It's about time: novel drug discovery concepts for the molecular pharmacological characterization fo the cannabinoid CB2 receptor

Bouma, J.

Chapter 2

Cellular assay to study β-arrestin recruitment by the cannabinoid CB_1 and CB_2 receptor

Jara Bouma, Marjolein Soethoudt, Noortje van Gils, Lizi Xia,
Mario van der Stelt, Laura H. Heitman

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Abstract

Cannabinoid CB_1 and CB_2 receptors (CB_1R and CB_2R) are G protein-coupled receptors (GPCRs) that activate a variety of pathways upon activation by (partial) agonists, including the G protein pathway and the recruitment of β-arrestins. Differences in activation level of these pathways leads to biased signaling. In this chapter, we describe a detailed protocol to characterize the potency and efficacy of ligands to induce or inhibit β-arrestin-2 recruitment to the human CB_1R and CB_2R using the PathHunter® assay. This is a cellular assay that uses a β-galactosidase complementation system which has a chemiluminescent read-out and can be performed in 384-well plates. We have successfully used this assay to characterize a set of reference ligands (both agonists, antagonists, and an inverse agonist) on human CB_1R and CB_2R, of which some examples will be presented here.

2.1 Introduction

Cannabinoid CB_1 and CB_2 receptors (CB_1R, CB_2R) are members of the G protein-coupled receptor (GPCR) superfamily[1]. Due to their localization, they regulate distinct physiological processes such as the control of cognition, memory and motor function for CB_1R, while CB_2R modulates (neuro)inflammatory processes[2]. Both receptors couple mostly to $G\alpha_{i/o}$ proteins to inhibit the activity of adenylate cyclase, activate the mitogen-activated protein kinase (MAPK) pathway and influence several ion channels[3,4]. Additionally, upon agonist stimulation CB_1R and CB_2R both recruit β-arrestin-2 to inactivate the receptors due to desensitization and internalization, but they only have a weak interaction with β-arrestin-1[5–7].

Following GPCR activation, some ligands preferentially modulate specific pathways over others. This concept of biased signaling, biased agonism or functional selectivity may increase drug effectiveness by selectively targeting the signaling pathways involved in the therapeutic effects and to move away from on-target side effects through other pathways[8–10]. This concept has also been thoroughly investigated and reviewed for both CB_1R and CB_2R, indicating biased signaling between G proteins and β-arrestin-2[11–18].

To characterize the potency and efficacy of cannabinoid receptor ligands to recruit β-arrestin-2, we have employed the previously reported β-arrestin PathHunter® assay[19,20]. This assay is an easy-to-use endpoint assay that has shown to deliver reproducible results and was successfully applied to measure β-arrestin-2 recruitment for a variety of cannabinoid receptor ligands[21–25]. In this assay, β-arrestin-2 activity is measured in live cells by using a

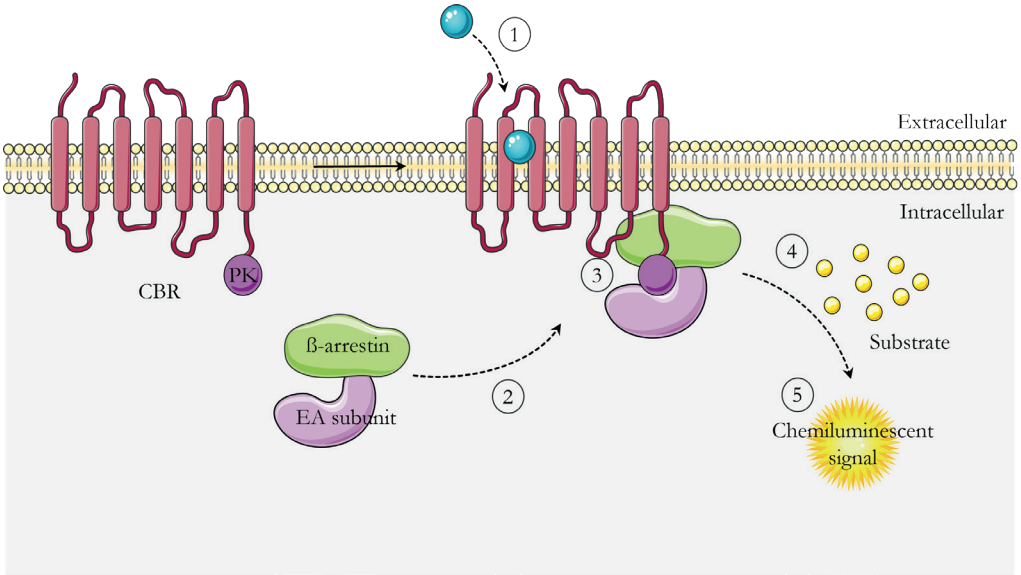

Figure 2.1 Schematic representation of the β-arrestin recruitment assay.
Upon activation of the receptor by a ligand (1), the inactive β-arrestin-EA complex is recruited to the receptor (2). This induces the complementation with the ProLink™ enzyme fragment (PK) to form an active β-galactosidase enzyme (3). The enzyme then reacts with the substrate in the PathHunter® detection reagent mixture (4), which results in a chemiluminescent signal that is directly related to the amount of β-arrestin-2 recruited to the activated receptor (5). The complemented enzyme remains receptor bound, but this is not shown in the figure for clarity reasons. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

β-galactosidase fragment complementation technology and a chemiluminescent read-out (**Figure 2.1**)[26,27]. The DiscoverX PathHunter® cell lines overexpress a GPCR of interest, in this case either the human CB_1R or CB_2R, which are tagged at the C-terminus by a small donor fragment of β-galactosidase called ProLink™ (PK). Additionally, these cells stably co-express β-arrestin-2, which is fused with the catalytically inactive N-terminal deletion mutant fragment of the β-galactosidase, i.e. the enzyme acceptor (EA)[20,26]. Activation of CB_1R or CB_2R will cause recruitment of the β-arrestin-EA complex to the PK-tagged receptor, which induces the complementation of the two enzyme fragments. Subsequently, this results in the formation of an active β-galactosidase. The active enzyme is able to convert an added substrate into a chemiluminescent product[19,20,26]. The light emission by this product is directly related to the activity of the β-galactosidase, and thus the level of β-arrestin-2 recruitment to the receptor after ligand binding[23,26,28]. In addition, its 384-well format enables high-throughput screening, a useful feature in early drug discovery research.

Of note, recent observations have shown that kinetics of cell signaling processes are important for the interpretation of agonist behavior and biased signaling[29–32]. As a consequence, novel β-arrestin assays have been developed which focus on a kinetic readout such as the bioluminescence resonance energy transfer (BRET) assays[5,33] and the luminescent NanoBiT assay[34]. In these assays, the receptor of interest is transfected into a chosen cell system. This provides an added advantage, since the effect of "system bias", i.e., bias caused by differential expression or amplification of second messengers depending on tissue or cell background, can be eliminated by the use of one cell system[8,35]. We envision that the field of cannabinoid receptor research will also move into the direction of kinetic signaling assays to further explore the concept of biased signaling.

2.2 Materials

All buffers and solutions are prepared using Millipore water (deionized using a MilliQ A10 Biocel™, with a 0.22 μm filter) and analytical grade reagents and solvents. Buffers are prepared at room temperature (rt) and stored at 4 °C, unless stated otherwise. Cannabinoid receptor reference ligand CP55,940 was obtained from Sigma Aldrich (St. Louis, MO), JWH133 was from Tocris BioScience (Bristol, United Kingdom), SR144528 and SR141716A were from Cayman Chemical Company (Ann Arbor, MI) and WIN55,212-2 was received from Hoffman-La Roche (Basel, Switzerland).

2.2.1 Cell Culture

1. Cells: PathHunter® CHO-K1 β-Arrestin Cell Line CNR1 or CNR2 (DiscoverX), overexpressing the CB_1R or CB_2R respectively. In this thesis, these cells are named $CHOK1hCB_1$_bgal or $CHOK1hCB_2$_bgal.
2. Phosphate buffered saline (PBS): 1.9 mM KH_2PO_4, 136.9 mM NaCl, 8.0 mM Na_2HPO_4, 3.4 mM KCl.
3. Trypsin solution: 0.25% trypsin in PBS, containing 0.44 mM EDTA.

4. Cell culture medium: Ham's F12 Nutrient Mixture supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM Glutamax, 50 µg/mL Penicillin/Streptomycin, 800 µg/mL G418 and 300 µg/mL Hygromycin. The antibiotic stock solutions can be pre-made and stored at -20 °C. Once the medium is made, store at 4 °C, but warm to 37 °C before use.
5. 10 cm ø culture dishes.
6. To count cells prior to seeding into plates, an automatic cell counter can be used, such as a TC10™ automated cell counter.

2.2.2 Assay

1. Ligands are prepared as DMSO stock solutions prior to testing, for example as 10 mM stocks. However, the endocannabinoids (anandamide and 2-arachidonoylglycerol) are unstable in DMSO, and therefore stock solutions (e.g., 10 mM) are prepared in acetonitrile. Stock solutions of most ligands are stable at -20 °C, but one should always be cautious with using stock solutions for longer periods of time. Product stability and purity should be checked regularly.
2. Cells are seeded into white walled, solid bottom 384-well assay plates with low fluorescence background (*see* **Note 1** and **2**).

2.2.3 Detection and data processing

1. Detection is done with the PathHunter detection kit® (DiscoverX). The detection mixture is prepared as follows (*see* **Note 3**): Cell assay buffer = 19 parts, Substrate Reagent 1 = 5 parts, Substrate Reagent 2 = 1 part, according to the manufacturer's protocol[36].
2. Measurement of the plates can be done by any multimode, or luminescence plate reader, for example a Wallac EnVision™ 2104 Multilabel reader.
3. Analysis of raw experimental data can be performed using the nonlinear regression curve fitting program GraphPad Prism 9.0.

2.3 Methods

All procedures should be carried out at rt, unless specified otherwise.

2.3.1 Cell culture

1. The CHOK1hCB$_1$_bgal and CHOK1hCB$_2$_bgal cells are subcultured twice a week when reaching ~85-90% confluency on 10 cm ø plates in cell culture medium in a humidified atmosphere at 37 °C and 5% CO_2.

2. Remove medium from the plate and wash the cells with 5 mL PBS.
3. Add 1 mL trypsin solution and incubate at 37 °C for maximally 5 minutes.
4. Gently detach the cells and resuspend in 4 mL cell culture medium.
5. Subculture the cells according to their confluence in fresh cell culture medium, for example in a ratio between 1:12-1:20. Each subculture step is one passage.
6. For the assay, cells should be passaged at least 3× and no more than 24× (max. 3 months in culture). Additionally, do not let the cells grow >90% confluency prior to harvesting for the assay.

2.3.2 Ligands under investigation

1. High throughput screening can be performed using a single high concentration of each ligand (Z' factor > 0.7[37]). Often a final concentration of 10 μM is used. Full dose-response curves of ligands are necessary for determination of their potency and efficacy.
2. Stock solutions of ligands under investigation are made in DMSO or acetonitrile. These stock solutions should be diluted to the desired concentration in cell culture medium. Make sure there is an equal amount of organic solvent (e.g., DMSO or acetonitrile) present in every dilution (*see* **Note 4**). For (inverse) agonistic assays, compound dilutions should be 5× the desired final concentration, while for antagonistic assays (and the EC_{80} of a reference agonist) the pre-made concentrations should be 10x the desired final concentration.
3. In all assays the E_{max} of a reference full agonist should be taken along (e.g., CP55,940 at 1 μM final concentration).
4. When investigating activation by the endocannabinoids, cells should be preincubated for 30 minutes with 50 μM phenylmethylsulphonyl fluoride (PMSF, final concentration) prior to endocannabinoid treatment (*see* **Note 5**).

2.3.3 Cell seeding

1. Remove the medium and wash the cells with 5 mL PBS.
2. Harvest the cells with 1 mL trypsin solution and incubate for maximally 5 min. at 37 °C and 5% CO_2.
3. Inactivate the trypsin solution by addition of 4 mL cell culture medium.
4. Transfer the cells to a Falcon tube and spin down (5 min. at 1000 rpm/200 × g).
5. Resuspend the pellet well in 1 mL cell culture medium and count living cells in an automated cell counter by addition of 10 μL cell suspension to 10 μL Trypan Blue.
6. Dilute the cells further with cell culture medium to get a density of 250,000 cells/mL (= 5,000 cells/well).
7. Add 20 μL of cell suspension per well of the 384-well plate. Make sure that you fill enough wells for control measurements: only medium (negative control),

unstimulated cells with organic solvent equal to assay (basal activity), cells stimulated by a full agonist, e.g., CP55,940 (positive control), cells stimulated by EC_{80} of reference agonist (positive control in case of an antagonistic assay).

8. Incubate cells overnight (between 16-18 hours) at 37 °C and 5% CO_2.

2.3.4 Cell stimulation and incubation

2.3.4.1 Antagonistic assay

1. Dilute antagonists of interest into cell culture medium to 10× the desired final concentration (*see* **Note 4**).
2. Add 2.5 µL antagonist solution per well.
3. Incubate 30 min. at 37 °C and 5% CO_2.
4. Add 2.5 µL of a 10× EC_{80} solution of CP55,940 to all wells (EC_{80} of CP55,940 needs to be determined prior to this assay), including wells for background measurements.
5. Incubate 90 min. at 37 °C and 5% CO_2.

2.3.4.2 Agonistic assay

1. Dilute agonists of interest into cell culture medium to 5× the desired final concentration (*see* **Note 4**).
2. Add 5 µL agonist solution per well.
3. Incubate 90 min. at 37 °C and 5% CO_2.

2.3.4.3 Inverse agonistic assay

1. Dilute inverse agonists of interest into cell culture medium to 5× the desired final concentration (*see* **Note 4**).
2. Add 5 µL inverse agonist solution per well.
3. Incubate 6 hours at 37 °C and 5% CO_2.

2.3.5 Detection and measurements

1. Add 12.5 µL/well of detection mixture to all wells.
2. Incubate 60 min. **in the dark** at rt.
3. Measure the chemiluminescent response by a multimode or luminescence plate reader (*see* **Note 6**).

2.3.6 Data analysis

Raw experimental data is analyzed using the nonlinear regression curve fitting program GraphPad Prism. All data points are corrected for all background conditions (negative control and basal activity). The response of (inverse) agonists is normalized to the maximum effect of a reference agonist (e.g., 1 µM CP55,940) and the response of antagonists is normalized to the EC_{80} of a reference full agonist (e.g., CP55,940). Basal activity of the cells is set at 0%. Potency, inhibitory potency, or efficacy values (EC_{50}, IC_{50} or E_{max}, respectively) of ligands can be obtained by choosing the nonlinear regression option "log (agonist or inhibitor) vs response".

2.3.7 Results

We applied the protocol described here to characterize the well-known cannabinoid receptor reference ligands CP55,940, WIN55,212-2, JWH133, SR141716A (Rimonabant) and SR144528 (see **Figure 2.2** and **Table 2.1**). We found that our findings correlate well with literature[18,38,39].

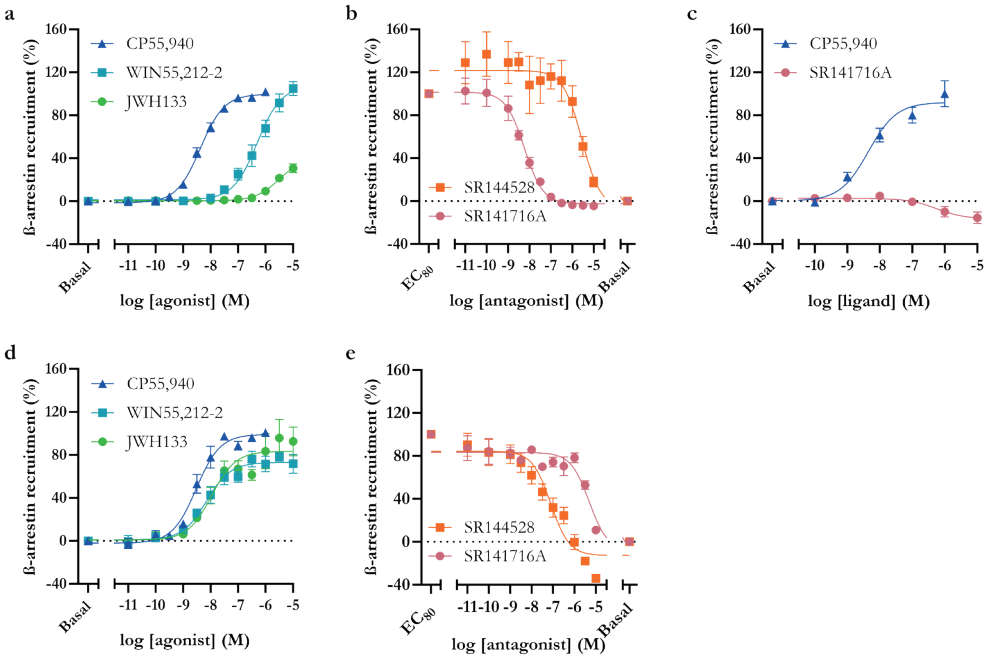

Figure 2.2 Dose-response curves of cannabinoid ligands on β-arrestin recruitment in PathHunter® CHOK1hCB$_1$_bgal or CHOK1hCB$_2$_bgal cells.

β-arrestin recruitment after activation for 90 min with increasing concentrations of (a, d) agonists, (b, e) pre-incubation with the EC_{80} concentration of CP55,940 followed by antagonist treatment or (c) stimulation with (inverse) agonist for 6 hours on human CB_1R or CB_2R. Basal activity of the cells was set at 0% and the efficacy was calculated as (a, c, d) a percentage of the maximum effect induced by 1 µM CP55,940 or (b, e) EC_{80} concentration of CP55,940 (25 or 46 nM for CB_1R and CB_2R, respectively). Data represent the mean ± SEM from at least three independent experiments performed in duplicate. Figure adapted from Soethoudt *et al.* (2016) and Xia *et al.* (2018)[40,41].

Table 2.1 **Potency and efficacy data of cannabinoid receptor ligands obtained with the PathHunter® β-arrestin recruitment assay.**

	hCB_1R		hCB_2R	
Agonists	**pEC_{50} ± SEM**	**E_{max} (%) ± SEM[a]**	**pEC_{50} ± SEM**	**E_{max} (%) ± SEM**
CP55,940	8.37 ± 0.08	102 ± 1	8.33 ± 0.18	101 ± 1
WIN55,212-2	6.30 ± 0.17	105 ± 7	8.04 ± 0.31	72 ± 9
JWH133	5.50 ± 0.01	31 ± 4	7.97 ± 0.15	93 ± 13
Antagonists	**pIC_{50} ± SEM[b]**		**pIC_{50} ± SEM**	
SR141716A	8.24 ± 0.06		5.31 ± 0.05	
SR144528	5.56 ± 0.11		7.12 ± 0.19	
Inverse agonist	**pIC_{50} ± SEM[c]**	**E_{min} (%) ± SEM[d]**	**pIC_{50} ± SEM**	**E_{min} (%) ± SEM**
SR14716A	6.25 ± 0.18	-15 ± 5	N.D.	N.D.

[a] Maximum effect (E_{max}) is determined as percentage of the maximum effect induced by 1 μM CP55,940. [b] Inhibitory potency (pIC_{50}) determined in the antagonistic assay by pre-incubation with EC_{80} of CP55,940 followed by antagonist treatment. [c] Inhibitory potency (pIC_{50}) as obtained from the inverse agonistic assay in the absence of any agonist. [d] Maximum inhibition (E_{min}) is determined in an inverse agonistic assay and normalized to the maximum effect induced by 1 μM CP55,940. Data represent the mean ± SEM from at least three independent experiments performed in duplicate. N.D. = not determined.

2.4 Notes

1. Perkin Elmer's white walled, solid bottom CulturPlate 384-well assay plates are delivered in a sterile environment. They should be kept that way by opening them only in an appropriate flow cabinet. Do not touch the plates at the bottom since greasy fingerprints can give extra background and/or decrease reading efficiency of the plate reader. Also make sure there is no dust in the wells which will give extra background and will increase well-to-well variation.
2. When working with 384-well plates, edge (or well-position) effects might occur due to differing evaporation rates of the outer wells compared to the central wells. This problem could be overcome by for example pre-incubation of the seeded cells at rt for at least 30 minutes before transferring the plate to the incubator. Alternatively, the less insulated outer wells could be filled with a fluid (sterile water/PBS/culture medium) and discarded from the experiment or a Breathe-Easy® sealing membrane (Sigma-Aldrich) could be used.
3. All PathHunter® detection reagents should be aliquoted in Eppendorf tubes and stored at -20 °C upon receipt. Reagents can only be thawed and refrozen twice. When the reagents are used for the detection mixture, they are thawed to **rt in the dark** and then mixed in the previously mentioned ratio. Once the detection mixture is made, it is stable for 24 hours at **rt in the dark**.
4. Stock solutions of ligands under investigation are made in DMSO or acetonitrile. Always make sure that there is an equal amount of organic solvent present in every dilution (≤1%) to avoid effects of the solvent. To obtain an equal concentration of organic solvent in every dilution, a dilution series in 100% DMSO or

acetonitrile should be made that is no less than 100× (agonist and inverse agonist) or 200× (antagonist or EC_{80} reference agonist) the desired final concentration. Dilute this series accordingly in cell culture medium to 5× or 10× the desired final concentration, depending on whether an agonistic or antagonistic assay is performed. Make sure there is also an equal amount of DMSO or acetonitrile in the wells for background measurements.

5. The endocannabinoids can be degraded by a variety of enzymes like fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). To prevent this degradation in the assay, the cells should be preincubated with a serine protease inhibitor, for example PMSF, before addition of the endocannabinoids.
6. The exposure time to read samples can depend on the instrument used. For example, the settings for a Wallac EnVision™ 2104 Multilabel reader should be:
    a. Instrument settings: EnVision Single emission with single emission mirror block.
    b. Filters:
        i. Emission filter: Luminescence 700
        ii. Mirror module: Luminescence
        iii. Install the emission filter in the Emission Filter Slide correctly. The filters must occupy adjacent slots in the Emission Filter Slide.
    c. The mirror needs to be manually changed to the top position within the machine and selected in the software before use.
    d. Allow the lamp to warm up for at least 10 min.
    e. Use protocol LUM Single (1.0 s read).

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