Published in final edited form as:
Med Chem Res. 2014 September 1; 23(9): 4295–4300. doi:10.1007/s00044-014-0972-6.

Evaluation of Phytocannabinoids from High Potency *Cannabis sativa* using *In Vitro* Bioassays to Determine Structure-Activity Relationships for Cannabinoid Receptor 1 and Cannabinoid Receptor 2

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Abstract

Cannabis has been around for thousands of years and has been used recreationally, medicinally, and for fiber. Over 500 compounds have been isolated from *Cannabis sativa* with approximately 105 being cannabinoids. Of those 105 compounds, Δ^9-tetrahydrocannabinol has been determined as the primary constituent, which is also responsible for the psychoactivity associated with *Cannabis*. Cannabinoid receptors belong to the large superfamily of G protein-coupled receptors. Targeting the cannabinoid receptors has the potential to treat a variety of conditions such as pain, neurodegeneration, appetite, immune function, anxiety, cancer, and others. Developing *in vitro* bioassays to determine binding and functional activity of compounds has the ability to lead researchers to develop a safe and effective drug that may target the cannabinoid receptors. Using radioligand binding and functional bioassays, a structure-activity relationship for major and minor cannabinoids was developed.

Keywords

Cannabis; tetrahydrocannabinol; structure-activity relationship; cannabinoid; cannabinoid receptor 1; cannabinoid receptor 2

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Introduction

Marijuana, also known as *Cannabis*, is defined as any preparation of the *Cannabis* plant used to elicit psychoactive effects whether it is recreational or medicinal. According to the 2004 World Drug Report, 3.7% of the population 15-64 years of age consumed marijuana from 2001-2003 (2004 World Drug Report). The use of marijuana is associated with numerous pharmacological effects; most, but not all may be attributed to Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam, 1964). The combination of Δ^9-THC and other compounds from *Cannabis*, such as cannabidiol (CBD), may exhibit specific pharmacological effects. Since Δ^9-THC is primarily responsible for the psychoactive effects of *Cannabis*, scientists have learned how to genetically increase the concentration of Δ^9-THC within plants to produce a higher percentage. Since 1993, the concentration of Δ^9-THC within marijuana has increased from 3.4% to approximately 8.8% in 2008 (Mehmedic et al., 2010).

Cannabis use has been reported for thousands of years and is not only associated with recreational or medicinal use, but it is also used for fiber and seeds. *Cannabis* produces a durable fiber, called hemp, for the manufacturing of rope and fabric. Along with the production of hemp, the seeds of *Cannabis* are rich in unsaturated fatty acids. The use of *Cannabis* dates back to around 2000 BC when the Chinese invented hemp paper (Peters and Nahas, 1999). In Dr. Mahmoud ElSohly's book published in 2010, *Marijuana and the Cannabinoids*, it is noted that *Cannabis* serves as a recreational drug and more importantly, as a potential therapeutic treatment for numerous diseases such as wasting syndrome, obesity, and multiple sclerosis (Clarke and Watson, 2010).

The CB1 receptor is encoded by the CNR1 gene, and is widely expressed throughout the brain. It is also expressed in the spinal cord, pituitary gland, thyroid gland, adrenal gland, fat cells, muscle cells, liver cells, digestive tract, lungs, kidneys, and male and female reproductive organs. Gerrard et al. cloned the rat cannabinoid receptor and shortly after, isolation of a human CB1 receptor cDNA was reported (Gerrard, 1991). The amino acid sequence showed 472 total amino acids, one less than other mammalian species (Matsuda, 1991). This receptor has been the target of much research due to the pharmacological effects associated with its activation (Pertwee, 1997).

Shortly after characterizing and cloning the human CB1 receptor, the CB2 receptor was cloned (Devane, 1992). The CNR2 gene encodes the CB2 receptor, and the amino acid sequence shows approximately 360 total amino acids. The CB1 and CB2 receptors have approximately 44% similarity of their amino acid sequences (Munro et al., 1993). The CB2 receptors are widely expressed throughout the peripheral tissues of the immune system, spleen, tonsils, thymus, and gastrointestinal system. Further investigation of CB2 receptors led to the discovery that these receptors are also expressed within the brain (Onaivi et al., 2006). The CB2 receptors play a major role in inflammatory diseases due to their interaction with these receptors in the immune system (Cabral and Griffin-Thomas, 2009).

This misuse of *Cannabis* negatively affects the people who need help with unwanted side effects associated with cancer chemotherapy and AIDS. *Cannabis* is not only used to help

those suffering from cancer chemotherapy and AIDS (Harrigan, 2001) (Berry and Mechoulam, 2002), but it also lowers intraocular pressure for those with glaucoma, acts as a pain reliever, and more recently has been found to help with symptoms of multiple sclerosis, Alzheimer's, and depression (Benito, 2003). Therefore, researchers are attempting to formulate synthetic cannabinoids that resemble the compounds isolated from *Cannabis*, but do not express psychotropic properties.

During the past century, and especially in the past 20 years, researchers have investigated Δ^9-Tetrahydrocannabinol (THC), the primary active constituent in marijuana, and it derivatives, for medical uses. These uses include wasting–syndrome in AIDS patients, anti-anxiety, antiemetic (in patients receiving cancer chemotherapy), analgetic (especially in cancer pain), anti-inflammatory, and neuroprotective effects, among others. The development of treatment strategies for these disorders remains a high priority. The broad effects associated with THC and other cannabinoids are directly related to the endocannabinoid system, which is a major regulatory system of the central and peripheral nervous system. The endocannabinoid system, which is comprised of cannabinoid receptors (CB1 and CB2), their endogenous ligands, and the enzymes responsible for metabolizing these ligands, is linked to the control of various physiological processes. These include depression, anxiety, and drug addiction, among others and it is clear that the endocannabinoid system provides a valuable new therapeutic target for a variety of disorders (Lambert, 2009).

Though much effort has been exerted in discovering and developing cannabinoid receptor ligands, there are still few marketed drugs in this category, and hence there is great potential and urgency for application of rational drug design for discovery of novel cannabinoid ligands. This study will help scientist gain a better understanding of the structure-activity relationship (SAR) of ligands binding affinity for these receptors.

Material and Methods

Plant material

Cannabis sativa plants were grown from high potency Mexican seeds. The seeds and plants were authenticated by Dr. Suman Chandra, The University of Mississippi, and a specimen (S1310V1) was deposited at the Coy Waller Complex, National Center for Natural Products Research, School of Pharmacy, the University of Mississippi. Whole buds of mature female plants were harvested, air-dried, packed in barrels and stored at −24°C.

Cell culture

Parental HEK293 cells were stably transfected via electroporation with full-length human recombinant cDNA for cannabinoid receptor subtypes 1 and 2. The human recombinant cDNA was obtained from Origene. Once transfected, the cells were maintained at 37°C and 5% CO_2 in a Dulbecco's Modified Eagle's medium (DMEM) nutrient mixture F-12 HAM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 0.5% penicillin-streptomycin, and G418 (Geneticin, 600 mg/mL). A single cell was picked from the parental plate and forced to replicate on its own in a fresh plate with the appropriate media.

Membranes were prepared by scraping the cells in a 50 mM Tris-HCl buffer, homogenized via sonication, and centrifuged for 40 minutes at 13,650 rpm at 4°C. The membranes were stored at −80°C. Protein concentrations for each membrane preparation were found using the Bradford protein assay.

Competitive binding assay

The binding assays were performed using slight modifications to previously published methods (Pertwee, 1999). Using 0.5 nM ^{3}H- CP-55940, 10 μM test compound (unless dose-response then first well is 100 μM followed by appropriate dilutions), and 10 μg protein of membrane for a total assay volume of 210 μL. Binding was initiated by the addition of 10 μg protein of CB1 or CB2 cell membranes. Assays were carried out at 37°C for 90 minutes before termination via rapid vacuum filtration through Whatman GF/C glass-fiber filters, presoaked with 0.3% BSA, using a Perkin Elmer 96-well Unifilter Harvester (Perkin Elmer Life Sciences Inc., Boston, MA, U.S.A.). Each assay plate was washed seven times with ice-cold wash buffer (50 mM Tris, 154 mM NaCl, 20 mM disodium ethylenediaminetetraacetic acid (EDTA), 0.2% BSA, and pH = 7.4). Filters were allowed to dry overnight at room temperature (25°C) and then radioactive counts were extracted from the filters using a scintillation cocktail before quantification using a Perkin Elmer TopCount (Perking Elmer Life Sciences Inc., Boston, Mass. U.S.A). There results were calculated using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.) to obtain K_i and IC_{50} values. Total binding was defined as binding in the presence of 0.1% dimethylsulfoxide (DMSO). Nonspecific binding was the binding observed in the presence of 0.1 μM CP-55940. Specific binding was defined as the difference between total and nonspecific binding.

GTPγS functional assay

The functional assays were performed using slight modifications to previously published methods (Xiong et.al., 2011). The assay buffer for the GTPγS functional assay consisted of 50 mM Tris-HCl, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 9 mM $MgCl_2$, 150 mM NaCl, and 1.4 g BSA. Binding took place under the following conditions: 50 μL compound diluted to the desired concentrations in the dose response curve were mixed with 20 μg CB1 or CB2 membrane, 50 μM GDP, 0.5 nM ^{35}S-labelled GTP, and 300 μL assay buffer for a total volume of 500 μL per well. Plates were incubated for 120 minutes at 37°C. The reaction was terminated via rapid vacuum filtration through Whatman GF/B filters using a Perkin Elmer 96-well Unifilter Harvester (Perkin Elmer Life Sciences Inc., Boston, MA, U.S.A.). Each assay plate was washed four times with ice-cold wash buffer (10 mM Tris-HCl, pH = 7.4). Filter plates were allowed to dry overnight at room temperature (25°C) and then radioactive counts were extracted from the filters using a scintillation cocktail before quantification using a Perkin Elmer TopCount (Perking Elmer Life Sciences Inc., Boston, Mass. U.S.A). Basal binding was defined as binding in the presence of assay buffer. Nonspecific binding was the binding observed in the presence of 40 μM unlabeled GTPγS salt. Emax binding was defined as binding in the presence of 1μM CP-55940. K_i and EC_{50} values were calculated using Graph Pad Prism (GraphPad Software, San Diego, CA, U.S.A.).

Results

The importance of developing a structure-activity relationship for the cannabinoid receptors is due to the lack of understanding of the receptor binding sites. Developing *in vitro* bioassays to evaluate binding affinity and functional activity for each of the cannabinoid receptors is critical to understanding the pharmacology behind these receptors. Currently, there is no crystal structure that exists for the cannabinoid receptors active binding site. This structure-activity relationship of phytocannabinoids may help further understand the pharmacology of these receptors along with requirements for their binding.

In most cases, if a ligand binds to a receptor then it would also functionally activate the receptor; however, this is not always true. Although compounds may bind tightly to a specific receptor, they do not always produce a biological response. It is not uncommon for GPCRs to dissociate upon binding of a ligand (Carlsson, 2010). Conversely, some ligands may not bind to the specific receptor yet cause a functional response. This is thought to be because of an allosteric binding site, a binding site other than the two known cannabinoid receptors, in which the ligand still produces a functional effect via the endocannabinoid system pathway.

Binding

The use of radiolabeled competitive binding assays is a common technique for evaluating compounds and their binding affinities to specific receptors (Table 1). Δ^9-THC displayed binding affinity within the low nanomolar range for both CB1 and CB2 receptors, 18 nM and 42 nM, respectively. The isolated compounds within the Δ^9-THC family all displayed weaker affinity for CB1 and CB2. Δ^8-THC displays slighter lesser binding affinity than Δ^9-THC for the CB1 receptor, suggesting that the location of the double bond has a role in binding affinity. Interestingly, **11** displayed strong binding affinity for the CB2 receptor with a Ki value of 11 nM. Therefore, substitutions at the C-8 position of cannabinol may have an influential effect on selectivity when binding to CB2. Compounds **12**-**23** did not warrant significant binding affinities.

Functional

Using radiolabeled functional bioassays allows for easily determining if a compound is acting as an agonist, partial agonist, antagonist, or inverse agonist. All phytocannabinoids mentioned in this study were determined to act as agonists using the GTPγS functional bioassay (Table 1). Three compounds displayed single nanomolar activity for either CB1 or CB2 receptors. Cannabichromanone D (**21**) warranted an EC_{50} of 8 nM for the CB1 receptor. This compound differs from the other compounds in the cannabichromanone class because the aliphatic chain cyclizes with the phenolic hydroxy to form a third ring. Though the cannabichromanone derivatives have not been evaluated for their ability to induce psychoactivity, it is safe to say that these compounds will induce psychoactivity, depending on the dose, because of their ability to functionally stimulate the CB1 receptor. However, cannabidivarin (**23**) displayed preferential activity for CB2 in the low nanomolar range, 3 nM. The importance of showing preference for the CB2 receptor is ideal in order to negate the side effects associated with activation of CB1, such as psychoactivity. Contrary to the

side chain length associated with Δ^9-THC, a decrease in the side chain length to three carbons of cannabidivarin causes a dramatic increase in selectivity, and potency for the CB2 receptor.

Discussion

Cannabinoid receptors portray different pharmacological properties when activated by an agonist, antagonist, or inverse agonist. Agonists that stimulate CB1 cause some of the unwanted side effects associated with *Cannabis*, such as psychoactivity (Hensen, 2005). Furthermore, inverse agonists that stimulate CB1 cause a loss of appetite, which in turn helps with the treatment of obesity. Rimonabant is an example of CB1-selective inverse agonist, and was marketed in Europe for the treatment of obesity until three years later when it was removed from the market. Removal of Rimonabant was due to those patients taking the drug having suicidal thoughts (Katoch-Rouse, 2003). Since it was selective for CB1, it is hypothesized that stimulation of the CB1 receptor may be the link to causing any type of negative side effects associated with the endocannabinoid system, the system responsible for activating the cannabinoid receptors.

The results presented in this manuscript indicate that phytocannabinoids have the potential to become lead therapeutic compounds to help patients suffering from cancer chemotherapy, AIDS, multiple sclerosis, glaucoma, and several other major diseases, while negating unwanted side effects associated with CB1 stimulation. Some side effects associated with *Cannabis* use include, but are not limited to psychoactivity, dependence, and increased heart rate. This manuscript identifies and evaluates 23 isolates from *Cannabis sativa*. The effort to develop compounds useful for medication requires structural diversity in order to achieve therapeutic success. Using *in vitro* bioassays has led to two potential compounds that need further testing to determine pharmacological effects *in vivo*.

Acknowledgments

This study was supported by Grant Number P20GM104932 from the National Institute of General Medical Sciences (NIGMS), a component of the National Institutes of Health (NIH) and its contents are solely the responsibility of the authors and do not necessarily represent the official view of NIGMS or NIH. This investigation was conducted in a facility constructed with support from research facilities improvement program C06RR14503 from the NIH National Center for Research Resources.

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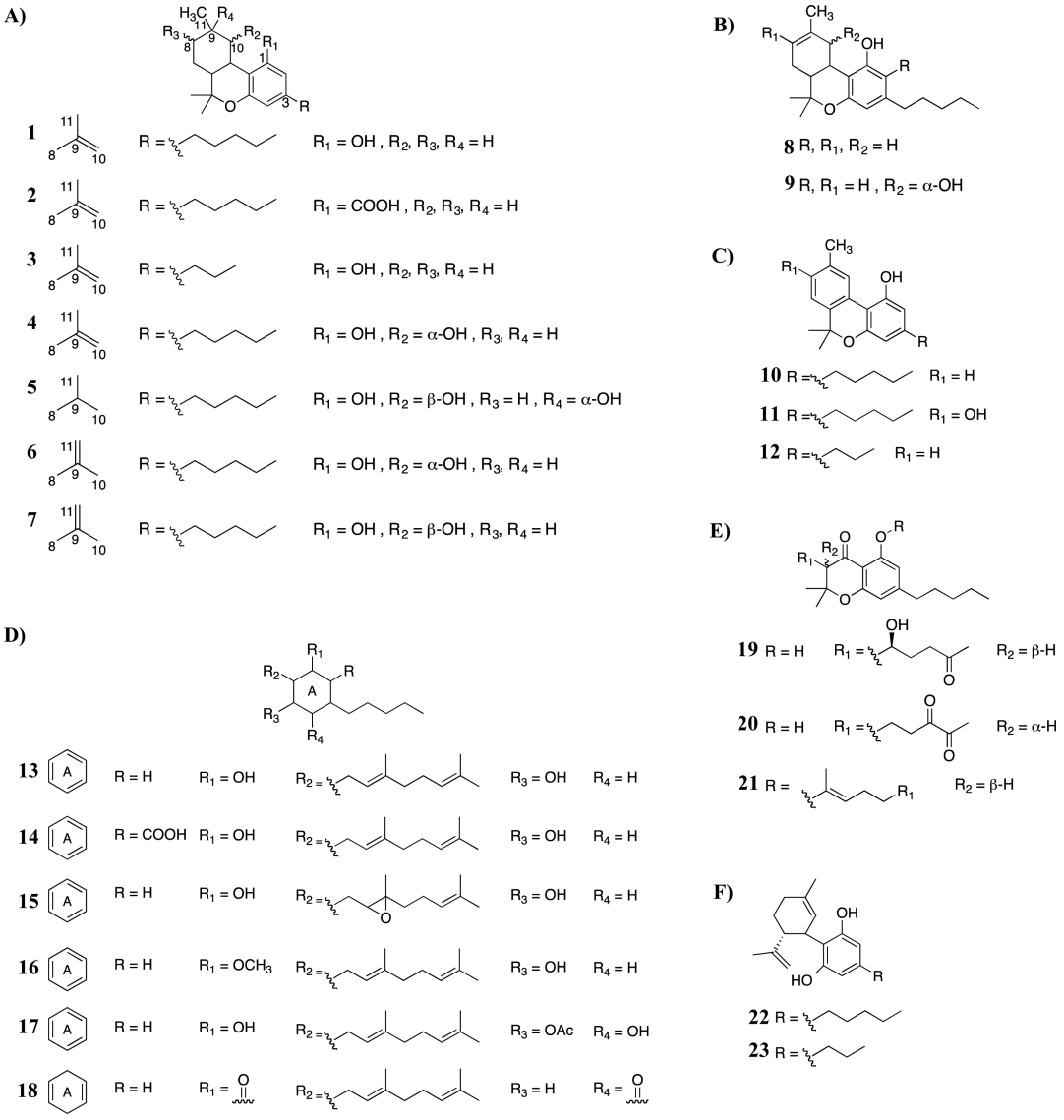

Figure 1.

Chemical structures of compounds isolated from *Cannabis sativa* organized via family substructure. A) Δ^9-THC B) Δ^8-THC C) Cannabinol D) Cannabigerol E) Cannabichromanone F) Cannabidiol.

Table 1

Binding affinities and functional activities of all compounds isolated from *Cannabis sativa*. All compounds evaluated displayed agonistic activity in the GTPγS functional assay for both CB1 and CB2 receptors.

Compound	Binding Affinity (nM)		Functional Activity (nM)	
	CB1	CB2	CB1	CB2
$\triangle^9$-THC, **1**	18 ± 4	42 ± 9	269 ± 36	327 ± 43
$\triangle^9$-tetrahydrocannabinolic acid, **2**	1292 ± 89	1650± 163	> 10,000	> 10,000
$\triangle^9$-tetrahydrocannabivarin, **3**	22 ± 5	105 ± 21	> 10,000	> 10,000
10-α-OH-THC, **4**	3293 ± 445	2771 ± 488	4425 ± 1229	7264 ± 1565
Cannabiripsol, **5**	5668±1324	2143 ± 353	> 10,000	> 10,000
10-α-OH-$\triangle^{9,11}$ -hexahydrocannabinol, **6**	117 ± 16	129 ± 13	> 10,000	> 10,000
10-β-OH-$\triangle^{9,11}$-hexahydrocannabinol, **7**	> 10,000	> 10,000	> 10,000	> 10,000
$\triangle^8$-THC, **8**	78 ± 5	12 ± 2	5820 ± 782	524 ± 70
10-α-OH-$\triangle^8$-THC, **9**	31 ± 6	30 ± 4	> 10,000	2622 ± 352
Cannabinol, **10**	75 ± 4	73 ± 4	307 ± 29	289 ± 38
8-OH-cannabinol, **11**	8063 ± 1986	11 ± 1	1438±399	5099 ± 725
Cannabivarin, **12**	565 ± 138	4780 ± 331	> 10,000	> 10,000
Cannabigerol, **13**	3090 ± 583	2919±752	> 10,000	1158±221
Cannabigerolic acid, **14**	4526 ± 953	> 10,000	182 ± 32	118 ± 27
6,7-epoxy-cannabigerol, **15**	> 10,000	4718± 87	1192 ± 330	> 10,000
5-Methoxy cannabigerol, **16**	> 10,000	3989 ± 772	235 ± 51	1572 ± 376
4-OH-5-acetoxy-cannabigerol, **17**	1409 ±162	388 ± 67	618±106	1743 ± 443
2-geranyl-5-n-pentyl-1,4-benzoquinone, **18**	> 10,000	> 10,000	> 10,000	2592 ± 519
Cannabichromanone B, **19**	3470±601	4371 ± 1119	965 ± 268	> 10,000
Cannabichromanone C, **20**	8681 ± 1404	5789 ± 685	483 ± 121	138 ± 36
Cannabichromanone D, **21**	7117±1090	2828 ± 569	8 ± 0.9	3945± 1106
Cannabidiol, **22**	151 ± 28	4582 ± 613	1469 ± 197	104 ± 14
Cannabidivarin, **23**	503 ± 58	3970 ± 976	> 10,000	3 ± 0.8