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Development and preliminary validation of a plate-based CB1/CB2 receptor functional assay

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Abstract

Cannabinoids receptors (CB) are being targeted therapeutically for the treatment of anxiety, obesity, movement disorders, glaucoma, and pain. More recently, cannabinoid agonists have displayed anti-proliferative activity against breast cancer and prostate cancer in animal models.

In order to study cannabinoid receptor ligands, we have developed a novel plate-based assay that measures internalization of CB1/CB2 receptors by determining the change in the intracellular levels of the radiolabeled agonists: [³H]-Win 55-212-2 for CB1 and [³H]-CP 55-940 for CB2. The developed plate-based assay was validated by determining IC₅₀ values for known antagonists: AM251, AM281, AM630 and AM6545. The data obtained was consistent with previously reported values, therefore confirming that the assay can be used to determine the functional binding activities (IC₅₀) of antagonists for the CB1 and CB2 receptors.

In addition, we demonstrated that the plate-based assay may be used for screening against complex matrices. Specifically, we demonstrated that the plate-based assay was able to identify which extracts of several species of the genus *Zanthoxylum* had activity at the CB1/CB2 receptors.

Keywords

plate-based assay; *Zanthoxylum*; isosteviol; CB1 antagonists; CB2 antagonist

1. Introduction

Cannabinoid (CB) receptors are 40 kDa integral membrane G-protein-coupled receptors that contain seven transmembrane domains. To date, three subtypes of cannabinoid receptors have been identified: CB1 [1], CB2 [2] and the novel cannabinoid receptor GPR55 [3]. These three G protein-coupled receptors play an important role in many physiological processes, including metabolic homeostasis, craving, pain, anxiety, bone growth, and

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immune function [4,5]. CB1 and CB2 receptors mediate their effects via a pertussis toxin-sensitive GTP-binding regulatory protein G_i while the novel cannabinoid-like receptor GPR55 does not couple to $G(i/o)$ or $G(q)$ but to $G\alpha_{13}$ [3].

CB1 receptors are abundant in the central nervous system particularly in regions involved in cognition and short-term memory, and with motor function and movement [6,7]. They are also expressed in peripheral areas, including but not limited to the pituitary gland, immune cells, heart, lung, bone marrow and gastrointestinal tissues [2]. CB2 receptors are mainly expressed in immune cells, such as macrophages, lymph nodes and microglia, although they have also been detected in non-immune cells [2, 8].

Prolonged exposure of cannabinoid receptors to an agonist results in desensitization of the receptor due to a decrease in agonist sensitivity [9]. The molecular mechanism of receptor desensitization involves several distinct events, including a decrease in the number of receptors on the cell surface, which can occur through internalization, thereby down regulating receptor signaling [10,11] or by degradation. Measurement of receptor internalization can be considered as a direct assessment of agonistic ligand binding activity for the CB1 and CB2 receptors. The efficacy in receptor internalization is dependent on the agonist used, for example, while Win55,212-2, CP55,940, and HU210 induce rapid internalization of the CB1 receptor, methanandamide (MA) and Δ^9 -THC had a much less pronounced effect, if any [11]. Further, Win 55,212-2 failed to induce CB2 internalization, whereas CP55,940 internalized the CB2 receptor robustly [10,11]. The direct assessment of antagonistic activity for the CB1 and CB2 receptors can be determined in the presence of the appropriate agonist.

The majority of the CB receptor-directed drugs currently in clinical use are phytocannabinoids or endogenous cannabinoids [4]. The importance of CB receptors as drug development targets is reflected in the number of programs now targeting cannabinoid receptors [4,5]. As a result, methods have been developed to screen compound libraries to identify novel ligands for the CB1/CB2 receptors. For example, Thermo Scientific reported a CB1 redistribution assay based on cellular translocation of GFP-tagged proteins in response to compounds or other stimuli (Thermo Scientific, Lafayette USA). This method was designed to assay compounds for their ability to modulate internalization of CB1. However, the CB1 protein was a fusion protein with a GFP-tag. Although there are no reports on the effect of a GFP-tag on CB1 receptors, it has been previously reported that the use of GFP-tagged proteins may induce alteration in functional activity of the receptor [12,13], its membrane localization [14] or rate of membrane incorporation [15]. In addition to this method, there are several commercially available techniques currently in use to screen CB2 activity. For example, the DiscoverX PathHunter HEK293 CB2 β -arrestin assay [16] was developed as a screening assay for both CB1 and CB2 receptors, while other methods used the MAPK cascade, forskolin-stimulated cyclic AMP production or Ca^{2+} influx [17] and the [^{35}S]Guanosine-5'-*O*-(3-thiotriphosphate), a ([^{35}S]GTP γ S binding assay [18], to measure the functional activity of cannabinoid receptors. While each of these methods have advantages and disadvantages, a major drawback is that these methods measure activity through a secondary mechanism. Direct measurement of binding to the CB1/CB2 receptors has been carried out with filtration assays, centrifugation or classical binding assays (K_d), and by frontal affinity chromatography using a CB1 and CB2 receptor column [19]. The binding affinity provides important information about the compounds ability to bind to the receptor, however, binding does not necessarily effect G_i coupling and therefore may not result in any functional activity. Direct measurement of functional activity of the cannabinoid receptors provides additional information as to whether the compounds binding will result in a functional response. To this end, we have developed a novel plate-based assay that measures the ligand effect on internalization of the CB1/CB2 receptors by

determining the change in intracellular levels of a radiolabeled agonist. We have also demonstrated that this method can be used to study the functional activity of a ligand, as well as to screen complex matrices. Specifically, expanding on our previous work [19], we show that the novel plate-based assay was able to identify extracts of plants of the genus *Zanthoxylum* with activity at the CB1 and CB2 receptors.

2. Materials and methods

Materials

The radioligands [³H]-CP55,940(144mCi/mmol) and [³H]-Win 55,212-2(40.9mCi/mmol), were provided by PerkinElmer Life Science (Boston, MA, USA). AM251, AM630, AM6545, isosteviol and G418 were purchased from Sigma-Aldrich (Milwaukee, Wisconsin). The molecular structure of the studied ligands is shown in Figure 1. Dulbecco's modified Eagles' medium nutrient mixture F12 Ham, (1:1) with L-glutamine and 2.438g/L sodium bicarbonate, trypsin solution, phosphate-buffered saline, FBS, sodium pyruvate solution (100 mM), L-Glutamine (200 mM), and penicillin/streptomycin solution (containing 10,000 units/ml penicillin and 10,000 µg/ml streptomycin) were obtained from Quality Biological (Gaithersburg, MD, USA).

All tested compounds were dissolved in DMSO. The treatment solutions were obtained from stock solutions by using 0.5 fold serial dilutions with final higher concentration of DMSO less than 0.1% in the media.

CHO cells

Chinese Hamster Ovary (CHO) cells stably transfected with cDNA encoding human cannabinoid CB1 or CB2 receptors were kindly donated from Dr. Paul Hollenborg (University of Michigan Medical School, Ann Arbor, MI 48109). They were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagles's medium nutrient mixture F12 Ham, (1:1) with L-glutamine and 2.438g/L sodium bicarbonate supplemented with 1% penicillin/Streptomycin solution and 10% fetal bovine serum (v/v) and 0.4mg/mL G418. This CHO-CB1/CB2 was passaged twice weekly using trypsin (0.05%)-EDTA (0.1%) in phosphate buffered saline without calcium and magnesium with a maximum of 50 passages.

Plate based assay

Chinese hamster ovary cells, transfected with CB1 or CB2, were cultured in 96 well plates at a density of 2.0×10^5 cells/mL for 24 h in a media consisting of DMEM/F12 (1:1) with L-glutamine and 2.438g/L sodium bicarbonate supplemented with 1% penicillin/streptomycin solution and 10% fetal bovine serum (v/v). However, the cells treatment media was simply DMEM/F12.

To assess antagonist effects mediated by CB1 receptor internalization, the cells were pre-incubated with two-fold serial dilutions of each compound tested (AM251 (0.25-500nM), AM630 (5-4000nM), AM281 (20-20000nM), AM6545 (0.05-10000nM), Rimonabant(10-10000nM), JD5001-050(0.2-500nM), JD5037-030(0.005-10nM), Otenabant(1-2000 nM), AB001 (1.0-2000nM), UR144 (1-2000nM) and isosteviol (0.5-1000 nM) for one hour prior to treatment with the radioligand tracer [³H]-Win 55,212-2 (10 nM).

To assess antagonist effect mediated by CB2 receptor internalization, the cells were pre-incubated with 0.5 fold serial dilution of each compound tested (AM251 (5-10000nM), AM630 (0.5-250nM), AM281 (0.4-40000nM), AM6545 (10-20000nM), Rimonabant(0.2-20000nM), JD5001-050(40-40000nM), JD5037-030(10-20000nM), Otenabant(0.4-40000nM), AB001 (10-20000nM), UR144 (10-2000nM) and isosteviol

(0.2–400 nM)) for one hour prior to treatment with the radioligand tracer [³H]-CP55, 940 (2 nM).

CHO-CB1/CB2 cells were incubated for one hour with the radiolabeled agonist following a pre-incubation of 1 hour with the tested antagonist. Then, the media was discarded and the cells were washed twice with PBS and 50 μ L of 1.0 N NaOH was used to lyse the cells and determine agonist uptake (radioligand tracer) after an hour of plate shaking. Radioactivity was measured by liquid scintillation counting.

Data analysis

Data were analysed with non-linear regression and the sigmoid dose-response curve using GraphPad Prism software. The results are presented as the mean $\pm$ SD (n=6).

Zanthoxylum preparation—*Zanthoxylum* spp. ripe pericarp material was collected from cultivated plants in China and Korea, while wild collected material was obtained from Nepal. The pericarps of *Z. armatum* DC. (Rutaceae) was collected from Kaski District, Nepal on November, 2010 at an altitude of 1600 m. A voucher specimen no. KD 109/2010 was deposited in the Central Department of Botany, Tribhuvan University, Kathmandu, Nepal and identified by taxonomist Krishna K. Shrestha of the same department. The pericarps of *Z. piperitum* DC. were collected in Sangcheong-gun, Gyeongnam Province, Korea on September, 2011 by Young Ho Kim of Chugnam University. Pericarps of *Z. schinifolium* Siebold & Zucc. were collected by Guanghua Lu of the Chengdu University of Traditional Chinese Medicine in Chongqing City, Sichuan Province in June, 2011. Two collections of pericarps of *Z. bungeanum* Maxim. were made. The first was collected by Guanghua Lu of the Chengdu University of Traditional Chinese Medicine in Hanyuan County, Sichuan Province in October, 2011, while the second was collected by Jinao Duan of the Nanjing University of Traditional Chinese Medicine in Longnan City, Gansu Province in July, 2011. The taxonomy of the samples of *Z. bungeanum* was confirmed by ITS sequencing to have 99% identity with literature sequences.

Extraction—25.0 g of dried pericarps of each *Zanthoxylum* sp. was ground and soaked in 2 L methanol overnight. It was then heated to 70° C for 5 minutes and filtered. The filtrate was concentrated in a vacuum to yield a crude extract.

3. Results and discussion

The development of the CB1 and CB2 plate-based assay was carried out using the CHO-CB1 and CHO-CB2 cell line and the appropriate radiolabeled agonist. Win 55,212-2 effectively induced internalization of the CB1 receptor, however, it failed to induce CB2 internalization (data not shown), which is in agreement with the literature [10,11]. CB2 internalization was measured using radiolabeled CP55,940, which is an effective internalizing agonist for both the CB1 and CB2 receptors [10]. Although, the effect on agonists can also be studied using our plate based assay, for example, the EC₅₀s of Win 55,212-2 and CP55,940 were determined to be (3.29 $\pm$ 1.31) nM and (0.12 $\pm$ 0.02) nM for the CB1 and CB2 receptors, respectively, which is consistent with literature [17]. Herein we report only on the antagonist-inhibited internalization assay for both the CB1 and CB2 receptors.

As both CP55,940 and Win 55,212-2 are lipophilic drugs that tend to have a high level of non-specific binding, a series of experiments were initially carried out to determine the level of non-specific binding for this assay. In the absence of any cells, low levels of CP55,940 (450 cpm) and Win 55,212-2 (1300cpm) were observed on the 96 well plates. The assay was then performed using the untransfected CHO-CAR cell line and the amount of non-specific

binding averaged at (218 ± 44) cpm for CP 55,940 and (442 ± 16) cpm for Win 55,212-2. The addition of the antagonists 10 μ M AM-630 and 10 μ M JD-5037 resulted in no change in the level of non-specific binding, with levels of (281 ± 73) cpm and (451 ± 45) cpm obtained for CP 55,940 and Win 55,212-2, respectively. While there was non-specific binding observed, the amount of specific binding obtained with the cell based assay ranged from 3000-5000 cpm for the maximal response for CP 55,940 and 3000 cpm for Win 55,212-2, well above what was observed on the untransfected CHO-CAR cell lines, suggesting that non-specific binding is not a factor for this assay.

The plate-based assay was optimized for a 96 well plate format, which provided an increase in throughput over our frontal chromatography system [20]. In the development of the antagonist-inhibited internalization assay, pre-incubation of the cells with the antagonist was tested at various time intervals (30 min to 120 min), and it was determined that 1 hour provided the most reproducible results for both the CB1 and CB2 receptors. In order to validate the antagonist-inhibited internalization assay for the CB1 and CB2 receptors, the IC_{50} values of a series of known antagonists for the CB1 and CB2 receptors were determined, namely, AM251, AM281, AM630 and the silent antagonist AM6545 (Table 1). Figure 2 A and B presents the normalized dose-response curves of AM281 obtained by plotting the internalization of CB1 and CB2 receptors in the presence of this antagonist.

While the IC_{50} values for AM-251, AM-6545 and AM-281 were very similar to previously reported values, AM-630 showed a discrepancy for the CB1 receptor, where we report IC_{50} values ~15 fold lower than previously reported [20] (Table 1). While this is a large difference, the rank order of the compounds was consistent with the literature. Considering that our method is a direct measurement of functional activity, and as the IC_{50} reported in the literature was determined using a filtration assay, a 10 fold difference is unsurprising. With respect to the CB2 assay, the largest difference between our plate-based assay and previously reported assays was less than ~3 fold. The ratio of the IC_{50} values for CB1 and CB2 (CB2/CB1) receptors was also determined and was consistent with the literature [20-23] except for AM-630, where a ~6 fold difference is seen, which is due to the large difference in the IC_{50} for the CB1 receptor [20]. Therefore, a series of additional diarylpyrazoles was also studied.

AM251 and AM281 were developed as a result of the first identified diarylpyrazole, Rimonabant a CB1 inverse agonist, designed as an anti-obesity drug. These diarylpyrazoles replace the p-chlorophenyl group at position 5 with a p-iodophenyl moiety. In addition, for AM281 the piperidine group was replaced with a morpholino group. There was no significant difference between AM-251 and Rimonabant for either the CB1 or CB2 receptor. A significant increase in selectivity for the CB1 receptor over the CB2 receptor was observed for AM-281, however, the affinity for CB1 was similar. This indicates that the morpholino group has a pronounced effect on the functional activity at the CB2 receptor. A more detailed analysis has been previously reported [24].

JD5037, a newly developed antiobesity drug candidate, which acts as a peripherally-restricted cannabinoid inverse agonist at CB1 receptors [25], was also tested. Tam et al [25] recently reported the binding affinity of JD5037 to be 0.35 nM for CB1 and 245 nM for CB2, which is similar to our results obtained with the plate-based assay, 0.2 and 162 nM, respectively (Table 1). The replacement of the methyl butyl amide group (JD5037) with a methyl group (JD5001), results in a significant decrease in affinity for both the CB1 and CB2 receptor (Table 1), consistent with previously reported values [26].

Otenabant, originally developed and underwent pre-clinical testing as an anti-obesity drug from Pfizer, was reported to be a selective CB1 antagonist, which is consistent

with what we obtained using the developed plate based assay, where 100 fold selectivity for CB1 was observed [26]. While the majority of the results were consistent with previously reported values [27], the results obtained with UR144 were not consistent. Indeed, the tetramethylcyclopropyl group reportedly confers selectivity for the peripheral CB2 receptor over the central CB1 receptor [28], however, in our assay, we did not observe any selectivity between the CB1 and CB2 receptors. To date, no reports have been published on the functional activity of AB001, a synthetic cannabis (cannabinimimetic) blend found in Europe in tobacco blends and head shops [29,30]. Using our plate-based assay we found that AB001 is a selective antagonist for the CB1 receptor (29.5 nM (CB1) vs 1700nM (CB2)). For AB001, the substitution of the tetramethylcyclopropyl group by an adamantyl group greatly improved the selectivity for the CB1 receptor. Similar results have been reported by Lu et al [31] using Δ^9 -THC analogs with adamantly substituted for the carbon side chain to increase selectivity.

Isosteviol, a diterpene glycoside isolated from the leaves of *Stevia rebaudiana*, has been reported to have anti-diabetic properties in rodents and humans [32-34]. It appears to increase insulin sensitivity, making it a candidate supplement in insulin resistant states for preventing type 2 diabetes. In addition, it has lipid-lowering properties. To date, the mechanism of action remains unknown, however, as its use *in vivo* lead to similar results observed with the application of CB1 antagonists, we investigated the possibility that isosteviol may be acting through cannabinoid receptors. We demonstrated that isosteviol was active at both the CB1 and CB2 receptors, however, it was ~40 times more selective for CB2 (37.5 nM vs 0.9 nM, respectively) (Table 1).

3.2. Screening of Complex Matrix

One of the major groups of CB receptor-directed drugs currently in use are phytocannabinoids. Further, Newmann and Cragg determined that more than 70% of all drugs approved from 1981 and 2006, were either derived from or structurally similar to organic material or nature based [36]. Therefore, a logical extension is to screen plant extracts to identify potentially unknown modulators of the cannabinoid receptors. To this end, we have previously reported that cannabinoid activity was observed with extracts of *Zanthoxylum clava-herculis* L. (Rutaceae). In order to determine whether the developed plate-based assay would function with a complex matrix, we tested extracts of four other species of *Zanthoxylum* against both the CB1 and CB2 plate-based assays: *Zanthoxylum bungeanum* (two samples), *Z.schinifolium*, *Z.piperitum* and *Z. armatum* (Figure 3).

As can be seen in fig. 3A a decrease in the uptake of [³H]-Win-55,212-2 was observed for both *Z. bungeanum* A and B extract, however, the decrease was larger for *Z.bungeanum* A, indicating the presence of a CB1 antagonist(s) in the extract. On the other hand, both *Z.schinifolium* and *Z. armatum* had a dose-dependent statistically significant increase of uptake over the control. This could be indicative of the presence of CB1 agonist(s) in *Z. schinifolium* and *Z. armatum*. Of interest, is that for the CB2 plate based assay, only *Z. bungeanum* A extract showed a slight decrease in [³H]-CP55, 940 uptake (cf. Fig.3B), while *Z. bungeanum* (B), *Z. schinifolium* and *Z. piperitum* all produced a pronounced dose dependent increase in uptake of [³H]-CP55,940. Currently, *Z. bungeanum* (A and B) are being evaluated against both the CB1 and CB2 receptors to purify novel phytocannabinoids and the results will be reported elsewhere. The identification of a novel phytocannabinoid that acts as a CB1 antagonist and CB2 agonist could be of great therapeutic relevance in the treatment of diabetes.

4. Conclusion

A plate-based assay has been developed for screening for CB1/CB2 receptor antagonists. The results obtained with the assay correlated with previously reported values. In addition, the identification of AB-001 as a CB1 antagonist has been demonstrated for the first time, as well as a potential mechanism of action for isosteviol. Further, it was demonstrated that the plate-based assay could be used in screening against complex matrices, namely extracts from the genus *Zanthoxylum*.

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Figure 1.
Chemical structures of tested compounds

Figure 2.

Dose-response curve of the selective CB1 cannabinoid receptor antagonist AM-281 on the inhibition of the uptake of [3H]-Win-55,212,2 for the CB1 receptors (A) or uptake of [3H]-CP55,940 for the CB2 receptors (B). Each experiment was run using 0.5 fold serial dilutions, starting at 20 μ M for CB1 and 40 μ M for CB2.

Figure 3. Effect of 10 µg/ml and 0.1 µg/ml of *Z. bungeanum*-A (A); *Z. bungeanum*-B (B); *Z. schinifolium* (C); *Z. piperitum* (D); *Z. armatum* (E) on the uptake of [3 H]-Win-55,212,2 for the CHO-CB1 cell line (i) or [3 H]-CP55, 940 uptake for the CHO-CB2 cell line (ii).

IC₅₀ values of tested ligands for the inhibition of the internalization of uptake of 10 nM [³H]-Win 55,212-2 and 2 nM [³H]-CP55, 940 for the CB1 and CB2 receptors, respectively.

Table 1

	CB1(IC ₅₀ ±SD) (literature) nM	inhibition of internalization	CB2 (IC ₅₀ ±SD) nM (Literature)	inhibition of internalization	CB2/CB1 (literature)	Reference
AM251	3.00±0.46 (8)	39.6%	1160±148 (2290)	60.5%	387(306)	[21]
AM630	331.3±46 (5152)	40.6%	12.3±2.47 (31.2)	51.4%	0.04(0.006)	[20]
AM6545	4.2 ±0.24 (1.7)	43.7%	1300±670 (523)	50.7%	309(307.6)	[22]
AM281	9.91±0.99(12)	32.1%	13000±240(4200)	44.2%	1311(350)	[23]
JD5037-030	0.2±0.05(0.35)	64.4%	162±17 (245)	52.1%	810(700)	[25]
UR144	27.2±6.6 (150)	29.6%	83.58±22 (1.8) i	38.1%	3 (0.01)	[27]
Rimonabant	11.3±2.3(25)	30.0%	1400±238 (1580)	37.7%	124(63)	[27]
JD5001-050	13.27±3.4 (7.8)	32.1%	1200±227(7943)	39.2%	90(1018)	[25]
Otenabant	13.1±9.1(0.7)	46.2%	1298±194(7600)	37.6%	99(10857)	[26]
AB001	29.5±2.23	32.6%	1700±863	30.3%	58	
Isosteviol	37.5 ± 6.9	71.1%	0.89 ± 0.16	56.8%	0.02	