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Cellular Assay to Study β -Arrestin Recruitment by the Cannabinoid Receptors 1 and 2

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

Abstract

Cannabinoid receptor 1 (CB₁R) and cannabinoid receptor 2 (CB₂R) 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 the activation level of these pathways lead to biased signaling. Here, we describe a detailed protocol to characterize the potency and efficacy of ligands to induce or inhibit β -arrestin recruitment to the human CB₁R and CB₂R 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 CB₁R and CB₂R, of which some examples will be presented here.

Key words GPCRs, Cannabinoid receptors, Receptor signaling, β -arrestin, Desensitization, Internalization, Biased signaling, Functional selectivity, DiscoverX PathHunter[®]

1 Introduction

Cannabinoid receptor 1 (CB₁R) and cannabinoid receptor 2 (CB₂R) 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 CB₁R, while CB₂R 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, CB₁R and CB₂R 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 CB₁R and CB₂R, 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 -arrestins, we have employed the previously reported β -arrestin PathHunter[®] assay [19, 20]. This assay is an easy-to-use endpoint assay that has been shown to deliver reproducible results and was successfully applied to measure β -arrestin-2 recruitment for a variety of cannabinoid receptor ligands [12, 21–24]. In this assay, β -arrestin-2 activity is measured in live cells by using a β -galactosidase fragment complementation technology and a chemiluminescent read-out (*see* Fig. 1) [25, 26]. The DiscoverX PathHunter[®] cell lines overexpress a GPCR of interest, in this case either the human CB₁R or CB₂R, which are tagged at the

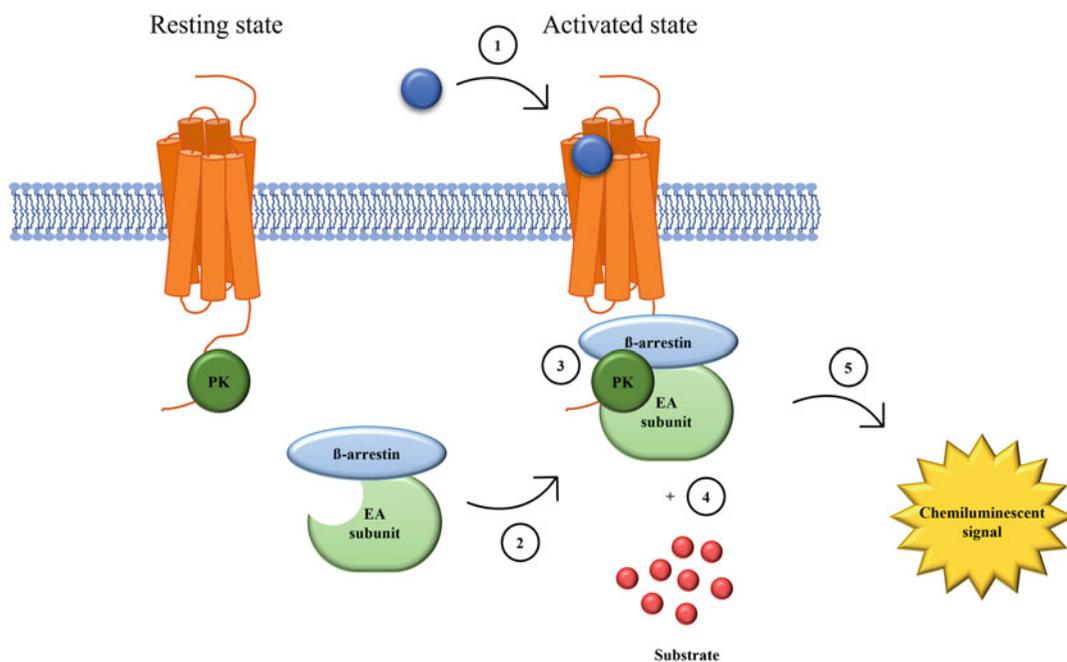


Fig. 1 Schematic representation of the β -arrestin recruitment assay. Upon activation of the receptor by a ligand (1), the inactive β -arrestin-EA complex is recruited to the receptor (2). This induces the complementation with the ProLink[™] enzyme fragment (PK) to form an active β -galactosidase enzyme (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

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, 25]. Activation of CB₁R or CB₂R 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, 25]. 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 [22, 25, 27]. 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 [28–31]. 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, 32] and the luminescent NanoBiT assay [33]. 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, 34]. We envision that the field of cannabinoid receptor research will also move in the direction of kinetic signaling assays to further explore the concept of biased signaling.

2 Materials

All buffers and solutions are prepared using Millipore water (deionized using a MilliQ A10 Biocel™, with a 0.22 μ m 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 CP55940 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 WIN55212-2 was received from Hoffman-La Roche (Basel, Switzerland).

2.1 Cell Culture

1. Cells: PathHunter® CHO-K1 β -Arrestin Cell Line CNR1 or CNR2 (DiscoverX), overexpressing the CB₁R or CB₂R respectively. In this chapter, these cells are named CHOK1hCB₁_bgal or CHOK1hCB₂_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, 100 IU/mL Penicillin, 100 $\mu\text{g}/\text{mL}$ Streptomycin, 800 $\mu\text{g}/\text{mL}$ G418, and 300 $\mu\text{g}/\text{mL}$ Hygromycin. The antibiotic stock solutions can be premade and stored at -20°C . Once the medium is made, store at 4°C , but warm to 37°C before use.
5. 10 cm \varnothing culture dishes.
6. To count cells prior to seeding into plates, an automatic cell counter can be used, such as a TC10TM automated cell counter.

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*).

2.3 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 [35].
2. Measurement of the plates can be done by any multimode, or luminescence plate reader, for example, a Wallac EnVisionTM 2104 Multilabel reader.
3. Analysis of raw experimental data can be performed using the nonlinear regression curve fitting program GraphPad Prism 9.0.

3 Methods

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

3.1 Cell Culture

1. The CHOK1hCB₁_bgal and CHOK1hCB₂_bgal cells are sub-cultured twice a week when reaching ~85–90% confluency on

10 cm \varnothing plates in cell culture medium in a humidified atmosphere at 37 °C and 5% CO₂.

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 a maximum of 5 min.
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 and 1:20. Each subculture step is one passage.
6. For the assay, cells should be passaged at least 3 times and no more than 24 times (max. 3 months in culture). Additionally, do not let the cells grow >90% confluency prior to harvesting for the assay.

3.2 Ligands Under Investigation

1. High throughput screening can be performed using a single high concentration of each ligand (Z' factor > 0.7 [36]). Often, a final concentration of 10 μ M is used. Full dose-response curves of ligands are necessary for the 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 \times the desired final concentration, while for antagonistic assays (and the EC₈₀ of a reference agonist) the premade concentrations should be 10 \times the desired final concentration.
3. In all assays, the E_{max} of a reference full agonist should be taken along (e.g. CP55940 at 1 μ M final concentration).
4. When investigating activation by the endocannabinoids, cells should be preincubated for 30 min with 50 μ M phenylmethylsulphonyl fluoride (PMSF, final concentration) prior to endocannabinoid treatment (*see Note 5*).

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% CO₂.
3. Inactivate the trypsin solution by the addition of 4 mL cell culture medium.
4. Transfer the cells to a Falcon tube and spin down (5 min at 200 \times g).

5. Resuspend the pellet well in 1 mL cell culture medium and count living cells in an automated cell counter by the addition of 10 μ L cell suspension to 10 μ L Trypan Blue.
6. Dilute the cells further with cell culture medium to get a density of 250,000 cells/mL (= 5000 cells/well).
7. Add 20 μ L 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. CP55940 (positive control), cells stimulated by EC₈₀ of reference agonist (positive control in case of an antagonistic assay).
8. Incubate cells overnight (between 16 and 18 h) at 37 °C and 5% CO₂.

3.4 Cell Stimulation and Incubation

3.4.1 Antagonistic Assay

1. Dilute antagonists of interest into cell culture medium to 10 \times the desired final concentration (*see Note 4*).
2. Add 2.5 μ L antagonist solution per well.
3. Incubate 30 min at 37 °C and 5% CO₂.
4. Add 2.5 μ L of a 10 \times EC₈₀ solution of CP55940 to all wells (EC₈₀ of CP55940 needs to be determined prior to this assay), including wells for background measurements.
5. Incubate 90 min at 37 °C and 5% CO₂.

3.4.2 Agonistic Assay

1. Dilute agonists of interest into cell culture medium to 5 \times the desired final concentration (*see Note 4*).
2. Add 5 μ L agonist solution per well.
3. Incubate 90 min. at 37 °C and 5% CO₂.

3.4.3 Inverse Agonistic Assay

1. Dilute inverse agonists of interest into cell culture medium to 5 \times the desired final concentration (*see Note 4*).
2. Add 5 μ L inverse agonist solution per well.
3. Incubate 6 h at 37 °C and 5% CO₂.

3.5 Detection and Measurements

1. Add 12.5 μ L/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*).

3.6 Data Analysis

Raw experimental data are 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 μ M CP55940), and

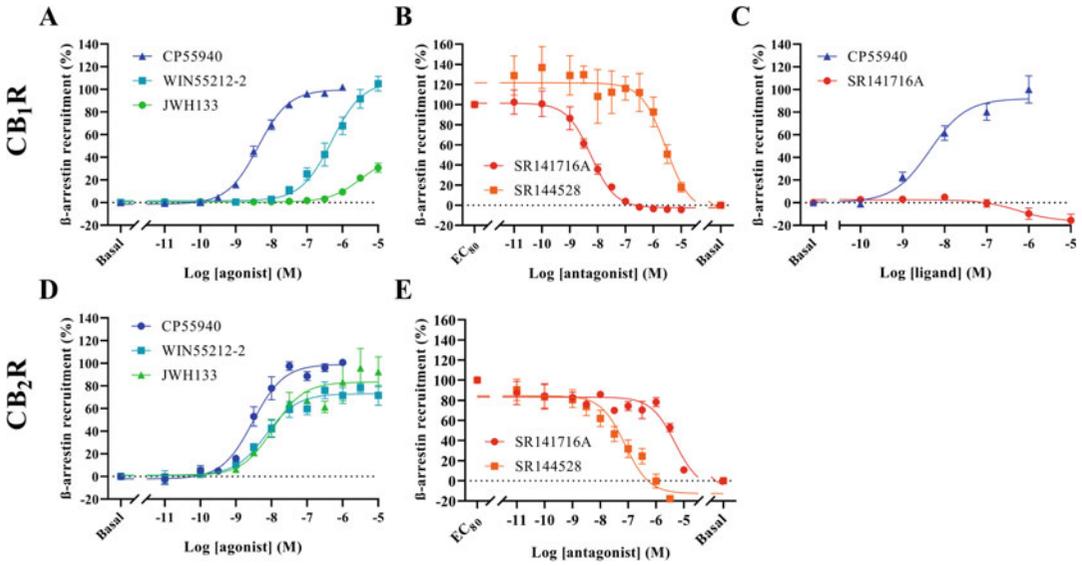


Fig. 2 Dose–response curves of cannabinoid ligands on β -arrestin recruitment in PathHunter[®] CHOK1hCB₁_b-gal or CHOK1hCB₂_bgal cells. β -Arrestin recruitment after activation for 90 min with increasing concentrations of agonists (A, D), preincubation with the EC₈₀ concentration of CP55940 followed by antagonist treatment (B, E), or stimulation with (inverse) agonist for 6 h (C) on human CB₁R or CB₂R. Basal activity of the cells was set at 0%, and the efficacy was calculated as a percentage of the maximum effect induced by 1 μ M CP55940 (A, C, D) or EC₈₀ concentration of CP55940 (25 or 46 nM for CB₁R and CB₂R, respectively) (B, E). Data represent the mean \pm SEM from at least three independent experiments performed in duplicate. Figure adapted from Soethoudt et al. (2016) and Xia et al. (2018) [21, 38]

the response of antagonists is normalized to the EC₈₀ of a reference full agonist (e.g. CP55940). Basal activity of the cells is set at 0%. Potency, inhibitory potency, or efficacy values (pEC₅₀, pIC₅₀, or E_{max}, respectively) of ligands can be obtained by choosing the nonlinear regression option “log (agonist or inhibitor) vs. response”.

3.7 Results

We applied the protocol described here to characterize the well-known cannabinoid receptor reference ligands CP55940, WIN55212-2, JWH133, SR141716A (Rimonabant), and SR144528 (see Fig. 2 and Table 1). We found that our findings correlate well with the literature [18, 22, 37].

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

Table 1
Potency and efficacy data of cannabinoid receptor ligands obtained with the PathHunter[®] β -arrestin recruitment assay

	hCB ₁ R		hCB ₂ R	
Agonists	pEC ₅₀ ± SEM	E _{max} (%) ± SEM ^a	pEC ₅₀ ± SEM	E _{max} (%) ± SEM
CP55940	8.37 ± 0.08	102 ± 1	8.33 ± 0.18	101 ± 1
WIN55212-2	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. ^e	N.D.

Data represent the mean ± SEM from at least three independent experiments performed in duplicate

^aMaximum effect (E_{max}) is determined as the percentage of the maximum effect induced by 1 μ M CP55940

^bInhibitory potency (pIC₅₀) determined in the antagonistic assay by preincubation with EC₈₀ of CP55940 followed by antagonist treatment

^cInhibitory potency (pIC₅₀) as obtained from the inverse agonistic assay in the absence of any agonist

^dMaximum inhibition (E_{min}) determined in an inverse agonistic assay and normalized to the maximum effect induced by 1 μ M CP55940

^eN.D., not determined

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.

- 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, preincubation of the seeded cells at RT for at least 30 min 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.
- 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.
- 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

($\leq 1\%$) 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 \times (agonist and inverse agonist) or 200 \times (antagonist or EC₈₀ reference agonist) the desired final concentration. Dilute this series accordingly in a cell culture medium to 5 \times or 10 \times 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 the 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.
 - (c) Install the emission filter in the Emission Filter Slide correctly. The filters must occupy **adjacent** slots in the Emission Filter Slide.
 - (d) The mirror needs to be manually changed to the top position within the machine and selected in the software before use.
 - (e) Allow the lamp to warm up for at least 10 min.
 - (f) Use protocol LUM Single (1.0 s read).

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