Chapter 5

Assay of CB_1 Receptor Binding

Valeria M. Catani and Valeria Gasperi

Abstract

Type-1 cannabinoid receptor (CB_1), one of the main targets of endocannabinoids, plays a key role in several pathophysiological conditions that affect both central nervous system and peripheral tissues. Today, its biochemical identification and pharmacological characterization, as well as the screening of thousands of novel ligands that might be useful for developing CB_1-based therapies, are the subject of intense research. Among available techniques that allow the analysis of CB_1 binding activity, radioligand-based assays represent one of the best, fast, and reliable methods.

Here, we describe radioligand binding methods standardized in our laboratory to assess CB_1 binding in both tissues and cultured cells. We also report a high-throughput radioligand binding assay that allows to evaluate efficacy and potency of different compounds, which might represent the basis for the development of new drugs that target CB_1 receptor-dependent human diseases.

Key words Type-1 cannabinoid receptor, CP55.940, Filtration assay, Radioligand-receptor binding, SR141716

1 Introduction

Type-1 cannabinoid receptor (CB_1), the first identified and cloned receptor of Δ^9-tetrahydrocannabinol [1], represents one of the main targets of endocannabinoids [2, 3]. It is highly expressed in brain, but also in peripheral organs (e.g., lung, liver, and kidney) and cells, including T lymphocytes and platelets [4]. Due to its broad localization, CB_1 is involved in the regulation of several physiological processes at both central nervous system and periphery, such as neurogenesis, reward, cognition, learning, memory, immune response, and body energy homeostasis [4–6]. In addition, several pathological conditions have been shown to correlate with up- or downregulation of this receptor. Overactive CB_1 signaling, for example, promotes the development of obesity, insulin resistance, and dyslipidemia [6]. Therefore, the design of novel CB_1 ligands that might be useful

Mauro Maccarrone (ed.), *Endocannabinoid Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1412,
DOI 10.1007/978-1-4939-3539-0_5, © Springer Science+Business Media New York 2016

therapeutics for prevention and/or treatment of CB_1-dependent human disorders is very active [6].

One of the main techniques used for the biochemical identification and pharmacological characterization of CB_1 is based on the use of a radioactive ligand, such as $[^3H]$CP55.940 [2]. The basic outline of radioligand-CB_1 binding assays is very easy and fast to perform: a preparation containing CB_1 is incubated with radioactive (hot) $[^3H]$CP55.940, thus forming the radioactive complex $[^3H]$CP55.940/CB_1. After reaching equilibrium, this complex is usually separated from unbound hot $[^3H]$CP55.940 by membrane filtration (still the most efficient, fast, and convenient method), and quantified by liquid scintillation counting. The use of an unlabeled (cold) CB_1 ligand, such as the antagonist/inverse agonist SR141716 or rimonabant [7], able to selectively displace hot $[^3H]$CP55.940 from CB_1 sites, allows then to assess CB_1 binding activity.

Noteworthy, we observed that, although CB_1 binding assay with filtration might work well with membrane homogenates from both tissues and cells, the most reproducible and reliable results are obtained by working with intact cultured cells without a filtration step.

Against this background, here we describe two relatively simple, but extremely powerful, methods that we have standardized in our laboratory and that we commonly employ to interrogate the presence of CB_1 in unknown samples, as well as to identify potential changes in its activity, under either physiological (e.g., in young versus aged tissues, or during cell differentiation) or pathological conditions (e.g., healthy versus diseased tissues). We also describe a standardized high-throughput CB_1 binding assay that, by employing human CB_1 over-expressing membranes, allows to screen large libraries of compounds. This method is a useful assay to identify novel CB_1 ligands that might represent the basis for developing innovative therapeutics against CB_1-dependent human diseases.

2 Materials

Prepare all buffers and solutions by using ultrapure water and analytical grade reagents. Prepare and store all reagents at –20 °C (unless indicated otherwise).

2.1 Labeled and Unlabeled Compounds

$[^3H]$CP55.940 [5-(1,10-dimethylheptyl)-2-[(1R,5R)-hydroxy-(2R)-(3-hydroxypropyl)-cyclo-hexyl]phenol].

CP55.940.

SR141716, rimonabant [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide] (Cayman Chemicals Ann Arbor, MI, USA).

2.2 Buffers and Equipments

2.2.1 CB_1 Binding Assay for Tissues

Buffer A: 2 mM Tris-EDTA, 320 mM sucrose, 5 mg/ml BSA, 5 mM $MgCl_2$, pH 7.4.

Buffer B: 50 mM Tris–HCl, 2 mM Tris-EDTA, 3 mM $MgCl_2$, pH 7.4.

Buffer C: 50 mM Tris–HCl, 2 mM Tris-EDTA, 3 mM $MgCl_2$, 5 mg/ml bovine serum albumin (BSA), 50 μM phenylmethylsulfonyl fluoride (PMSF) (to add immediately before use), pH 7.4.

Buffer D: 50 mM Tris–HCl, 1 mg/ml BSA, pH 7.4.

0.1 % Triton in Buffer D, pH 7.4.

Glass/Teflon Potter homogenizer.

Ultra-Turrax T 25.

15 ml polypropylene tubes.

Vortex mixer.

Water bath or Thermoblock for 15 ml tubes, or incubator set at 37 °C.

Whatman GF/C Glass Microfiber filters (binder free), diameter 25 mm, with 1.2 μm pore size.

Vacuum filtration manifold, Millipore® model 1225 (Merck-Millipore Co., Darmstadt, Germany).

Vacuum pump.

Liquid scintillation cocktail (Ultima Gold™ XR).

10 ml scintillation vials.

Liquid scintillation β-counter.

2.2.2 CB_1 Binding Assay for Intact Cells

1. Incubation buffer: 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 320 mM sucrose, 2 mg/ml BSA, pH 7.4.
2. Washing buffer: 50 mM Tris–HCl, 500 mM NaCl, 1 mg/ml BSA, pH 7.4.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
4. Sterile 24-well plates for cell cultures.
5. Incubator for cell cultures set at 37 °C.
6. Liquid scintillation cocktail (Ultima Gold™ XR).
7. 10 ml scintillation vials.
8. Liquid scintillation β-counter.

2.2.3 High-Throughput CB_1 Binding Assay

1. Binding buffer: 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mg/ml BSA, pH 7.4.
2. Pre-soaking buffer for FC-filter plate: 50 mM Tris–HCl, 0.33 % polyethylenimine (PEI), pH 7.4.
3. Washing buffer: 50 mM Tris–HCl, 500 mM $NaCl_2$, 1 mg/ml BSA, pH 7.4.
4. 0.1 % Triton in buffer D, pH 7.4.
5. Incubator set at 37 ° C.
6. Polypropylene 96-well plate with lid.
7. Human CB_1 over-expressing membranes (Merck-Millipore Co., Darmstadt, Germany).
8. MultiScreenHTS 96-well filter plate (Merck-Millipore Co., Darmstadt, Germany).
9. Vacuum pump.
10. FC-96-well plate.
11. Liquid scintillation cocktail (Ultima Gold™ XR).
12. 10 ml scintillation vials.
13. Liquid scintillation β-counter.

3 Methods

3.1 Protocol 1: CB_1 Binding Assay for Tissues

This protocol is recommended for low-throughput assays, or for just a few experiments with tissue samples.

3.1.1 Preparation of Membrane Homogenates

Perform all steps (Fig. 1) on ice and keep samples on ice or in a refrigerated centrifuge, in order to preserve CB_1 functionality.

1. Resuspend tissue (*see* **Note 1**) in pre-cooled buffer A (fresh weight/volume ratio = 1 g/4 ml), and homogenize it on ice (*see* **Note 2**).
2. Centrifuge the membrane homogenate at 1000 *g* and 4 °C for 10 min.
3. Save the supernatant (S1) in a tube on ice.
4. Resuspend the pellet with the same volume of buffer A used in **Step 1**, and centrifuge again at 1000 *g* and 4 °C for 10 min.
5. Save the supernatant (S2) and add it to S1.
6. Repeat **Steps 4** and **5** to obtain S3, and discard the pellet.
7. Centrifuge the collected volume (S1 + S2 + S3) at 39,000 *g* and 4 °C for 30 min.
8. Discard the supernatant.
9. Resuspend the resulting pellet in the smallest possible volume of cold buffer B.

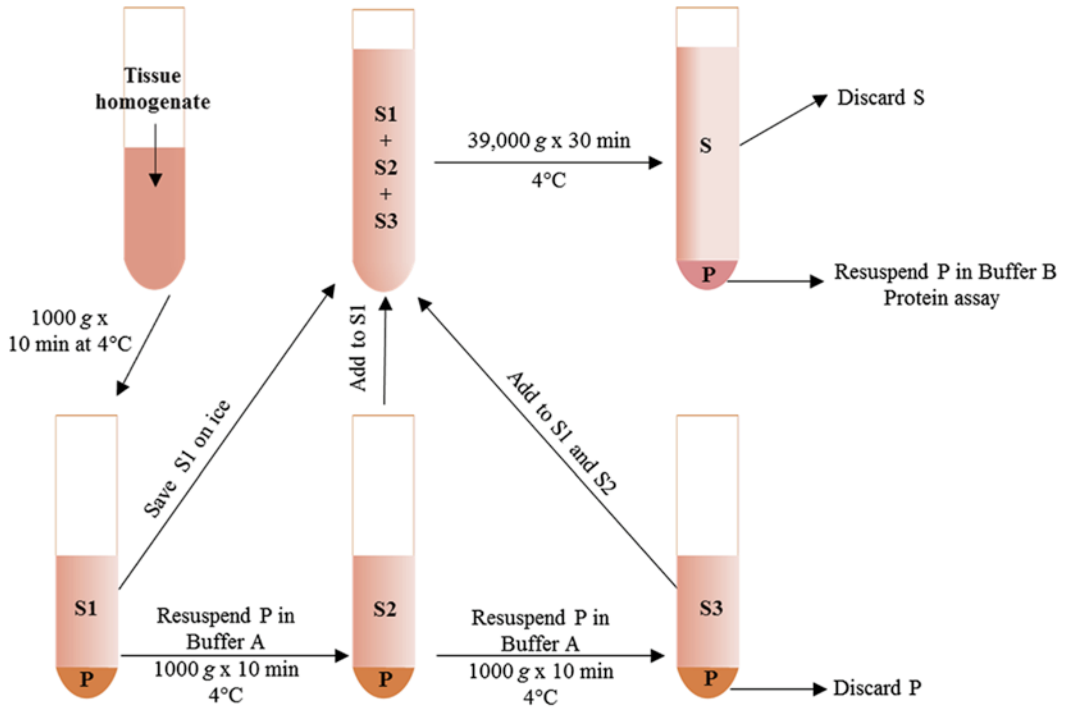


Fig. 1 Schematic representation of membrane homogenate preparation. *See* Subheading 3.1.1 for further details. *P* pellet; *S* supernatant

10. Calculate protein concentration with any commercially available assay, such as Bradford assay.
11. Membrane homogenate is now ready for CB_1 binding assay (*see* **Note 3**).

3.1.2 Assay Using Vacuum Filtration Manifold Millipore® Model 1225

Each condition in the assay must be repeated in triplicate, in order to calculate average and standard error of the mean (S.E.M.).

Prepare also three tubes that contain only pre-warmed buffer C and [^{3}H]CP55.940 (i.e., reaction blank, Bk) (Fig. 2). All incubations are carried out at 37 °C in pre-warmed buffer C, at a final reaction volume of 500 µl.

1. For each sample, prepare three 15 ml polypropylene tubes (in triplicate), marked as TB (total binding), NSB (nonspecific binding), and SR1 (CB_1 antagonist/inverse agonist SR141716). Add in each tube the reagents in the following order:
   (a) Pre-warmed buffer C (up to 500 µl).

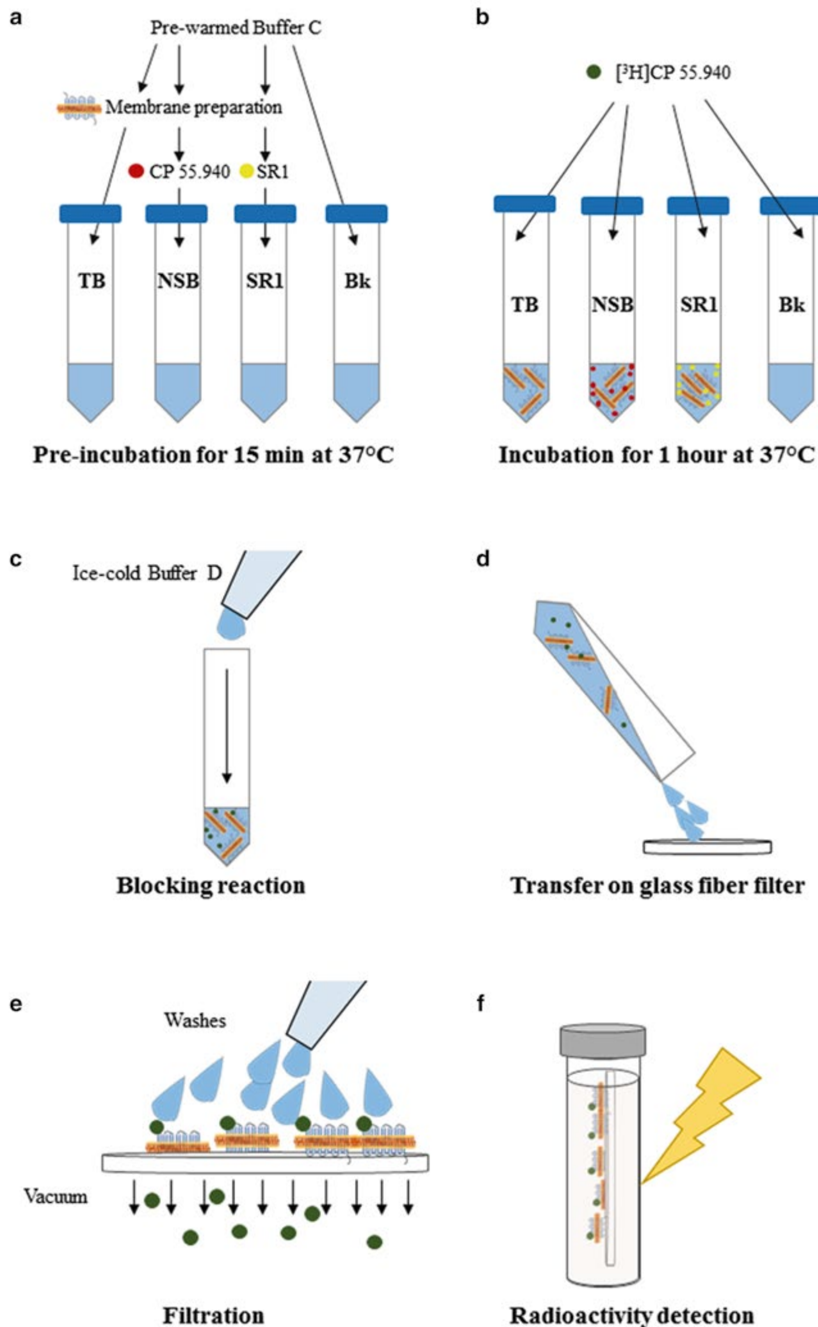


Fig. 2 Schematic representation of the procedure to analyze CB_1 binding activity in membrane homogenates by using filtration assay. (**a**) TB (total binding, that contains buffer C and membrane proteins), NSB (nonspecific binding, that contains buffer C, membrane proteins and cold CP55.940), SR1 (that contains buffer C, membrane proteins and the CB_1 antagonist/inverse agonist SR141716), and Bk (blank of reaction, that contains buffer C only) tubes are pre-incubated at 37 °C for 15 min. (**b**) After adding [^{3}H]CP55.940 to each tube, samples are incubated for 1 h at 37 °C. (**c**) At the end of the incubation, reaction is blocked by adding 2 ml of ice-cold buffer D to the tubes. (**d**) The content of each tube is then transferred to a GF/C Glass fiber filter, placed in the filter plate of vacuum filtration manifold, Millipore® model 1225. (**e**) Vacuum traps the complex [^{3}H]CP55.940/CB_1 receptor onto the glass fiber filter, while unbound radioligand is removed through cycles of washes with ice-cold buffer D and vacuum. (**f**) Each filter is transferred to 10 ml scintillation vial, and residual radioactivity is measured in a liquid scintillation β-counter. *See* Subheading 3.1.2 for further details

(b) Membrane homogenate. For murine brain membranes, we usually use 50–100 μg/test (*see* **Note 4**).

(c) CP55.940 at a final concentration of 1 μM, for NSB (*see* **Note 5**), or SR141716 at a final concentration of 0.1 μM, for SR1 (*see* **Note 6**).

2. Vortex and incubate for 15 min at 37 °C (Fig. 2a).
3. Add [^{3}H]CP55.940. For murine brain membranes, we use it at the final concentration of 0.4 nM (*see* **Note 7**).
4. Vortex and incubate for 1 h at 37 °C (Fig. 2b).
5. In the meantime, place in a Petri dish, with tweezers, as many GF/C glass fiber filters as the number of tubes, and pre-soak them in cold buffer D (*see* **Note 8**).
6. 10 min before terminating the reaction, place (with tweezers) each filter in the numbered filter plate of the vacuum filtration manifold.
7. Turn on vacuum to remove excess of buffer D from glass fiber filters.
8. Block the vacuum.
9. Terminate reaction by adding 2 ml of ice-cold buffer D to each tube (*see* **Note 9**) (Fig. 2c).
10. Vortex and transfer each sample to the corresponding filter plate (Fig. 2d).
11. Apply vacuum (for not more than 2 min) to trap the complex [^{3}H]CP55.940/CB_1 receptor onto the filter, and remove unbound radioligand (*see* **Notes 10** and **11**) (Fig. 2e).
12. Block the vacuum.
13. Wash each tube with 2 ml of ice-cold buffer D.
14. Repeat **steps 11–13**.
15. Wash each filter twice with 2 ml of ice-cold buffer D (Fig. 2e).
16. Apply vacuum.
17. Block the vacuum and allow filters to dry at room temperature (*see* **Note 12**).
18. Transfer with tweezers each dried filter in the corresponding scintillation vial, containing 500 μl of 0.1 % Triton in buffer D.
19. Add 3.5 ml of liquid scintillation cocktail, and read radioactivity by a scintillation β-counter (*see* **Note 13**) (Fig. 2f).

3.2 Protocol 2: CB_1 Binding Assay for Adherent Living Cells

Each condition in the assay must be repeated in triplicate, in order to calculate average and S.E.M.

All incubations are carried out at 37 °C in pre-warmed incubation buffer (final volume of reaction for 24-well plate = 300 μl).

1. Plate cells in order to reach 90–100 % confluence on the day of the assay (*see* **Note 14**). For each sample, prepare three wells (in triplicate), marked as TB (total binding), NSB (nonspecific binding), and SR1 (CB_1 antagonist/inverse agonist). For blanks (Bk), add culture medium to three wells, without cells.
2. On the day of the experiment, carefully wash each well twice with 1 ml of PBS.
3. After removing PBS, add to each well incubation buffer up to 300 μl.
4. Add CP55.940 for NSB (at a final concentration of 1 μM for HeLa cells) (*see* **Note 5**), or SR141716 (at a final concentration of 0.1 μM for HeLa cells), for SR1 (*see* **Note 6**).
5. Incubate for 10 min at 37 °C.
6. Add $[^3H]$CP55.940 (at a final concentration of 2.5 nM for HeLa cells) (*see* **Note 7**).
7. Incubate for 1 h in an incubator set at 37 °C.
8. At the end of the incubation, carefully remove the incubation buffer.
9. Carefully rinse cells 4 times with 1 ml of ice-cold washing buffer.
10. After removing washing buffer, add 300 μl of 0.5 M NaOH, and pipet up and down for several times to lyse cells.
11. Transfer solution to a 10 ml scintillation vial.
12. Add 3.5 ml of liquid scintillation cocktail, and immediately read radioactivity in a scintillation β-counter.

3.3 Protocol 3: CB_1 Binding Assay for High-Throughput Screening

Each condition in the assay must be repeated at least in triplicate, in order to calculate average and S.E.M. Prepare also three wells that will contain only pre-warmed binding buffer and $[^3H]$CP55.940 (i.e., blank of reaction, Bk). All incubations are carried out at 37 °C in pre-warmed binding buffer, at a final reaction volume of 200 μl.

1. Add the reagents to each well of a polypropylene 96-well plate, in the following order:
   (a) Pre-warmed binding buffer (up to 200 μl).
   (b) CB_1 over-expressing membranes (10 μg/well, except for the blanks).
   (c) CP55.940 for NSB, SR141716 for SR1 (*see* **Notes 4** and **5**), or test compounds (*see* **Note 15**).
2. Cover the lid and carefully shake the plate.
3. Incubate for 20 min at 37 °C.
4. Add $[^3H]$CP 55.940 at the final concentration of 2.5 nM, to start the reaction.

5. Cover the lid and carefully shake the plate.
6. Incubate for 1 h at 37 °C.
7. In the meantime, pre-soak FC-filter plate with 200 μl of 50 mM Tris–HCl containing 0.33 % PEI (*see* **Note 8**).
8. 10 min before terminating the reaction, turn on vacuum to remove PEI from filters.
9. Wash filters 3 times in washing buffer.
10. Transfer reaction solution from 96-well plate to the FC-filter plate, and filter by turning on vacuum.
11. Wash 4 times with 300 μl of washing buffer.
12. Allow filters to dry, by placing the plate for 30 min in an incubator at 37 °C.
13. Transfer each filter to the corresponding scintillation vial containing 500 μl of 0.1 % Triton in buffer D.
14. Add 3.5 ml of liquid scintillation cocktail and read radioactivity in a scintillation β-counter (*see* **Note 13**).

3.4 Data Analysis

It is recommended to repeat each condition in the assay at least in triplicate, in order to calculate mean and S.E.M.

3.4.1 Protocols 1 and 2

To calculate CB_1 binding activity (usually expressed as fmol or pmol per mg of membrane protein), do as follows:

1. Subtract disintegrations per minute (DPM) mean value of blank (Bk) from every other mean value.
2. Calculate SB by subtracting DPM relative to NSB from those relative to TB.
3. Knowing that 1 nCi corresponds to 2200 DPM, convert DPM in nCi.
4. To calculate moles of bound [^{3}H]CP55.940 to CB_1, divide the obtained nCi for the specific activity of [^{3}H]CP55.940 (such a value, expressed as Ci/mmol, is reported in the datasheet provided by the manufacturer) (*see* **Note 16**).
5. Divide the moles by the mg of proteins used in the assay (*see* **Note 17**).
6. Calculate SR141716 displacement according to the formula:

$$\%\ \text{of inhibition} = 100 - \frac{[3\text{H}]\text{CP55.940 SB} - \text{SR141716 SB}}{[3\text{H}]\text{CP55.940 SB}}$$

7. Calculate differences among two or more groups by inferential statistics: Student's *t*–test (unpaired or paired data) or

Mann Whitney *U* test/Wilcoxon' signed ranks test; ANOVA (or repeated-ANOVA) with Bonferroni *post hoc* test or Kruskal–Wallis test, followed by Mann–Whitney *U* test.

3.4.2 Protocol 3

To calculate IC_{50} and Ki values of novel CB_1 ligands (agonists/antagonists/inverse agonists), calculate SB of $[^3H]$CP55.940 (see above how to convert DPM of SB into fmol or pmol per mg of membrane proteins), in the presence of unlabeled test compound at each concentration tested. By using GraphPad Prism software for Science (San Diego, CA), plot each value and determine IC_{50} and Ki values by nonlinear regression analysis of one-site competition binding curve. It should be stressed that Ki is dependent on IC_{50} value, as indicated by the Cheng and Prusoff equation:

$$\mathrm{Ki} = \mathrm{IC}_{50} / \left[1 + \left([L] / K_D\right)\right]$$

where $[L]$ is the fixed concentration of $[^3H]$CP55.940 used in the assay, and K_D is its equilibrium dissociation constant (*see* Table 1 and Fig. 3). Therefore, prior to performing competition binding studies, you should know (or eventually calculate through saturation binding assay) K_D value of the radioligand used.

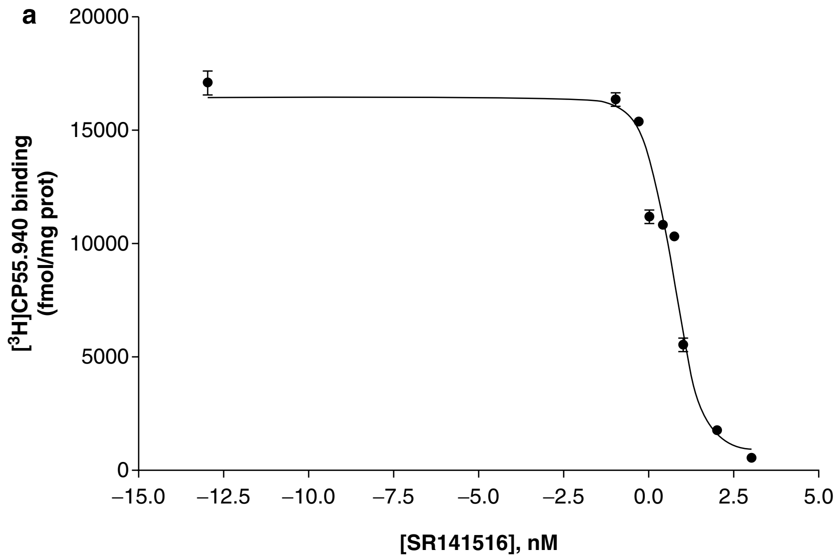
Table 1
Experimental data of heterologous competition binding studies performed by incubating membranes over-expressing human CB_1 receptor with 2.5 nM $[^3H]$CP55.940, and increasing concentrations (0–1000 nM) of SR141716

[SR141716][a]	Specific binding of $[^3H]$CP 55.940[b]
0.00	17,088.23 ± 558.42
0.10	16,344.45 ± 281.93
0.50	15,353.87 ± 128.72
1.00	11,165.90 ± 272.95
2.50	10,851.56 ± 92.94
5.00	10,288.43 ± 203.93
10.00	5500.12 ± 291.70
100.00	1725.14 ± 77.32
1000.00	526.40 ± 18.21

Values are the means ± S.E.M. of three independent experiments, each performed at least in quintuplicate

[a]The concentration of SR141716 is expressed as nM

[b]Binding values are expressed as fmol/mg of membrane protein



b

Best-fit values	
Bottom	820.2
Top	16397
$LogIC_{50}$	0.6836
IC_{50}	4.826
Ki	3.666
[^{3}H]CP 55.940 (Constant)	2.500
[^{3}H]CP 55.940 K_D *(Constant)	7.900
Std. Error	
Bottom	474.9
Top	375.8
$LogIC_{50}$	0.06064
95% confidence Intervals	
Bottom	–145.5 to 1786
Top	15633 to 17161
$LogIC_{50}$	0.5603 to 0.8069
IC50	3.633 to 6.411
Ki	2.760 to 4.870
Goodness of Fit	
Degrees of Freedom	34
R^2	0.9569

Fig. 3 Representative heterologous competition curve of SR141716 against [^{3}H]CP55.940 binding to membrane preparations over-expressing CB_1. Binding assays were performed in our laboratory by using MultiScreenHTS 96-well filter plate, as described in Subheading 3.3. (**a**) Human CB_1 over-expressing membranes (10 μg/test) were incubated with 2.5 nM [^{3}H]CP55.940 in the presence of increasing concentrations (0–1000 nM) of unlabeled SR141716. (**b**) Experimental data (reported in Table 1) were used to calculate IC_{50} and Ki values by nonlinear regression analysis of one-site competition binding curve, through GraphPAD Software for Science version 5 (San Diego, CA). *To calculate IC_{50} and Ki values of SR141716, we have previously calculated K_D (obtained value = 7.9 ± 1.9 nM) of hot CP55.940 through saturation binding assays

4 Notes

1. We recommend to work with fresh tissues; if it is not possible, keep tissues frozen at −80 °C until the day of the assay, and thaw them on ice in the presence of pre-cooled buffer A.

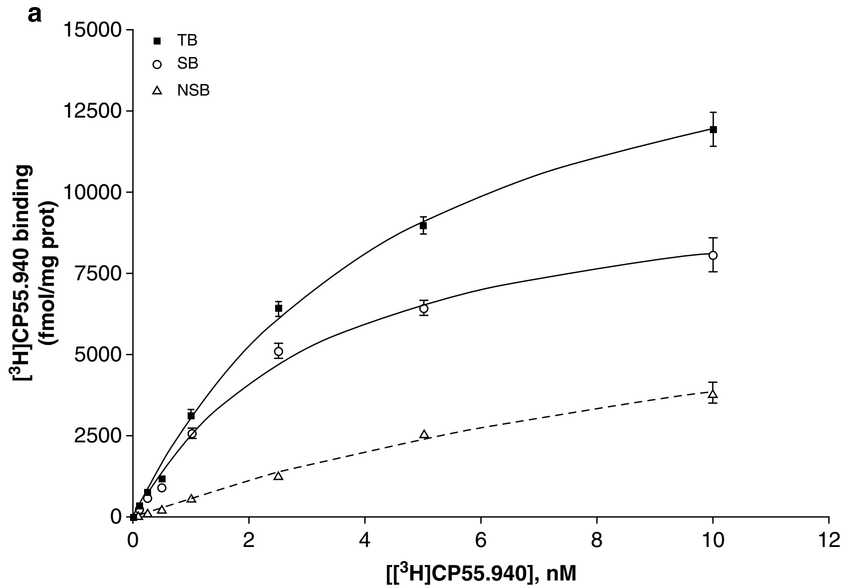
2. For soft tissues (like brain), homogenize samples with a Glass/Teflon Potter homogenizer. To facilitate homogenization of fibrous tissues (e.g., muscle), prior1 to using Glass/Teflon Potter homogenizer, mince samples with scissors and, then, with an Ultra-Turrax T25.
3. Membrane homogenates from fresh tissues can be aliquoted, frozen on dry ice, and stored at −80 °C for no longer than one week. In order to preserve receptor binding activity, do not store membranes from frozen tissues, but proceed immediately with CB_1 receptor binding assay.
4. Protein concentration to be used in the assays depends on CB_1 receptor expression levels; thus we recommend to perform a dose–response curve for unknown biological samples.
5. In addition to binding to receptors of interest (specific binding, SB), radioligands can also interact nonspecifically (nonspecific binding, NSB) with proteins that are not of interest, as well as with the tube walls (during incubation) or with the filters. To overcome this problem, use an excess of CP55.940 at a final concentration that is at least 1000-fold higher than that used for [^{3}H]CP55.940. Indeed, in the presence of an excess of cold CP55.940, CB_1 receptors are virtually all occupied by the unlabeled ligand, and hence the hot ligand can only bind to nonspecific sites. Ideally, NSB should be less than 50 % of the Total Binding (TB).
6. SR141716 is a highly potent antagonist/inverse agonist of CB_1 that displays almost 300-fold selectivity for CB_1 over type-2 cannabinoid receptor (CB_2); Ki values are 1.800 ± 0.075 nM and 514 ± 30 nM, respectively [2]. Therefore, we recommend to use SR141716 at a concentration lower than the Ki value for CB_2.
7. [^{3}H]CP55.940, as other tritiated ligands, has high specific activity; therefore, we recommend to use it at a concentration equal or below its K_D value. For unknown samples, calculate [^{3}H]CP55.940 K_D through saturation binding experiments that measure TB and NSB at various concentrations of hot CP55.940. Calculate K_D and Bmax for specific binding, by using a one-site binding hyperbola nonlinear regression analysis (i.e., with GraphPad Prism), as shown in Table 2 and Fig. 4.
8. To minimize ligand binding to filters, you can also pre-soak glass fiber filters in 0.1–0.5 % PEI (a cationic polymer that neutralizes the negative charge of the glass fiber filter) in buffer D. Prior to filtration of receptor sample, filter PEI solution away, and then wash with ice-cold buffer D for at least three times.

Table 2
Experimental data of saturation binding assay of [^{3}H]CP55.940 to murine brain membrane homogenate

([^{3}H]CP 55.940)[a]	Total binding (TB)[b]	Nonspecific binding (NSB)[b]	Specific binding (SB)[b]
0.10	372.50 ± 17.65	77.49 ± 5.95	295.01 ± 14.62
0.25	751.46 ± 29.94	152.94 ± 11.09	598.52 ± 27.84
0.50	1199.12 ± 19.33	272.49 ± 39.45	926.63 ± 20.10
1.00	3175.15 ± 145.71	596.71 ± 51.70	2578.44 ± 154.68
2.50	6418.74 ± 233.15	1288.23 ± 50.84	5130.51 ± 245.02
5.00	8997.04 ± 267.42	2556.41 ± 85.48	6440.63 ± 376.47
10.00	11,941.20 ± 536.31	3849.46 ± 312.21	8091.74 ± 653.32

Values are the means ± S.E.M. of three independent experiments, each performed in triplicate
[a]The concentration of [^{3}H]CP 55.940 is expressed as nM
[b]Binding values are expressed as fmol/mg of membrane protein

9. A drastically reduced temperature (with ice-cold buffer D) during the washing step will prevent and/or slow down dissociation of bound ligands from CB_1.
10. Control vacuum pump in order to have enough pressure to rapidly filter the samples and prevent ligand dissociation, yet without affecting filter integrity or the amount of membranes retained on the filter. If possible, use a control pressure valve.
11. Time of filtration negatively correlates with ligand K_D; it can range from 20 min for ligands with a $K_D = 0.1$ nM, to 10 s for ligands with a $K_D = 10$ nM.
12. Dry filters require less liquid scintillant to achieve maximum signal than wetted filters. If filters are not completely dried prior to the addition of scintillation liquid cocktail, the residual water present in the filters can interact with the scintillant, thus reducing counting efficiency.
13. Since radioligand embedded within the filter requires some time to become accessible to the liquid scintillant, read radioactivity in the vial at least after 6 h of incubation.
14. We usually plate HeLa cells the day before the experiment, at a concentration of 100,000 cells/well, in a 24-well plate. We recommend to set up a growth curve with different cell types, in order to optimize cell concentration.
15. We advise to use at least 6–8 concentrations for each test compound: the lowest concentration should be at least 1/10 of K_D for CP55.940, while the highest concentration should be approximately 10-fold over such K_D (see Table 2 and Fig. 4).



b

Best-fit values	
Bmax	10773
Kd	3.224
Std. Error	
Bmax	560.8
Kd	0.4104
95% confidence Intervals	
Bmax	9637 to 11909
Kd	2.393 to 4.055
Goodness of Fit	38
Degrees of Freedom	0.9682

Fig. 4 Representative saturation curve of [^{3}H]CP55.940 binding to whole brain membrane homogenates, performed in our laboratory. Binding assays were performed by using a vacuum filtration manifold, Millipore® model 1225, as described in Subheading 3.1.2. (**a**) Increasing concentrations (0–10 nM) of the synthetic agonist of CB receptors [^{3}H]CP55.940 were incubated with whole brain membrane homogenates (50 μg/test), in the absence [Total Binding (*Filled square* TB)] or in the presence of 1 μM unlabeled CP55.940 [Nonspecific Binding (*triangle* NSB)]. NSB mean values were subtracted to relative TB mean values, in order to obtain Specific Binding (circle SB). (**b**) Experimental data (reported in Table 2) were used to calculate Bmax (expressed as fmol/mg membrane protein) and K_D (expressed as nM), by using nonlinear regression analysis for one-site binding hyperbola, through GraphPAD Software for Science version 5 (San Diego, CA)

16. If data are expressed as CPM (counts per min), you need to know the efficiency of your β-counter for [^{3}H], in order to apply the following formula:

$$\text{Efficiency} = \text{Net CPM of standard} / \text{known DPM of standard}.$$

17. In case of CB_1 receptor binding assay performed with intact living cells, data may also be reported as fmol or pmol per 10^6 cell. In this case, it is important to know the exact amount of confluent cells in the well.

References

1. Matsuda LA, Lolait SJ, Brownstein MJ et al (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564
2. Pertwee RG, Howlett AC, Abood ME et al (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. Pharmacol Rev 62:588–631
3. Tantimonaco M, Ceci R, Sabatini S et al (2014) Physical activity and the endocannabinoid sytem: an overview. Cell Mol Life Sci 71:2681–2698
4. Gasperi V, Evangelista D, Savini I et al (2015) Downstream effects of endocannabinoid on blood cells: implications for health and disease. Cell Mol Life Sci 72:3235–3252
5. Mechoulam R, Parker LA (2013) The endocannabinoid system and the brain. Annu Rev Psychol 64:21–47
6. Gatta-Cherifi B, Cota D (2015) Newinsights on the role of the endocannabinoid systemin the regulation of energybalance. Int J Obes (Lond). doi:10.1038/ijo.2015.179
7. Rinaldi-Carmona M, Barth F, Heaulme M et al (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett 350:240–244