Published in final edited form as:

Anal Biochem. 2011 May 1; 412(1): 85–91. doi:10.1016/j.ab.2010.12.034.

Development and Characterization of Immobilized Cannabinoid Receptor (CB1/CB2) Open Tubular Column for On-line Screening

R. Moaddel^{1,*}, A. Rosenberg¹, K. Spelman¹, J. Frazier¹, C. Frazier¹, S. Nocerino², A. Brizzi², C. Mugnaini², and I.W. Wainer¹

¹ Gerontology Research Center, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA

² Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Via A. Moro, snc 53100, Siena, Italy

Abstract

Cannabinoid Receptors, CB1 and CB2, are therapeutic targets in the treatment of anxiety, obesity, movement disorders, glaucoma and pain. We have developed an on-line screening method for CB1 and CB2 ligands, where cellular membrane fragments of a chronic myelogenous leukemia cell line, (KU-812), were immobilized onto the surface of an open tubular capillary to create a CB1/CB2-OT column. The binding activities of the immobilized CB1/CB2 receptors were established using frontal affinity chromatographic techniques. This is the first report that confirms the presence of functional CB1 and CB2 receptors on KU-812 cells. The data from this study confirm that the CB1/CB2-OT column can be used to determine the binding affinities (K_i values) for a single compound and to screen individual or a mixture of multiple compounds. The CB1/CB2-OT column was also used to screen a botanical matrix, *Zanthoxylum clava-herculis*, where preliminary results suggest the presence of a high affinity phytocannabinoid.

Keywords

Biochromatography; basophils; *Zanthoxylum*; cannabinoids; KU-812

1. Introduction

Cannabinoid (CB) receptors are 40 KDa integral membrane G-Protein coupled receptors that contain 7 transmembrane domains [1]. Two CB receptor subtypes, CB1 and CB2, have been identified and characterized [2] and both are coupled to the G_i , G-protein signal transduction pathway. CB2 receptors are predominantly expressed in immune cells [2–4], where they negatively affect the onset of various parameters of the immune response [5] and play a role in the stimulation of growth in several hematopoietic lineages [6]. CB1 receptors are abundant in the Central Nervous System and are also expressed in peripheral areas including but not limited to the pituitary gland, immune cells, heart, lung and gastrointestinal tissues.

*Corresponding Author: Ruin Moaddel, PhD, Bioanalytical and Drug Discovery Unit, National Institute on Aging, National Institutes of Health, Gerontology Research Center, 5600 Nathan Shock Drive, Baltimore, MD. 21224-6825, (410)-558-8294 Phone; moaddelru@grc.nia.nih.gov, (410)-558-8409 FAX.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

They also suppress the release of a number of excitatory and inhibitory neurotransmitters [7,8].

The CB receptors are therapeutic targets in the treatment of anxiety, obesity, movement disorders and glaucoma [2]. The majority of the CB receptor-directed drugs currently in clinical use are phytocannabinoids or endogenous cannabinoids [9]. The importance of the CB receptors as drug development targets is reflected by the development of a number of methods for the screening of compound libraries for CB agonists and antagonists. There are several commercially available techniques currently in use to screen for CB2 agonist activity, for example, the DiscoverRx PathHunter HEK293 CB2 β -arrestin assay [10] and a screening method established for CB1 and CB2 [11]. Other approaches include membrane binding assays using Win-55,1212 as markers [12] and intracellular cAMP assays [13].

We now report an alternative liquid chromatographic approach to the online screening for CB1 and CB2 ligands based upon cellular membrane affinity chromatography (CMAC) [14]. In this method, cellular membrane fragments containing the targeted transmembrane protein are immobilized via biotin-streptavidin strategy onto the surface of open tubular capillaries to create OT columns. This approach has been previously described [15]. In the previous studies, membrane fragments from a cell line expressing the P-glycoprotein drug transporter (Pgp) were used to create a Pgp-Open Tubular (Pgp-OT) columns. Subsequent studies with the Pgp-OT column demonstrated that the approach could be used to rapidly screen for Pgp substrates and that chromatographic retention on the Pgp-OT correlated to data obtained using the Caco-2 monolayer efflux assay, the standard industry screen [c.f. 16].

In this study, membrane fragments obtained from KU-812 cells were used to create an OT column. The immobilization of the KU-812 membrane fragments resulted in a CB1/CB2-OT column. The activities of the immobilized CB1/CB2 receptors were established using frontal affinity chromatographic techniques. The data from this study confirm that the CB1/CB2-OT column can be used to determine the binding affinities (K_i values) for single compound and to screen chemical libraries and botanical extracts for components with CB receptor affinities.

2. Experimental

2.1. Materials

Tris buffer, NaCl, $MgCl_2$, $CaCl_2$, benzamidine, pepstatin A, aprotinin, leupeptin, phenylmethanesulfonyl fluoride (PMSF), glycerol, CHAPS detergent, amino propyl trimethoxy silane (APTS), gluteraldehyde aqueous solution, avidin, biotin-X (6-[(biotinoyl)amino]hexanoic acid), dialysis tubing, Win-55,1212 mesylate, and R-(+)-methanandamide (MA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Arachidonyl-2'-chloroethylamide (ACEA) was obtained from Tocris Bioscience (Ellisville, MO). [3H]-Win-55,1212 mesylate (250 mCi/mol) was purchased from PerkinElmer Life Sciences Products, Inc. (Boston, MA), and stored in ethanol at $-20^\circ C$. Open tubular capillaries (100 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ). *Zanthoxylum clav-herculis* L. (Rutaceae) was purchased from Galens Way (Sebastopol, CA). The COR and LOR series of compounds were synthesized as previously described: COR607, COR608, COR611, COR612 [17]; COR609, COR610, COR613 [18]; COR614 [19]; LOR20, LOR19, LOR53 [20] (Chart 1).

2.2 Cell lines

The human basophil cell line KU-812 was obtained from American Type Tissue Culture (Manassas, Va) and were maintained in EMEM (Invitrogen, Carlsbad, CA, USA)

supplemented with 10% fetal bovine serum, 1mM sodium pyruvate in a humidified incubator at 37°C with 95% O₂ and 5% CO₂. The cells were split 1:10 every 7 days and sub-cultured into new flasks.

2.3. RT-PCR

Total RNA was extracted from human immune cell lines using the RNeasy midi kit (Qiagen, Valencia, CA). The RNA was digested with DNase using the DNA-free kit (Ambion, Austin, TX), 2.5 volumes anhydrous ethanol and 0.1 volume 3M sodium acetate were added and the solution stored at -20 °C overnight. The resulting mixture was centrifuged at 20,800 ×g for 30 min at 4 °C, the pellet was collected, washed once with 70% ethanol, air dried for 10 min and re-suspended in 20 µl DEPEC treated water (Invitrogen) and quantified. Total normal human brain RNA was purchased as the control from Ambion.

cDNA was generated from 5 µg of RNA, using a modification of the GeneFilters protocol from Research Genetics (Huntsville, AL). For the priming step 5 µg of RNA was suspended in 8 µl of DEPEC treated water (Invitrogen) and 2.0 µl of 5 µM Oligo dT (Invitrogen) and the mixture was kept at 70 °C for 10 min and then placed on ice for 2 min. For the elongation step the 10 µl mixture was added to 20 µl of a solution containing: 6 µl of 5X First Strand buffer, 1.0 µl of 0.1 M DTT, 1.5 µl of 2.5 mM dNTP mixture and 1.5 µl of Reverse Transcriptase (200 U/µl), and 10 µl of DEPEC treated water (Invitrogen) and kept at 37°C for 2.5 h.

The forward and reverse primers for the CB1 and CB2 receptors, CB1: 5'-Primer 5'-CGTGGGCAGCCTGTTCTCA-3'; 3'-Primer 5'-CATGCGCGCTTG GTCTGG-3' Product size 408; CB2: 5'-Primer 5'-CGCCGGAAGCCCTCATACC-3'; 3'-Primer 5'-CCTCATTCGGGCCATTCTG-3' Product size 522; β-actin: 5'-Primer 5'-GAGCACAGAGCCTCGCCTTT-3'; 3'-Primer 5'TGGTGGTGAAGC TGTAGCCG-3' Product size 666, had been previously described by Carracedo, et al.[21] and were purchased from Integrated DNA Technologies (Coral Ville, IA). The forward and reverse primers for β-actin, Table 1, were also obtained from Integrated DNA Technologies. For each PCR reaction, 5 µl of cDNA was added to a solution containing 2.0 µl of 2.5 mM dNTP, 1.0 µl Taq polymerase (5 U/µl), (Clontech, Mountain View, CA), 1.0 µl of the primer (20 pmol/µl), 5.0 µl of 10X buffer and 35 µl of DEPEC treated water (Invitrogen) and run on PTC-100 Thermo Cycler (Waltham, MA). For CB1 the reaction conditions were 94°C for 1 min, 65°C for 1 min 72°C for 1min for 36 cycles; for CB2 the reaction conditions were 94°C for 1 min, 67°C for 1 min 72°C for 1min for 35 cycles; for β-actin the reaction conditions were 94°C for 1 min, 65°C for 1 min 72°C for 1min for 35 cycles. A 20 µl aliquot of the PCR reaction was analyzed using a 0.8% agarose gel and quantified with UV detection.

2.4. Preparation of the CB1/CB2-OT column

The CB1/CB2-OT column was synthesized using a previously described protocol [14,15]. Briefly, 10⁷ KU812 cells were homogenized on a Model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland) in 10 ml of Tris buffer [50 mM, pH 7.5] containing 100 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 100 µM benzamidine, 4 µM pepstatin A, 100 µg aprotinin, 100 µg leupeptin, and 1mg PMSF. The homogenate was centrifuged at 700 × g for 5 min, the pellet discarded and the supernatant centrifuged at 100,000 × g for 30 min at 4°C. The resulting pellet was suspended in 2 ml of Tris buffer [50 mM, pH 7.5] containing 2% (w/v) CHAPS, 100 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 100 µM benzamidine, 100 µg aprotinin, 100 µg leupeptin, 1mg PMSF, 4 µM pepstatin A, 10% glycerol and the resulting mixture was rotated at 150 rpm using an orbit shaker for 18 h at 4°C. The mixture was then centrifuged at 100,000 × g for 25 min and the supernatant was used for the immobilization onto the open tubular capillary.

The open tubular capillary (25 cm × 100 µm i.d.) was primed with 1 M NaOH for 20 min, rinsed with water, and dried at 95°C for 1 h. A 10% aqueous solution of APTS was passed through the capillary for 5 min followed by a 30 min incubation at 95°C in an oven and the process was repeated. After 18 h, a 1% aqueous solution of glutaraldehyde was passed through the capillary for 1 h followed by water and a solution of avidin (25 mM, 5 mg in 2 ml water) was recycled through the capillary for 5 min. Both tips of the capillary were submerged in the avidin solution for 7 days at 4°C. Then a solution of biotin-X (3.5 mM, 5 mg in DMSO:water (3:1 v/v) was run through the capillary for 1 h. The solution containing the solubilized KU812 membranes was recycled through the column for 30 min. The open tubular capillary was then dialysed against Tris buffer [50 mM, pH 7.4] containing 100 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, and 1 mM EDTA and rotated overnight at 4 °C. The following day, solubilized KU812 membranes were again passed through the capillary for 30 min and the dialysis was repeated.

2.5. Frontal chromatography with radio-labeled markers

2.5.1. Chromatographic system—The CB1/CB2-OT column was connected to a LC-10AD isocratic HPLC pump (Shimadzu, Columbia, MD, USA). The mobile phase consisted of ammonium acetate [10 mM, pH 7.4]: methanol (90:10, v/v), delivered at a rate of 50 µl/min at room temperature. Detection was accomplished using an on-line scintillation detector (IN/US system, β-ram Model 3, Tampa, FL, USA) with a dwell time of 1 s using Laura Lite 3 software and a 50 µl flow cell with the scintillation flow rate set at 150 µl/min.

2.5.2. Chromatographic studies—The marker ligand used in the frontal chromatographic studies was [³H]-Win-55,1212 added to the mobile phase at a concentration of 0.25 nM for studies with CB2 receptor ligands or 0.5 nM for studies with CB1 receptor ligands. A 50 ml sample Superloop (Amersham Pharmacia Biotech) was used to apply the marker ligand and a series of displacer ligands. For the CB1: Win-55,1212: 10, 20, 25 and 40 nM; methanamide (MA): 10, 25, 30, 40 and 60 nM; Arachidonyl-2'-chloroethylamide (ACEA): 0.5, 0.75, 1.25, 10 and 30 nM. For the CB2: Win-55,1212: 0.15, 0.25, 0.5 and 5 nM; MA: 0.25, 0.75, 1 and 10 µM; ACEA: 0.5, 1, 10, 25, 50 and 100 µM.

2.5.3. Data Analysis—The dissociation constants for the marker (K_{dM}) and displacer ligands (K_i) were calculated using a previously described non-linear approach [22]. In brief, the dissociation constants of displacer ligand (K_i) can be calculated using Eq. (1), assuming that the $[M] \ll K_i$ of Marker, so that $[M]/K_{dM} \ll 1$:

$$[D](V - V_{\min}) = P[D](K_d + [D])^{-1} \quad (1)$$

where $[D]$ is the concentration of displacer ligand, V is the retention volume of displacer ligand and $V_{\min}$ is the retention volume of displacer ligand when the specific interaction is completely suppressed and P is the product of the $B_{\max}$ (the number of active binding sites) and (K_i/K_{dM}) [21]. From the plot of $[D](V - V_{\min})$ versus $[D]$, dissociation constant values (K_i), for displacer ligand can be obtained. The data was analyzed by non-linear regression with a rectangular hyperbolic curve using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) running on a personal computer.

2.6. Screening of chemical libraries and plant extracts

2.6.1. Screening of novel CB2 analogs—A series of compounds (Chart 1) LOR20, LOR19, LOR53, COR607, COR608, COR609, COR610, COR611, COR612, COR613 and COR614 were screened on the KU-812 open tubular columns. A 2 mL frontal sample containing 0.25 nM [³H]-Win-55,1212 with a mixture of 10 nM of 3–4 compounds was

carried out. The groupings were as follows: Group A: LOR20, LOR19, LOR53; Group B: COR609, COR611, COR613, COR614; Group C: COR607, COR608, COR610; Group D: COR607, COR612.

2.6.2. Screening of *Zanthoxylum clava-herculis* ethanol extract—A standard herbal extract was obtained from a company that produces extracts following Good Manufacturing Practices (Galens Way, Sebastopol, CA). Briefly, fresh, dormant roots of *Z. clava-herculis* were air dried in the dark. Species was verified by microscopic identification. Roots were washed thoroughly, cut into small pieces (≤ 1 cm wide) and extracted in 65% ethanol (root to solvent ratios 1:3). After 3 weeks maceration the solvent was recovered. Species was verified by microscopy. A 1% solution of the *Zanthoxylum clav-herculis* extract in 2 mL frontal sample with 0.25 nM [3 H]-Win-55,1212 was injected on the KU-812 OT column at 50 μ L/min.

3. Results and Discussion

3.1. The Identification of the CB1/CB2 receptors in KU-812 cells

The KU-812 cell line is a chronic myelogenous leukemia cell line, and related to cells found in the immune system. While it has been previously reported that basophils express both CB1 and CB2 receptors [4], this expression was not definitively confirmed in the KU-812 cells. The presence of CB1 and CB2 mRNA in the KU-812 cell line was confirmed using RT-PCR, Fig 1.

3.2. Characterization of the CB1/CB2-OT column

The presence of functional CB1 and CB2 receptors in the KU-812 cell line was confirmed by the data from frontal affinity studies on the CB1/CB2-OT. In these studies, the CB1/CB2 receptor agonist, [3 H]-Win-55,1212 was used as the marker ligand. A representative chromatogram obtained on the CB1/CB2-OT is presented in Fig. 2A. As previously described [14], the initial flat portion of the chromatographic trace represents specific and non-specific binding to the immobilized KU-812 membrane fragments and the rapid vertical breakthrough and plateau represents the specific saturation of the CB1/CB2 receptors. When 0.5 nM [3 H]-Win-55,1212 was chromatographed alone, the midpoint of the breakthrough curve was observed at 8.07 min, representing a breakthrough volume of 0.404 ml, which is equivalent to 200 column volumes. The columns lifetime averaged between 2–3 weeks, with continuous use, with a noticeable decrease in retention time from 8.1 min to 3.3 min (0.17 ml) after the 3rd week.

It has been previously reported that Win-55,1212 is an agonist at both CB1 and CB2 and is 15x more selective for CB2, therefore making it a potential chromatographic marker ligand for the differentiation between CB1 and CB2 binding on the CB1/CB2-OT column. This was demonstrated using [3 H]-Win-55,1212 mobile phase concentrations of 0.25 nM (for CB2) and 0.50 nM (a saturating concentration for CB2) for CB1. Using these conditions, increasing concentrations of Win-55,1212, from 0.1 nM to 50 nM produced dose-dependent decreases in retention volume which were used to calculate the binding affinities, K_d values, of Win-55,1212 for the CB1 and CB2 receptors. When the [3 H]-Win-55,1212 mobile phase concentration was 0.25 nM (for CB2) the calculated K_d for Win-55,1212 was 0.37 nM and when the [3 H]-Win-55,1212 mobile phase concentration was 0.50 nM (for CB1) the calculated K_d for Win-55,1212 was 8.6 nM, Table 1. These results are consistent with previously reported CB2 and CB1 K_d values for Win-55,1212 of 0.76 nM and 11.9 nM, respectively [23]. The B_{max} values calculated from the displacement studies also indicate that there was a 10-fold greater immobilization of CB1 receptor versus CB2 receptor from

the KU-812 membrane fragments, Table 1, which is consistent with the data from the RT-PCR.

The use of a single marker in the chromatographic characterization of two isoforms of a receptor has been previously demonstrated using a cellular membrane affinity chromatography column (CMAC) containing membrane fragments from the 1321N1 astrocytoma cell line, the CMAC (1321N1) column [24]. In these studies, [³H]-epibatidine was used as the marker ligand on the CMAC (1321N1) column to characterize the expression of two neuronal nicotinic receptor (nAChR) subtypes, $\alpha 4\alpha \beta 2$ nAChR and $\alpha 7$ nAChR.

In addition to Win-55,1212, multiple concentrations of CB1 selective agonists, MA and ACEA were also run on the CB1/CB2-OT to determine their binding affinities, K_i values, Table 1. The calculated K_i values correlated with previously reported data, $r^2 = 0.907$. The results demonstrate that the approach can be used to determine the binding affinities of CB1/CB2 receptor ligands.

3.3. The use of the CB1/CB2-OT column for screening of chemical libraries

Previous studies with CMAC($\alpha 3\beta 4$ nAChR) and CMAC($\alpha 4\beta 2$ nAChR) columns have demonstrated that the relative displacement of [³H]-epibatidine, expressed as Δt values (ie. change in retention volume between the [³H]-epibatidine alone and [³H]-epibatidine in the presence of a displacer) could be used to determine whether a compound had affinity for the nAChR [25]. In addition, frontal affinity chromatography on a CMAC(GPR17) column has been used to identify compounds with affinity for the GPR17 receptor within a chemical library [26]. Based on these observations, we determined if a single differential displacement experiment on the CB1/CB2-OT was able to determine if a mixture of compounds contained CB1 and/or CB2 ligands.

The initial studies determined the displacement of 0.25 nM [³H]-Win-55,1212 by 10 nM concentrations of known CB1/CB2 receptor ligands, Win-55,1212, MA, ACEA, COR-615, dodeca-2(E),4(E)-dienoic acid, isobutylamide and β -caryophyllene and compounds with no known affinity for the CB1/CB2 receptors, nicotine and diphenhydramine (Table 2). We have previously demonstrated [15] that a $\Delta t > 0.5$ min can be used as an accurate method to classify a compound as a ligand for the immobilized receptor. In this case, each of the CB1/CB2 ligands decreased the [³H]-Win-55,1212 retention volume, equivalent to Δt , by > 0.5 min while the non-ligands produced no significant decrease in [³H]-Win-55,1212 retention volume. In order to determine that there was no loss in activity for the CB1/CB2 column, 0.25 nM [³H]-Win-55,1212 was run every four days to confirm that observed retention volume did not decrease. A mixture of the known CB1/CB2 ligands produced a $\Delta t > 0.5$ min while a mixture of the non-ligands produced no significant displacement indicating that the screen can be carried out with mixtures as well as single compounds (Table 2).

The simultaneous screening of multiple compounds was used to screen 11 recently synthesized compounds for affinity for the CB1/CB2 –OT column, Table 3. The compounds were screened in 4 groups (Group A: LOR20, LOR19, LOR53; Group B: COR609, COR611, COR613, COR614; Group C: COR607, COR608, COR610; Group D: COR607, COR612) and the chromatographic results correctly predicted the groups with compounds with CB2 activity.

3.5. Screening of a complex matrix (plant extract)

As phytocannabinoids are one of the major groups of CB receptor-directed drugs currently in clinical use, a logical extension is to screen plant extracts for the potential to identify new/unknown compounds that have CB1/CB2 activity. To this end, we investigated the

screening potential of the CB1/CB2-OT column for complex matrices using the plant extract of *Zanthoxylum clava-herculis* L. (Rutaceae), which is known to be a rich source of alkylamides. Targeting alkylamides as a potential source of cannabinoid ligands is reasonable as it has been previously reported the 15 alkylamides from *Echinacea* spp. [27], at least 2 are known to be high affinity CB2 ligands [28,29]. To date, there have been no reports of the *Zanthoxylum* species having cannabinoid activity, although, they are known to have affinity for the TRPV1 and TRPA1 [30,31]. As seen in Figure 3, a significant displacement of 0.25 nM [3H]-Win-551212 was observed with a 1% solution of the *Zanthoxylum* extract, indicating the presence of ligands with high affinity at the CB1/CB2 receptors. The isolation and characterization of the active components is currently being carried out and will be reported elsewhere.

Acknowledgments

This research was supported in part by the Intramural Research Program of the National Institute on Aging/NIH.

References

1. Shire D, Calandra B, Delpech M, Dumont X, Kaghad M, Le Fur G, Caput D, Ferrara P. Structural Features of the Central Cannabinoid CB1 Receptor Involved in the Binding of the Specific CB1 Antagonist SR 141716A. *Journal of Biol Chem* 1996;271(12):6941–6946. [PubMed: 8636122]
2. Merighi S, Simioni C, Gessi S, Varani K, Borea PA. Binding thermodynamics at the human cannabinoid CB1 and CB2 receptors. *Biochemical Pharmacology* 2010;79:471–477. [PubMed: 19766600]
3. Tyagi V, Philips BJ, Su R, Smaldone MC, Erickson VL, Chancellor MB, Yoshimura N, Tyagi P. Differential Expression of Functional Cannabinoid Receptors in Human Bladder Detrusor and Urothelium. *The Journal of Urology* 2009;181:1932–1938. [PubMed: 19237176]
4. Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P. Expression of central and cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 1995;232:54–61. [PubMed: 7556170]
5. Parolaro D, Massi P, Rubino T, Monti E. Endocannabinoids in the immune system and cancer. *Prostaglandins Leukot Essent Fatty Acids* 2002;66:319–32. [PubMed: 12052046]
6. Derocq JM, Jbilo O, Bouaboula M, Segui M, Clere C, Casellas P. Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. *J Biol Chem* 2000;275:15621–15628. [PubMed: 10821843]
7. Pertwee RG. The Pharmacology of Cannabinoid CB1 and CB2 Receptors. *Pharmacol Ther* 1997;74(2):129–180. [PubMed: 9336020]
8. Ong WY, Mackie K. A light and electron microscopic study of the CB1 cannabinoid receptor in the primate spinal cord. *J Neurocytol* 1999;28(1):39–45. [PubMed: 10573606]
9. Mackie K. Cannabinoid Receptors as Therapeutic Targets. *Annu Rev Pharmacol Toxicol* 2006;46:101–22. [PubMed: 16402900]
10. McGuinness D, Malikzay A, Visconti R, Lin V, Bayne M, Monsma F, Lunn CA. Characterizing Cannabinoid CB2 Receptor Ligands Using DiscoveRx PathHunter™ 2-Arrestin Assay. *J Biomol Screen* 2009;14:49. [PubMed: 19171920]
11. Drmota, T.; Greasley, P.; Groblewski, T. Screening Assays for Cannabinoid-Ligand-Type Modulators of GPR55. US2006/0194260 A1. 2006.
12. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacol Rev* 2002;54:161–202. [PubMed: 12037135]
13. Faubert Kaplan BL, Rockwell CE, Kaminski NE. Evidence for Cannabinoid Receptor-Dependent and -Independent Mechanisms of Action in Leukocytes. *JPET* 2003;306(3):1077–1085.

14. Moaddel R, Wainer IW. The Preparation and Development of Cellular Membrane Affinity Chromatography Columns. *Nat Protocol* 2009;4(2):197–205.
15. Moaddel R, Bullock P, Wainer IW. Development and characterization of an open tubular column containing immobilized P-glycoprotein for rapid on-line screening for P-glycoprotein substrates. *JChromB Analyt Technol Biomed Life Sci* 2004;799(2):255–63.
16. Moaddel R, Hamid R, Patel S, Wainer IW, Bullock P. Comparison of an open tubular column containing immobilized P-glycoprotein with Caco-2 cell monolayers for the purpose of investigating interactions between drug candidates and Pgp. *Anal Chimica Acta* 2006;578(1):25–30.
17. Silvestri R, Ligresti A, La Regina G, Piscitelli F, Gatti V, Brizzi A, Pasquini S, Lavecchia A, Allarà M, Fantini N, Carai MA, Novellino E, Colombo G, Di Marzo V, Corelli F. Synthesis, cannabinoid receptor affinity, molecular modeling studies and in vivo pharmacological evaluation of new substituted 1-aryl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamides. 2. Effect of the 3-carboxamide substituent on the affinity and selectivity profile. *Bioorg Med Chem* 2009;17:5549–5564. [PubMed: 19595596]
18. Silvestri R, Cascio MG, La Regina G, Piscitelli F, Lavecchia A, Brizzi A, Pasquini S, Botta M, Novellino E, Di Marzo V, Corelli F. Synthesis, cannabinoid receptor affinity, and molecular modeling studies of substituted 1-aryl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamides. *J Med Chem* 2008;51:1560–1576. [PubMed: 18293908]
19. Brizzi A, Brizzi V, Martinelli A, Tuccinardi T, Cascio MG, Bisogno T, Di Marzo V. Design, Synthesis and binding studies of new potent ligands of cannabinoid receptor (II). *Bioorg Med Chem* 2007;15:5406–5416. [PubMed: 17561406]
20. Pasquini S, Botta L, Semeraro T, Mugnaini C, Ligresti A, Palazzo E, Maione S, Di Marzo V, Corelli F. Investigations on the 4-Quinolone-3-carboxylic Acid Motif. 2. Synthesis and Structure-Activity Relationship of Potent and Selective Cannabinoid-2 Receptor Agonists Endowed with Analgesic Activity *in Vivo*. *J Med Chem* 2008;51:5075–5084. [PubMed: 18680276]
21. Carracedo A, Gironella M, Lorente M, Garcia S, Guzma M, Velasco G, Iovanna JL. Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. *Cancer Research* 2006;66:6748–6755. [PubMed: 16818650]
22. Kimura T, Perry J, Anzai N, Pritchard J, Moaddel R. Development and Characterization of immobilized human organic anion transporter based liquid chromatographic stationary phase: hOAT1 and hOAT2. *J ChromB* 2007;859(2):267–271.
23. Ross RA, Brockie HC, Stevenson LA, Murphy VL, Templeton F, Makriyannis A, Pertwee RG. Agonist-inverse agonist characterization at CB1 and CB2 cannabinoid receptors of L759633, L759656, and AM630. *Br J Pharmacol* 1999;126(3):665–672. [PubMed: 10188977]
24. Kitabatake T, Moaddel R, Cole R, Gandhari M, Frazier C, Hartenstein J, Rosenberg A, Bernier M, Wainer IW. Characterization of a multiple ligand-gated ion channel cellular membrane affinity chromatography (CMAC) column and identification of endogenously expressed receptors in astrocytoma cell lines. *Anal Chem* 2008;80(22):8673–8680. [PubMed: 18847217]
25. Moaddel R, Jozwiak K, Yamaguchi R, Cobello C, Whittington K, Sarkar T, Basak S, Wainer IW. On-line screening of conformationally constrained nicotines and anabasines for agonist activity at the $\alpha 3\beta 4$ and $\alpha 4\beta 2$ – nicotinic acetylcholine receptors using immobilized receptor based liquid chromatographic stationary phases. *JChromB* 2004;813(1–2):235–240.
26. Calleri E, Ceruti S, Cristalli G, Martini C, Temporini C, Parravacini C, Volpini R, Daniele S, Caccialanza G, Lecca D, Lambertucci C, Trincavelli ML, Marrucci G, Wainer IW, Ranghino G, Fantucci P, Abbracchio M, Massolini G. Frontal Affinity Chromatography-Mass Spectrometry Useful for Characterization of New Ligands for GPR17 Receptor. *J Med Chem* 2010;53:3489–3501. [PubMed: 20394377]
27. Spelman K, Wetschler MH, Cech NB. Comparison of alkylamide yield in ethanolic extracts prepared from fresh versus dry *Echinacea purpurea* utilizing HPLC–ESI-MS. *J Pharm Biomed Anal* 2009;49:1141–9. [PubMed: 19321283]
28. Gertsch J, Schoop R, Kuenzle U, Suter A. *Echinacea* alkylamides modulate TNF- α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS Letters* 2004;577(3):563–569. [PubMed: 15556647]

29. Raduner S, Majewska A, Chen J-Z, Xie X-Q, Hamon J, Faller B, Altmann K-H, Gertsch J. Alkylamides from Echinacea Are a New Class of Cannabinomimetics: Cannabinoid Type 2 Receptor-Dependent and -Independent Immunomodulatory Effects. *J Biol Chem* 2006;281:14192–14206. [PubMed: 16547349]
30. Koo JY, Jang Y, Cho H, Lee C-H, Jang KH, Chang YH, Shin J, Oh U. Hydroxy-3-sanshool activates TRPV1 and TRPA1 in sensory neurons. *European Journal of Neuroscience* 2007;26(5): 1139–1147. [PubMed: 17767493]
31. Riera CE, Menozzi-Smarrito C, Affolter M, Michlig S, Munari C, Robert F, Vogel H, Simon SA, Le Coutre J. Compounds from Sichuan and Melegueta peppers activate, covalently and non-covalently, TRPA1 and TRPV1 channels. *British Journal of Pharmacology* 2009;157(8):1398–140. [PubMed: 19594761]

Figure 1.

A. RT-PCR of CB1 and CB2 expression in Bone marrow (1), Human Mast cell (2), KU-812 (3) and blank (4). B. RT-PCR β -actin primers.

Figure 2.

The chromatographic traces obtained for 0.5 nM [^3H]-Win-55,1212 on the KU-812-OT column in the presence of varying concentrations of ACEA. The mobile phase was composed of ammonium acetate [10 mM, pH 7.4] and methanol (90:10, v/v) and delivered at a flow rate of 50 $\mu\text{L}/\text{min}$. In panel A, the column was injected with 0.5 nM [^3H]-Win 55-1212 with 0.5 nM, 0.75 nM, and 1.25 nM ACEA (right to left). In panel B the column was injected with 0.25 nM [^3H]-Win-55,1212 with 0.05 μM , 10 μM , and 25 μM ACEA (right to left).

Figure 3.

Frontal Affinity Chromatogram showing the displacement of 0.25 nM [^3H]-Win 55,1212 (A) in the presence of 1 % *Zanthoxylum clava-herculis* (B) at a flow rate of 50 $\mu\text{l}/\text{min}$ and the mobile phase composed of ammonium acetate [10 mM, pH 7.4] and methanol (90:10, v/v).

LOR 19

LOR20

LOR53

COR607

COR608

Chart 1.
Chemical structure of compounds LOR19, LOR20, LOR53, COR607, COR608, COR609, COR610, COR611, COR612, COR613, COR614.

Table 1

The K_i of Win 55-1212, Methanamide (MA) and ACEA as determined on the KU-812 open tubular column and B_{max} , using frontal affinity chromatographic techniques compared to binding affinities reported in literature [12,23].

Agonist	K_i CB1 (nM) B_{max} (pmoles)	K_i CB2 (nM) B_{max} (pmoles)	K_i CB1 (lit)	K_i CB2 (lit)
Win-55,1212	8.6±8.3 3.5	0.37±0.12 0.14	11.9	0.76
MA	19.8±10.9 3.9	653±182 0.12	17.9	868
ACEA	0.74±0.22 2.0	5200 ±2.5 0.16	1.4	2000

Table 2

The screening of compounds for activity at the CB1/CB2 receptors using the KU-812 OT columns. 0.25 nM of [³H]-Win-55,1212 was used as the marker ligand with 10nM injections of a series of known binders and non-binders (A) and a mixture of compounds. ΔT greater than 0.5 minutes indicates activity and is represented by a (+).

A.		
Compound [10 nM]	ΔT > 0.5 (min)	ΔT (min)
Win -55,1212	+	2.0
ACEA	+	2.9
MA	+	1.5
COR 615	+	3.5
Dodeca-2(E),4(E)-dienoic acid isobutylamide	+	1.3
β-caryophyllene	+	3.0
Diphenhydramine	–	–0.1
Nicotine	–	0.1

B.		
Compounds [10 nM]	ΔT > 0.5 (min)	ΔT (min)
Nicotine, Cotinine, Diphenhydramine, Ketamine	–	0.04
Nicotine, Cotinine, Diphenhydramine, Ketamine, Win-55,1212	+	2.22

Table 3

The screening of compounds for activity at the CB1/CB2 receptors using the KU-812 OT columns. 0.25 nM of [³H]-Win-55,1212 was used as the marker ligand with 10nM injections of a series of compounds.

Compound	Ki CB1 (nM)	Ki CB2 (nM)	CB1/CB2-OT
LOR20	14.3	10.7	+++
LOR19	30.5	0.8	+++
LOR53	640	3.4	+++
COR607	70	>3940	–
COR608	30	>3940	–
COR609	28	3940	–
COR610	5.6	1.7	+
COR611	80	>3940	–
COR612	56	>3940	–
COR613	35	3940	–
COR614	190	2400	–