To investigate whether kinetic binding parameters of CB₂R agonists could be predictive for signaling parameters various correlation plots were generated (Fig. S16). A fast association with CB₂R was significantly correlated with kinetic potencies pIR₅₀ in both inhibition of cAMP production (R^2 0.66, $p < 0.0001$) and β -arrestin-2 recruitment (R^2 0.73, $p < 0.0001$) (Fig. S16a,i). In other words, the faster engaging agonists displayed higher kinetic functional potencies than the slowly engaging agonists. In contrast, no correlation was found between association rate constants and kinetic efficacy IR_{max} or the time-dependent increase in efficacy in either readout (Fig. S16b,d,j,l). Unexpectedly, a quick engagement did not correlate with a quick activation, evident by the lack of statistically significant correlations between association rate constant k_{on} and rate of signal onset k_1 for either inhibition of cAMP production ($R^2 < 0.01$, p 0.8045) or β -arrestin-2 recruitment (R^2 0.03, p 0.5064) (Fig. S16c,k).

Dissociation rate constants k_{off} were not correlated with pIR₅₀ nor IR_{max} in both inhibition of cAMP production (R^2 0.02, p 0.5736; R^2 0.12, p 0.1587) or β -arrestin-2 recruitment (R^2 0.04, p 0.4569; R^2 0.06, p 0.3127) (Fig. S16e,f,m,n). On the contrary, a statistically significant correlation was observed for k_{off} values and time-dependent differences in efficacy for inhibition cAMP production (R^2 0.34, p 0.0139), but not β -arrestin-2 recruitment ($R^2 < 0.01$, p 0.9673) (Fig. S16h,p). Specifically, fast dissociating agonists displayed an increase in cAMP efficacy over time (Fig. S16h). Furthermore, no statistically significant correlations were found between dissociation rate constants and the rate of signal decline k_2 in cAMP production ($R^2 < 0.01$, p 0.8798) or β -arrestin-2 recruitment (R^2 0.19, p 0.0705) (Fig. S16g,o). Although, a small trend was displayed for slow dissociating agonists and slow deactivation in β -arrestin-2 recruitment (Fig. S16o).

Altogether, these correlations highlight that optimization of

association rate constants rather than dissociation rate constants may contribute to high kinetic potencies but does not affect kinetic efficacy. Nevertheless, agonists with a fast dissociation rate constant might gain efficacy over time in inhibition of cAMP production and a fast k_{off} may play a role in deactivation of β -arrestin-2 recruitment. This emphasizes the importance of providing a full kinetic overview of agonist-mediated CB₂R signaling.

3.10. Determination of ligand bias at CB₂R

Ultimately, biased signaling at CB₂R was investigated by using the different methods of analysis, i.e., an endpoint, a semi-kinetic and a kinetic analysis. The operational model was used to derive transduction coefficients LogR from the inhibition of cAMP production and β -arrestin-2 recruitment dose-response curves. To investigate the time-dependency of bias and the impact of different analysis strategies, the model was applied to the dose-response curves generated after 7.5 and 60 min of agonist activation (endpoint), AUC (semi-kinetic) and IR (kinetic) determination. In all cases, the LogR values were normalized to most balanced agonist HU308 resulting in Δ LogR for each agonist per pathway. Subsequently, these ratios were compared per agonist between the cAMP and β -arrestin-2 pathways, which presented the relative bias Δ LogR (Fig. 6, Table S8). As mentioned earlier, CBD, GW-842166X and EHP-101 failed to activate (or bind to) CB₂R, and consequently no LogR values were calculated for these agonists.

No clear preference for either pathway was observed for Olorinab, S-777469, ART-27.13, NTRX-07 and Tedalinab relative to HU308 using any of the methods of analysis. HU910 displayed a slight preference for inhibition of cAMP production over β -arrestin-2 recruitment in the endpoint analysis after 7.5 and 60 min of activation, whereas in the semi-kinetic and kinetic determination no clear bias was observed. On the other hand, after 7.5 min of activation, Nabilone and PRS-211375 demonstrated a small bias towards β -arrestin-2 recruitment over inhibition of cAMP production relative to HU308, but the preference for β -

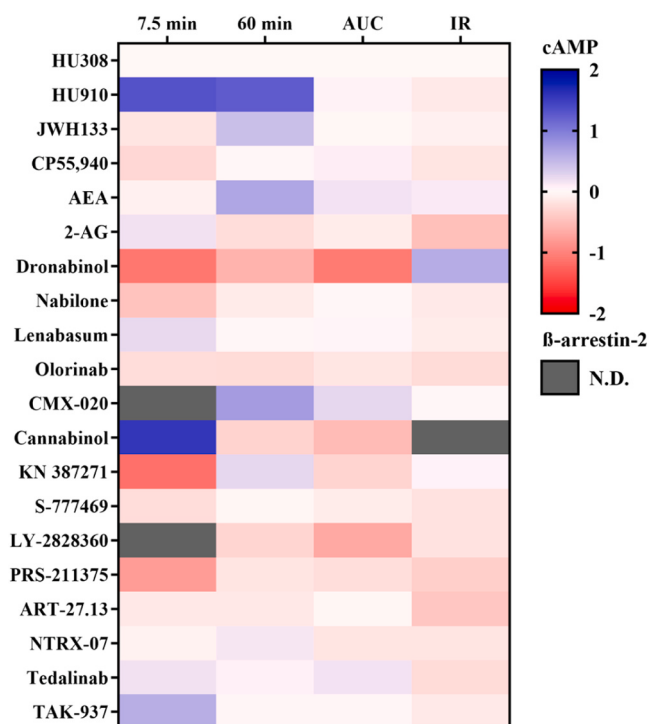


Fig. 6. Quantification of biased agonism between inhibition of cAMP production and β -arrestin-2 recruitment by benchmarks agonists, endocannabinoids and clinical agonists determined in the multiplex assay on CB₂R using an endpoint, semi-kinetic or kinetic analysis. The dose response curves of all agonists based on endpoint (i.e., 7.5 and 60 min after agonist addition), semi-kinetic (AUC) or kinetic (IR) analysis were analyzed using the operational model of agonism to obtain transduction coefficients LogR, which were normalized to the balanced agonist HU308 per pathway (Δ LogR ratios). The bias between the two pathways was reported as $\Delta\Delta$ LogR and is shown in the heatmap. Blue indicates bias towards cAMP production, whereas red indicates bias towards β -arrestin-2 recruitment. Agonists for which bias could not be determined (N.D.) due to absence of a dose response curve are presented in grey. CBD, GW-842166X and EHP-101 are omitted from the heatmap due to lack of receptor affinity and activation. One-way ANOVA was performed to analyze differences in $\Delta\Delta$ LogR values of agonists compared to HU308 (* $p < 0.05$). Data are mean from at least three independent experiments performed in duplicate. Bias factors can be found in Table S8.

arrestin-2 recruitment was attenuated after 60 min of activation, as well as in the semi-kinetic and kinetic analysis. Time-dependent bias was observed for JWH133, AEA, 2-AG, CBN, KN 387271 evident by switched preferences after either 7.5 or 60 min of activation. A bias towards β -arrestin-2 recruitment over inhibition of cAMP production was observed for Dronabinol in the endpoint and semi-kinetic analysis, whereas the bias was flipped towards inhibition of cAMP production in the kinetic analysis. No bias factor could be calculated for LY-2828360 after 7.5 min of activation due to the absence of a complete dose-response curve in the inhibition of cAMP production, while potency could be determined in β -arrestin-2 recruitment. This may suggest that at the early time point LY-2828360 is strongly biased towards β -arrestin-2 recruitment over inhibition of cAMP production, which is also observed in the semi-kinetic and kinetic analysis although slightly less pronounced. Although trends in biased signaling were observed for all other agonists, no statistically significant bias between inhibition of cAMP production and β -arrestin-2 recruitment was found relative to HU308 in our system independent of the method of analysis used. However, in general a small trend towards β -arrestin bias may be observed for several agonists (Fig. 6).

As a receptor's constitutive activity in kinetic context can play an influential role on functional profiles of agonists, we determined the

level of CB₂R constitutive activity between cAMP inhibition and β -arrestin recruitment in our multiplex system testing inverse agonist AM630, reference agonist CP55,940 and balanced agonist HU308. (Fig. S17) Differences in activity were found between vehicle and 1 μ M AM630 in both cAMP inhibition and β -arrestin-2 recruitment in varying levels of significance, therefore concluding detectable constitutive activity for both pathways.

Ultimately, we explored whether CB₂R binding kinetics could be predictive for the degree of bias. To maintain the kinetic context, correlation plots were generated for association and dissociation rate constants and the $\Delta\Delta$ LogR values obtained from kinetic analysis. No statistically significant correlation was found between k_{on} or k_{off} values and $\Delta\Delta$ LogR values of the agonists ($R^2 < 0.01$, p 0.9368; R^2 0.15, p 0.1296) (Fig. S16q,r).

Altogether, no statistically significant bias was observed for any of the agonists between the inhibition of cAMP production and β -arrestin-2 recruitment determined in our multiplex assay. Small differences were observed in trends for biased signaling, which were in some cases dependent on the method of analysis (Fig. 6, Table S8).

3.11. Determination of ligand bias at CB₁R

To further validate our novel multiplex assay's ability to reliably detect biased ligands, commercially available CB₁R biased compound JWH145 was tested and compared with reference agonist CP55,940. Results were analyzed similarly to prior CB₂R agonists (Fig. 7, Table 4). As inclusion of JWH145 in CB₁R was only for proof-of-concept purposes, potency and efficacy parameters as well as bias calculations were done exclusively according to the semi-kinetic quantification method, with LogR values normalized to CP55,940 as reference agonist.

For inhibition of cAMP production, JWH145 acted as a partial agonist in inhibition of cAMP production for CB₁R, reaching an E_{max} of 65 ± 4 and pEC_{50} of 6.27 ± 0.07 (Table 4).

JWH145 was inactive for β -arrestin-2 recruitment with a maximal activation of 5 % at 10 μ M agonist normalized to CP55,940 (Fig. 7, Table 4). Consequently, no $\Delta\Delta$ LogR values could be calculated for β -arrestin-2 recruitment, indicating JWH145 qualitatively demonstrates bias towards cAMP inhibition referred to as modality bias [11].

4. Discussion

Ligand bias is an emerging concept that holds great promise to advance drug discovery by selectively targeting therapeutic relevant signaling pathways [1–3]. However, examination of ligand bias *in vitro* is rather challenging, and experiments need to be designed with great caution to reduce system or observation bias [11]. Most multiplex applications rely on fluorescent labeling, but multiplex luciferase reporters have also emerged for detection of genes as indicator of downstream signaling [57,58]. Nevertheless, these technologies often rely on sequential analysis of two (or more) luciferases by quenching the light of the first enzyme before addition of the second substrate. Additionally, cells often need to be lysed, so measurements are typically measured at a single time point (endpoint assays), which removes the dynamic context of signaling events [58,59]. To this end, we developed a novel multiplex assay that simultaneously detects cAMP production and β -arrestin-2 recruitment in a kinetic manner in the same cells. We demonstrated the applicability of this kinetic multiplex assay on CB₂R by screening a large panel of diverse agonists (Fig. 1, Table S1). Subsequently, we validated our multiplex assay for detection of biased signaling with biased agonist JWH145 on CB₁R.

In the multiplex assay presented in this study, we successfully combined the GloSensor technology for capturing differences in cAMP production and NanoBIT for β -arrestin-2 recruitment to CB₂R (Fig. 2, Table S4), which generated similar potency values for reference agonist CP55,940 as previously seen in literature [28,35,60]. Biased signaling at CB₂R has never been investigated in a kinetic context and, to our

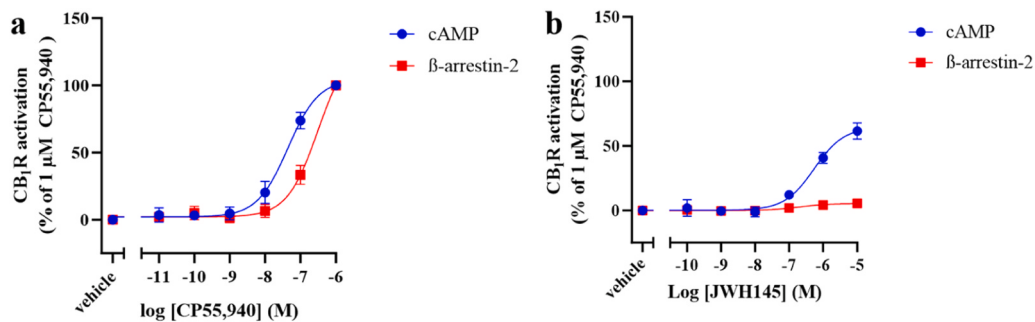


Fig. 7. Semi-kinetic (AUC) curves of inhibition of cAMP production and β -arrestin-2 recruitment to CB₁R by benchmark agonist CP55,940 and biased agonist JWH145 from the multiplex assay on HEK293T CB₁R-LgBiT SmBiT- β -arrestin-2-366 truncated cells. Inhibition of cAMP production and recruitment of β -arrestin-2 curves by (a) CP55,940 and (b) JWH145. Efficacy was calculated as percentage of the maximum effect induced by 1 μ M CP55,940. Data are shown as mean $\pm$ SEM from at least three independent experiments performed in duplicate.

Table 4
Inhibition of forskolin-induced cAMP production and β -arrestin-2 recruitment by benchmark agonist CP55,940 and biased agonist JWH145 determined in the multiplex assay on CB₁R.

Agonist	cAMP signaling				β -arrestin-2 recruitment				
	pEC ₅₀	E _{max} or receptor activation at 10 μ M (%)	AUC LogR	AUC Δ LogR	pEC ₅₀	E _{max} or receptor activation at 10 μ M (%)	AUC LogR	AUC Δ LogR	AUC $\Delta\Delta$ LogR (Bias factor)
CP55,940	7.36 $\pm$ 0.08	105 $\pm$ 1	7.45 $\pm$ 0.06	0.00 $\pm$ 0.09	6.5 $\pm$ 0.09	133 $\pm$ 7	6.82 $\pm$ 0.04	0.00 $\pm$ 0.06	0.00 $\pm$ 0.11 (1.00)
JWH145	6.27 $\pm$ 0.07	65 $\pm$ 4	6.07 $\pm$ 0.04	-1.37 $\pm$ 0.07	N.D.	5 $\pm$ 1 [#]	N.D.	N.A.	N.A. (modality bias)

Potency (pEC₅₀) and efficacy (E_{max}) values were determined from AUC dose-response curves, derived from the cAMP and β -arrestin-2 time traces in the multiplex assay. [#]In the absence of a DRC, maximal receptor activation (%) was determined at 10 μ M of agonist. Data are mean $\pm$ SEM from at least three independent experiments performed in duplicate. N.A. is not applicable, N.D. is not detectable.

knowledge, experiments have always been conducted in different functional (endpoint) assays with different cellular backgrounds, which could potentially introduce system bias [31,33–35,37]. Furthermore, reported agonist incubation times range from 5 min to 90 min and sometimes even differed between assays within the same study. To investigate the time-dependency of activation and the impact of data analysis approaches, we analyzed the multiplex data using three methods: 1) as endpoint, i.e., after 7.5 and 60 min, 2) semi-kinetic AUC analysis (both these methods provide the traditional potency and efficacy values) and 3) kinetic analysis using mathematical models that provided kinetic potency and kinetic efficacy based on initial rates (pIR₅₀, IR_{max}) and signaling rate constants k_1 and k_2 , representing the rate of signal onset and decline, respectively [20]. Since the recent introduction of these novel curve fitting models, they have been carefully being incorporated in pharmacological studies to aid more accurate characterization of ligand-receptor interactions for a variety of receptors and pathways [5,61–65]. We found different time-dependent effects in inhibition of cAMP production and β -arrestin-2 recruitment and that time-dependency varied per agonist used. Moreover, results from the semi-kinetic and kinetic analyses were well correlated, while the kinetic analysis additionally provided signaling rate constants k_1 and k_2 . Importantly, the impact of the chosen method of data analysis became clear in ligand bias analyses (Fig. 6, Table S8). A trend in increased bias towards cAMP over time was observed for HU910, while the slight cAMP bias of TAK-937 was attenuated and 2-AG switched from cAMP preference at 7.5 min to β -arrestin-2 preference after 60 min. These results are in line with results on the dopamine D₂ receptor, where it was demonstrated that apparent ligand bias could change over time [13].

To this end, the semi-kinetic and kinetic analyses were employed to reduce the influence of time and use the obtained signaling time traces to their fullest extent. In general, the observed potency values for inhibition of cAMP production and β -arrestin-2 recruitment were slightly lower compared to literature [28,35,66–68]. On the other hand, Lin *et al.* observed no β -arrestin-2 recruitment to CB₂R after activation by

LY-2828360, whereas in our multiplex assay we found a low potency for LY-2828360 although with low efficacy (Table 3) [68]. Moreover, the observed statistically significant correlations between the pIR₅₀ and IR_{max} values from inhibition of cAMP production and β -arrestin-2 recruitment suggest a lack of bias in the overall agonist set. However, the signal onset and decline rate constants k_1 and k_2 were not correlated and as such may contribute to the small trends in bias that were observed for Dronabinol and 2-AG. Thus, we envision that kinetic analysis, or early time point analysis, in *in vitro* assays generates parameters that better predict the *in vivo* pharmacological effects, i.e., starting GPCR activation and signal transduction without interference of artificial regulation mechanisms [20]. Interestingly, a faster engagement (k_{on}) of agonists with CB₂R correlated with both a higher affinity as well as a higher potency in both inhibition of cAMP production and β -arrestin-2 recruitment (Fig. S14b, S16a,i), and as such is a driving force for CB₂R binding and signaling. While a quick engagement with CB₂R did not result in a quick signal onset (k_1), we found that superagonists Olorinab, PRS-211375, ART-27.13 and Tedalinab in β -arrestin-2 recruitment were characterized by fast signal onset rate constants k_1 in the kinetic analysis. However, not all agonists with fast k_1 values behaved as superagonists. Although no correlation was found for k_{off} and affinity or potency (Fig. S14c, S16e,m), we observed that slowly dissociating agonists exhibited slow deactivation of β -arrestin-2 recruitment, which may suggest that extended agonist binding results in a longer receptor interaction with β -arrestin-2. Since the introduction of the target binding kinetics as an important concept for drug discovery [14], the primary focus has been on optimization of RT, rather than on association rate constants [69]. However, this study clearly indicates that optimization of the association rate constants for CB₂R agonists is equally valuable to improvement of the dissociation rate constants.

The applied kinetic signaling models do not only allow for quantification of signaling parameters, but the shape of the traces may also be indicative of cellular regulation mechanisms. For example, our cAMP data was best fit by the ‘Baseline then rise-and-fall to baseline’ model,

which was also previously reported for cAMP production after activation of the adenosine A₁ receptor and β_2 -adrenoceptor [62,64]. The simplest cellular mechanism that can produce this curve shape involves an initial signal burst from the receptor, followed by receptor desensitization to prevent further signaling, combined with decline of the cAMP inhibition signal itself, potentially resulting from inactivation of G α_i [20]. [61] Agonist-dependent differences were observed in the time traces. Some agonists, such as Tedalinab, displayed a steeper decline of the cAMP inhibition signal, which was reflected by higher signal decline rate k_2 values (Fig. 3d, Table 2). One potential mechanism to explain this effect is more rapid receptor desensitization, which requires further experimental investigation [62]. Other agonists, such as AEA and EHP-101 lack β -arrestin-2 recruitment but do show sustained cAMP profiles potentially due to the inability of the receptor to internalize (Fig. S2–S5), as opposed to other agonists that are able to activate receptor internalization via β -arrestin-2. Atwood *et al.* described how agonist-specific rates and differences in level of internalization in rat CB₂R contribute to functional selectivity. However, initiation of endosomal signaling (or lack thereof) can induce location bias mistakenly seen as signaling bias or *vice versa* [61].

The majority of these compounds have been reported as selective CB₂R agonists usually confirmed by broad selectivity profiling *in vitro* [70] Unexpectedly, this work shows CB₂R-independent cAMP responses in parental HEK293T cells (i.e. in absence of CB₂R), thus revealing off-target effects (Fig. S6). However, at the high concentration ranges used in our assay (up to 10 μ M) off-targets may be hit, albeit therapeutically irrelevant. The β -arrestin-2 recruitment time traces were best analyzed using the 'Baseline then rise-and-fall to steady state' model (Fig. 3, Table 3), which has been previously reported for β -arrestin-2 recruitment time traces to other GPCRs [5,61,64]. Several mechanisms can produce this curve shape, including receptor desensitization, internalization, recycling to the cell membrane or degradation [61]. Several studies have reported that agonist-stimulated CB₂R also undergoes these processes [71–78]. For example, Grimsey *et al.* described that HU308-induced internalization of CB₂R did not result in degradation of the receptor, but rather recycling of the receptor to the cell membrane. However, a remaining proportion of the receptors was not recycled nor degraded but remained in the cytoplasm [73]. To be able to assign signal onset and decline rate constants k_1 and k_2 to particular cellular regulation mechanisms, additional investigation to reveal differential agonist-dependency for initiation of β -arrestin-mediated internalization, recycling, or degradation at CB₂R needs to be done, alike results found at the β_2 -adrenergic receptor [79].

For proper use of the Black-Leff operational model for bias analysis the maximal response of the system needs to be accurately estimated [52,80]. Several agonists displayed superagonism in β -arrestin-2 recruitment compared to full agonist CP55,940, which was most prominent for Tedalinab (Table 2, 3). Therefore, Tedalinab was used as full agonist, which also improved the fits on all other agonists (data not shown). Similar to previous work, benchmark agonists HU308, HU910 and JWH133 remained balanced between the two studied pathways (Fig. 6, Table S8) [35]. Although some trends towards biased signaling were observed, none of the calculated $\Delta\Delta\text{LogR}$ values were significantly different from balanced agonist HU308 (Fig. 6, Table S8). To prove applicability of our multiplex assay in detection of biased signaling, we assessed biased compound JWH145 at CB₁R. Although no bias factor could be calculated for JWH145 due to antagonistic behavior in β -arrestin-2 recruitment, we can confidently confirm that our novel multiplex assay is able to detect biased signaling simultaneously in one well. Similar patterns of combined agonistic and antagonistic behavior for separate pathways resulting in ligand bias have been observed previously in literature and recent IUPHAR guidelines proposed to call this modality bias [1].

Possibly previously reported biased signaling of CB₂R agonists (bias factors ranging between 0.0015 and 90) may have been influenced by system and observational bias due to the use of a biased reference

agonist, different cellular backgrounds, time points and measurements used to report bias amongst pathways [52,80]. While our multiplex assay minimizes most of the issues regarding system and observational bias, not all potential factors are eliminated. Using inverse agonist AM630, we found significant receptor constitutive activity for cAMP inhibition in comparison to a small significant level of receptor constitutive activity for β -arrestin-2 recruitment, which would promote bias trends towards inhibition of cAMP recruitment (i.e. due to observational bias) (Fig. S17). However, the general trend in bias for the profiled CB₂R agonists was actually towards β -arrestin-2 recruitment. This suggests that the bias towards β -arrestin-2 might be even more pronounced than can be measured using our multiplex assay.

Interestingly, the FDA approved μ -opioid receptor biased agonist oliceridine only displayed a bias factor of 3 for G protein activation over β -arrestin-2 recruitment, while bias factors up until 85 have been reported on the same receptor [81,82]. Nevertheless, more recent studies have hypothesized that the partial agonism of oliceridine may be responsible for the beneficial therapeutic effect rather than its biased profile [83]. However to date, the mechanism of therapeutic effects at CB₂R and the potential importance of biased signaling is largely unknown. To prove whether signaling bias on CB₂R has therapeutic relevance, the mechanism should be further explored. This may be done by *in vitro* in relevant native cell background and/or *in vivo* by knockout studies of β -arrestin-2 or G α_i proteins to specify which signaling pathway is therapeutically relevant [1,11].

In conclusion, this study describes the development and application of a novel multiplex assay that simultaneously detects cAMP production and β -arrestin-2 recruitment in a time-dependent manner in the same cells. Using our multiplex assay, biased signaling can be detected in relation to a balanced reference ligand, while avoiding system or observational bias. We demonstrate that agonist-mediated CB₂R activation and biased signaling is time sensitive and dependent on the specific agonist used. Similar potency and efficacy parameters can be obtained from semi-kinetic and kinetic methods of analysis, while the latter additionally provides signaling rate constants that may accentuate differences between agonist-dependent signaling and bias. Moreover, this study demonstrates the importance of fast agonist engagement (k_{on}) with CB₂R for increased affinity and potency, while slowly dissociating agonists extended the interaction between CB₂R and β -arrestin-2. We envision that kinetic parameters that capture the early response, such as pIR_{50} , IR_{max} and signal onset rate constant k_1 , could be applied to *in vitro* studies as better predictors of their pharmacological effects *in vivo*. Ultimately, combining these parameters with binding kinetics results in more extensively profiled GPCR agonists to advance future drug discovery efforts.

CCRediT authorship contribution statement

Arne C. Rufer: Writing – review & editing, Conceptualization. **Uwe Grether:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Matthias B. Wittwer:** Methodology, Investigation, Formal analysis. **Wolfgang Guba:** Methodology, Investigation. **Samuel R.J. Hoare:** Formal analysis. **Sanjay S. Kumar:** Investigation. **Barry J.W. van den Berg:** Investigation. **Elisa J.M. de Koning:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Cas van der Horst:** Investigation, Formal analysis. **Mario van der Stelt:** Writing – review & editing, Supervision, Conceptualization. **Laura H. Heitman:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jara Bouma:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal

relationships which may be considered as potential competing interests: Laura Heitman reports financial support was provided by Dutch Research Council. S.R.J.H. was employed by the company Pharme-mechanics LLC. M.B.W., W.G., A.R. and U.G. are full employees of F. Hoffmann-La Roche Ltd. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.phrs.2025.108021](https://doi.org/10.1016/j.phrs.2025.108021).

Data Availability

Data will be made available on request.

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