1

CANNABINOIDS: INTERROGATING THE LABS

1.1

ENDOCANNABINOID SYSTEM AND CANNABINOIDS:

principles of pharmacology

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Cannabinoids

Introduction

History of the medical use of marijuana/traditional use (ancient world)

Humans have been using mind-altering substances since prehistoric times. Hemp (Cannabis sativa) is one of the first plant species cultivated by man for over 10,000 years.[1] The first records of the medical use of cannabis come from ancient China about 4000 years ago,[2] where its therapeutic properties were mainly valued, not intoxicating. The Chinese called it "the drug that takes away the mind;" they also used it for neurologic diseases and in religious rites. Cannabis was understood to have medical value for pain relief, as well as for gastrointestinal disorders, insomnia, blood clots and parasites. By the 8th century, cannabis was used for both medicinal and religious purposes in China, Egypt, Greece, India, and the Middle East, and possibly parts of Europe as well. Cannabis gained new attention in the Western medical world at the introduction of the Irish physician, William B. O'Shaughnessy, who is credited with bringing cannabis to the West. In the mid-19th century and early 20th century, cannabis was included in hundreds, if not thousands, of patent medicines, including tinctures, powders, and syrups. They quickly became available in pharmacies in the form of an over-the-counter drug. By 1890, cannabis plants were widely used for a variety of ailments, but perhaps their most notable usage was for dysmenorrhea (or menstrual cramps). It is during this time when the royal family physician, Sir J. Russel Reynolds, prescribed Queen Victoria a tincture of cannabis for her menstrual pains.[3] Scientific efforts to pinpoint the psychoactive ingredients that cause the mild euphoria began in the 19th century. But investigators were stymied by the complex, lipophilic nature of the plant, which required sophisticated technology to probe and parse. Although the first cannabinoid – cannabidiol (CBD) – was discovered in 1940 by Roger Adams, he was not aware of his incredible success until years later. A key turning point for modern cannabis research came in 1964, when Israeli scientists Raphael Mechoulam and Yechiel Gaoni isolated and identified tetrahydrocannabinol (THC). Mechoulam not only discovered THC, but he also identified the stereochemistry of CBD and THC, which revealed a direct relationship between cannabinoids and the euphoric effects associated with cannabis use.

Discovery of the canonical endocannabinoid system

The psychoactive effects of *Cannabis sativa* are primarily mediated through neuronal CB1 receptors, while its therapeutic immune properties are primarily mediated through CB2 receptors. Two endocannabinoids, N-arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG), have been identified, their action on CB1 and CB2 thoroughly characterized, and their production and inactivation elucidated. The above as we take for granted today started with a breakthrough in 1988, when scientists at the St. Louis University Medical School determined that a rat's brain has receptor sites that are activated

by THC.[4] Allyn Howlett and William Devane identified and cloned,[5] this cannabinoid CB1 receptor, which turned out to be far more abundant in the mammalian brain than any other G-protein-coupled receptor (GPCR). This discovery made scientists realize there had to be an endogenous, THC-like compound, that signaled through these receptors. The search was on for CB1's internal trigger. In 1992, researchers at Hebrew University in Jerusalem, such as Raphael Mechoulam, William Devane, and Lumir Hanus, isolated a novel lipid neurotransmitter that binds with the CB1 receptor in pig brain tissue. They called it "anandamide," Sanskrit for bliss, a word suggestive of its mood-altering effects.[6] Although anandamide and THC do not share a similar molecular structure, they behave in a similar way when they bind to the CB1 receptor. In 1993 scientists identified a second type of cannabinoid receptor: CB2, which is present throughout the immune system, the peripheral nervous system, metabolic tissue, and in many internal organs. Initially reported in Nature in 1993,[7] this discovery shed new light on how cannabinoid signaling regulates inflammation and why cannabinoid therapy could be a helpful treatment for a raft of autoimmune diseases. Another important Israeli contribution to the field was the identification by Mechoulam *et al.*, and independently in parallel by Japanese scientists,[8] of 2-AG in 1995.[8, 9] Compared to AEA, 2-AG proved to be more potent, more prevalent, and more broadly expressed throughout the body. 2-AG levels in the human brain are approximately 170 times higher[10] than those of AEA, and 2-AG binds efficiently to both cannabinoid receptors, CB1 and CB2. AEA and 2-AG are both lipid neurotransmitters that signal all over the brain and body to help maintain internal homeostasis among a barrage of ever-changing environmental inputs.

Metabolic enzymes: FAAH and MAGL discovery

This complex and pleiotropic endogenous signaling consists also of proteins and enzymes for the regulation of endocannabinoid levels and action at receptors. Endocannabinoids are born and broken down by various biosynthetic and catabolic enzymes and are made when needed and then degraded after serving their purpose.[11] AEA is degraded by fatty acid amide hydrolase (FAAH),[12] while 2-AG is deactivated primarily by monoacylglycerol lipase (MAGL). The molecular structure of FAAH was characterized by Ben Cravatt in 1996, and the following year Di Marzo's group identified MAGL as a key degradative enzyme for 2-AG.[13]

Endocannabinoidome: the modern view on the endocannabinoid system

The two cannabinoid receptor subtypes along with AEA, 2-AG, and their biosynthetic and degradative enzymes, comprised the basic components of the canonical or "classical" endocannabinoid system, which modulates most biological functions. By now we have learned a lot more about the endocannabinoid system and its interactions with many other lipids signaling molecules and receptor networks far beyond CB1 and CB2.[14]

Cannabinoids

Pharmacology of phytocannabinoids

Cannabis sativa has been called a "neglected pharmacological treasure trove" of bioactive compounds.[15] As many as 538 secondary metabolites were already mentioned in a review published in 2005,[16] and their number has increased significantly in recent decades due to a renewed interest in this plant. Nevertheless, it is surprising that the biological profile of most of them is still unknown. The troubled and controversial history of C. sativa and the regulatory complications associated, until recently, even with the study of its non-narcotic constituents are responsible for this discouraging state.[17, 18] The characteristic secondary metabolites of C. sativa are the phytocannabinoids, a class of C21 or C22 terpenophenolic compounds that can directly interact with cannabinoid receptors, such as CB1 and CB2, or share a chemical similarity with cannabinoids.[19] Guidelines will have to be established to determine certain criteria like the appropriate potency threshold. It was not until 2016 that Hanus *et al.* proposed a structural, rather than biological, definition for the term phytocannabinoid, implying that compounds from other sources, such as fungi and liver, would also be classified as "phytocannabinoids."[20] So far, over 90 phytocannabinoids have been identified. Their composition varies between cannabis chemotypes,[18, 21] such as Δ9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), cannabigerol (CBG) and cannabichromene (CBC) that are the most abundant phytocannabinoids (**Figure 1.1.1**).[22, 23] It is important to know that their concentration does not depends only by the these chemovars, but also on age, growth conditions, harvest time and storage conditions.[18] Indeed,

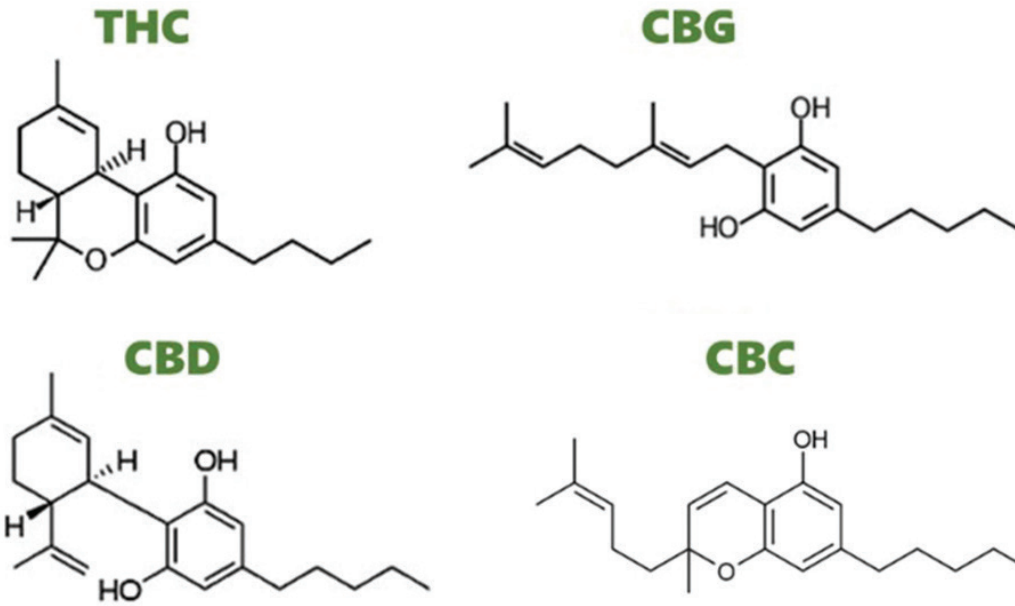

 Figure 1.1.1 Major phytocannabinoids.

according to Elsohly, 2017, THC concentration increases with plant age and plateaus at the budding stage of the plant. This plateau remains for 1-2 weeks and decreases thereafter. These imply that the cultivation of cannabis for medicinal use as either extracts or production of pharmaceutically active ingredients (API), must be executed under strict optimized conditions to ensure consistency and good manufacturing practice (GMP). This challenge also incentivizes the development of biotechnological systems such as recombinant yeast, for the production of phytocannabinoids.[24]

Major phytocannabinoids

Δ9-Tetrahydrocannabinol

THC is the principal psychoactive component of *Cannabis sativa*[25] and it is one of the early chemically elucidated constituents of this plant.[26, 27] It has been reported to exhibit activities at both cannabinoid and non-cannabinoid receptors. At the CB1 receptor, THC has a relatively lower efficacy compared to many synthetic cannabinoids, as well as AEA and 2-AG.[28] Therefore, it is unsurprising that there have been reports showing it is a full agonist, partial agonist, and antagonist, in different in-vitro assays and receptor expression systems. For example, THC was shown to stimulate [35S]GTPyS binding in rat cerebellum but with a lower efficacy than WIN55212-2 (a synthetic CB1 agonist).[29] THC agonism was also demonstrated in N18TG2 neuroblastoma and CB1-transfected COS (fibroblast-like) cell lines, through the inhibition of adenylyl cyclase, where it acted as a full agonist.[30] When applied to rat hippocampal neurons, THC antagonized 2-AG-induced inhibition of intracellular Ca2+ spikes. THC also inhibited glutaminergic transmission when used alone.[31] More so, THC was also able to antagonize R-(+)-WIN55212 inhibition of excitatory postsynaptic currents (EPSC) in mice hippocampal neurons. It was not able to inhibit EPSC by itself but desensitized CB1 receptors upon long incubation (18 h).[32] At CB2 receptors, THC also behaves as an agonist but with a lower efficacy than CB1 relative to CP 55,940.[33] It is unclear whether these observations are due to differences in levels of receptor expression or coupling efficiency. THC has also been shown to behave as an inverse agonist by inhibiting [35S]GTPyS binding to membranes obtained from CB2-transfected CHO (Chinese Hamster Ovary) cells.[34] In summary, THC is a low efficacy agonist of CB1 and CB2 receptor but with a lower intrinsic activity in the latter. THC has been shown to induce vasorelaxation through activation of the nuclear receptor, peroxisome proliferator-activated receptor-gamma (PPARy) in rat isolated arteries. PPARy activation was also demonstrated in a transiently transfected HEK293 (Human Embryonic Kidney) cells, using a luciferase reporter gene assay.[35] There is also evidence THC modulates T-type Calcium channels CaV3.1, CaV3.2, and CaV3.3 with low micromolar potency in transfected HEK293 cells and acutely isolated trigeminal ganglion sensory neurons.[36] THC (20 μM) is a lower efficacy activator of TRPA 1 than mustard oil isothiocyanates (20 μM) in rat sensory neurons, although it appeared to be more potent in a recombinant rat TRPA 1 transfected in HEK293 cells.[37, 38] De Petrocel-

lis *et al*.[37] demonstrated that THC blocks TRPM8 channels by measuring elevated intracellular [Ca2+] in rat dorsal root ganglia (DRG) sensory neurons. In addition, THC allosterically potentiates glycine-mediated activation of glycine receptor. This was reported in native glycine receptors in isolated rat ventral tegmental area neurons and recombinant human glycine receptors transfected in Xenopus laevis oocytes. Additional evidence suggest THC does not affect glycine receptor trafficking.[39] THC activity on glycine receptor potentially contributes to THC-induced analgesia. The antiemetic properties of THC are likely contributed by non-competitive antagonism of 5-HT3 receptors. The potency of THC inhibition of 5-HT3 was higher than AEA, WIN55,212-2 and CP 55,940.[40, 41] THC is currently used therapeutically for the treatment of nausea associated with cancer chemotherapy and anorexia associated with weight loss in AIDS patients and to improve symptoms associated with multiple sclerosis.

Cannabidiol

Cannabidiol (CBD) is the major non-psychotropic constituent of *Cannabis sativa*. It is the first reported phytocannabinoid to be structurally defined[42] and some of the most studied targets include cannabinoid CB1 and CB2 receptors, 5HT1a receptors, GPR55, and TRP channels. At the cannabinoid receptors, functional studies have shown CBD to behave as low-potency inverse agonist/antagonist at both CB1 and CB2 receptors through a non-competitive mechanism.[12] In C57BL/6 mouse brain membranes, CBD (10 μM) inhibited CB1-mediated [^{35}S]GTPyS binding to mice brain membranes with a greater effect compared to 10 μM Rimonabant, a selective CB1 antagonist. The inhibitory effect of CBD may not have been exclusive to CB1 activity, because similar response was obtained in a genetically CB1-deleted C57BL/6 mouse. When repeated in a heterologous CB1 and CB2-transfected CHO cells, CBD also inhibited [^{35}S]GTPyS binding and the response was absent in non-transfected CHO cells.[43] The non-competitive activity of CBD has been supported by the report that it is an allosteric modulator of CB1 receptors. Using an operational model of allosterism, the effect of CBD was compared to ORG2759 and PSNGBAM-1 (negative allosteric modulators). Cannabidiol reduced the efficacy and potency of 2-AG and THC on phospholipase CP3- and ERK.1/2-dependent signaling in cells heterologously (HEK 293A) or endogenously (STHdhQ7/Q7) expressing CB1 receptors.[44] While many studies have shown CBD activity at so many targets, few provide incontrovertible evidence of specificity. The fact that CBD tends to be an inhibitor of channels and receptors means it is more difficult to provide strong evidence for their involvement in given effects because unlike agonists, antagonists cannot reverse them. Furthermore, some reported effects of CBD often occur at very high concentrations. Clinical data shows that plasma concentration of CBD saturates at 77.9 ng/ml, following a single oral dose ranging between 400 mg and 800 mg and higher doses do not result in a significant increase in maximum concentration (Cmax).[45] Assuming all administered CBD is restricted to the plasma, this is equivalent to an in-vitro concentration of about 0.24 μM, which is considerably less than the potencies reported above. This raises the que-

stion as to how many of these targets really play significant roles in CBD behavioral effects that may be of therapeutic benefit. CBD is currently approved for the management of treatment-resistant Dravet syndrome, tuberous sclerosis complex and Lennox-Gastaut Syndrome in children.[46, 47] It is also combined with THC for the treatment of spasticity associated with multiple sclerosis.[48]

Cannabigerol

Like most previously discussed phytocannabinoids, cannabigerol (CBG) was also isolated and chemically elucidated by Gaoni and Mechaoulam. They supplied the compound to Grunfeld and Edery, who then reported its biological activity in 1969.[49] CBG is considered the initial product of cannabinoid biogenesis.[50] Early *in-vivo* studies in dogs and monkeys showed it had no psychotropic effect like THC.[49, 51] The lack of psychotropic effect may have dampened interest in the compound, leading to fewer studies investigating its *in-vitro* pharmacology. CBG was reported to have similar binding affinity to CB1 (Ki=440 nM) and CB2 (Ki=337 nM) receptor, in displacing [3H]CP 55,940 in mouse brain and spleen membranes respectively.[52] Rosenthaler *et al*. in 2014 also reported an affinity at human cannabinoid receptors in SF9 cells (CB1: 900 nM; CB2: 150 nM). There is evidence that concentrations between 10-100 nM CBG stimulated [35S]GTPyS binding in MF1 mouse brain membrane, but higher concentrations inhibited [35S]GTPyS binding.[53] In line with this, 10 μM CBG antagonized CP 55,940 and anandamide stimulation of [35S]GTPyS binding to MF1 mouse brain membrane. This effect was not observed with 1 μM CBG and they suggested CBG was a CB1 antagonist.[53] There was no evidence in this study about the CBG activity at CB2 receptors. CBG is also reported to be a CB2 partial agonist in HEK293T cells transfected with human CB2 receptors. However, there was no antagonism of this response with CB2 selective antagonist or data in wild type cells. Therefore, it is obvious that more investigation on the activity of CBG is necessary. In addition to actions at CB1 and CB2, CBG reportedly acts as a potent a2 adrenoceptor agonist by inhibiting electrically evoked contractions in MF1 mouse isolated vas deferens.[53] This study also showed that CBG moderately blocks 5HT 1A receptors at the orthosteric site. 5HT1A antagonism may explain its antinausea effect.[54] Like many phytocannabinoids, it is also a potent TRPA 1 agonist, weak agonist of TRPV 1 and TRPV 2. Finally, CBG also blocked icilin activation of TRPM8 channels.[55]

Cannabichromene

Cannabichromene (CBC) was independently isolated and its chemically elucidated by two research labs in 1966.[56] Alongside CBD, THC, and cannabinol (CBN), it is considered one of the most abundant phytocannabinoids[22] and it is the only phytocannabinoid with a substantial amount of both isomers in cannabis, where both CBC enantiomers exist in equal proportion.[20] However, recent evidence suggests it is a scalemic mixture that meaning a mixture of enantiomers at a ratio other than 1:1.[57] Given that receptor binding can depend on the stereochemistry of the ligand, it will be important to identify the active

CBC enantiomer because there is a possibility that both enantiomers may not have equal activity at the same receptor. In contrast to THC and CBD, there is limited information on the biological activity of CBC. At non-cannabinoid targets, CBC has been reported to potently activate the TRPA 1 channel by elevating intracellular Ca2+ in transfected HEK293 cells. A high concentration of the TRPA 1 antagonist AP18 (50 μM) inhibited maximal CBC activity by about 50%.55 The same concentration of antagonist also only partially inhibited the agonist activity of mustard oil, a potent TRPA 1 agonist,[58] CBC desensitized TRPA 1 receptor within 5 minutes. CBC also activated TRPV 3, TRPV 4, although less potently than TRPA 1, but it was inactive at TRPV 1, TRPV 2 channels and blocked TRPM8 activation.[55, 59]

Minor phytocannabinoids

Despite the very large number of phytocannabinoids isolated from Cannabis sativa L., bioactivity studies have long remained focused on the so called "Big Four" [Δ9-THC, CBD, CBG and CBC] because of their earlier characterization and relatively easy availability via isolation and/or synthesis. Bioactivity information on the chemical space associated with the remaining part of the cannabinome, a set of ca 150 compounds traditionally referred to as "minor phytocannabinoids" (**Figure 1.1.2**). As a result, the name "minor phytocannabinoids," including CBN, (-)-Δ9-tetrahydrocannabidivarin (THCV) and (-)-trans-Δ9-tetrahydrocannabiphorol (THCP), has been used to indicate cannabinoids whose biological profile is poorly investigated; therefore, "minor" refers not to their actual concentration in cannabis, but in the literature.

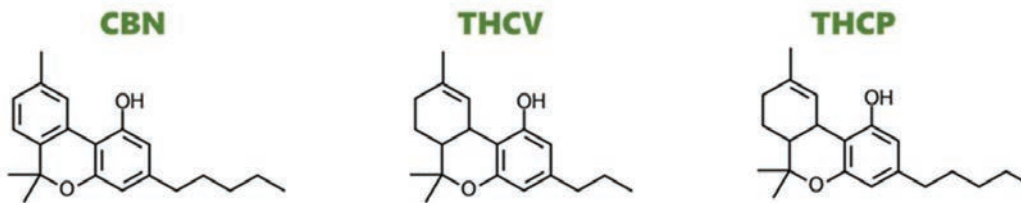

Figure 1.1.2 Minor phytocannabinoids.

Cannabinol

CBN was isolated in 1898 and its structure elucidated in the early 1930s.[60] It is the oxidative product of THC and mostly found in aged cannabis18 and also a decarboxylation product of cannabinolic acid.[61] CBN binds to CB1 and CB2 receptors less potently than THC.[62] At CB1 receptors, CBN was reported to be inactive, in assays activating [35S] GTPγS binding in CHO cell membranes and inhibiting adenylyl cyclase in the same