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Chapter 4

Evaluation of anti-TNF-α activity of eight major cannabinoids isolated from Cannabis sativa

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Abstract

We investigated the antiinflammatory effect of a series of cannabinoids in an in-vitro systems, human U937 cells stimulated with LPS to secrete immunoregulatory cytokine tumor necrosis factor alpha (TNF-α). Phytocannabinoids like the psychoactive delta-9- tetrahydrocannabinol (THC) and nonpsychoactive cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), cannabidiolic acid (CBDA), Δ9-tetrahydrocannabinolic acid (THCA), cannabigerolic acid (CBGA) showed activity which suggest that cannabinoids can potentially alter cytokine secretion of human U937 cell lines.

Keywords: Cannabinoids, inflammation, TNF-α, U937 cell lines
4.1. Introduction

Inflammation is a response of a tissue to injury, which can be caused (or followed by) pathogen invasion. It is characterized by redness, heat, swelling, pain and dysfunction of the organs involved. The process of inflammation is mediated by several pro-inflammatory and anti-inflammatory cytokines. Tumor necrosis factor alpha (TNF-α) is one of the most important pro-inflammatory cytokines which promotes inflammation. TNF-α is mainly produced by macrophages upon stimulation by a bacterial cell wall component lipopolysaccharide (LPS) at nanogram per milliliter concentration. Wounds can never heal without inflammation, so release of TNF-α plays an essential role in host defense against pathogens and tissue recovery. However, excessive production of TNF-α can lead to endotoxic shock, rheumatoid arthritis, and cachectic states associated with malignancies, chronic parasitic infections and several diseases related to autoimmunity. In these cases anti-TNF-α therapies are recommended for the treatment of several inflammatory diseases. Several protein based drugs are available for the inhibition of TNF-α but these are associated with high costs and side effects. Thus, it is important and even essential to develop safer and perhaps more-cost-effective TNF-α inhibitors. Many natural compounds belonging to various classes have been found to reduce TNF-α level (Paul et al., 2006).

Nature is a main source of compounds for pharmaceutical purposes. Because of the great structural diversity, natural products or natural product-derived compounds offer great opportunities for the development of anti-inflammatory drugs. Their origin extends to plants, fungi, bacteria, and marine organisms. Plants have been and continue to be the greatest source of natural compounds from which drugs can be synthesized. Of the 1184 new chemical entities registered as medicine in the period of 01/1981 to 06/2006, 60% are derived from or based on natural products. Natural products clearly play a dominant role in the discovery of leads for drug development (Gautam and Jachak, 2009).

Cannabis is considered one of the oldest psychotropic drugs known to humanity. It is difficult to trace the beginnings of its use by humans because it was cultivated and consumed long before the appearance of writing (McKim, 2000). There are several species of cannabis. The most relevant are Cannabis sativa, Cannabis indica and Cannabis ruderalis. Cannabis sativa, the largest variety, grows in both tropical and temperate climates. The two main preparations derived from cannabis are marijuana and hashish. Marijuana is a Mexican term initially attributed to cheap tobacco but referring today to the dried leaves and flowers of the hemp plant. Hashish, the Arabic name for Indian hemp, is the viscous resin of the plant (Ben Amar, 2006).
More than 460 different compounds have been identified from cannabis plants, around 60 of which are grouped under the name cannabinoids. The major psychoactive ingredient of cannabis is delta-9-tetrahydrocannabinol, commonly known as THC. Other cannabinoids present in Indian hemp include delta-8-tetrahydrocannabinol (\(\Delta^8\) THC), cannabinol (CBN), cannabidiol (CBD), cannabicyclol (CBL), cannabichromene (CBC) and cannabinerol (CBG), but they are present in small quantities and have no significant psychotropic effects compared to THC. However, they may have an impact on the product’s overall effect.

The therapeutic effects of cannabis and its derivatives have been extensively investigated, and they have been shown to exhibit a wide variety of beneficial properties, inhibiting cancer, neuropathic pain, multiple sclerosis, Alzheimer’s disease, atherosclerosis, rheumatoid arthritis, asthma and many inflammatory diseases (Alexander et al., 2009; Ligresti et al., 2009; Nolin et al., 2002; Pacher et al., 2006). Inflammation plays a crucial role in most of the mentioned health issues, and cannabinoids have been proven to influence these processes. Their biological activity is connected to the activation of specific receptors: CB1, expressed mostly in the central nervous system; and CB2, found mainly in peripheral tissues. CBD, a non-psychoactive cannabinoid, is responsible for the antiinflammatory activity of marihuana, acting mostly on the CB2 receptor in peripheral tissues (Rajesh et al., 2007; Zoratti et al., 2003).

Antiinflammatory studies are performed using the U937 cell line derived from a human histiocytic lymphoma (Sundström and Nilsson, 1976). This cell line is maintained as replicative non-adherent cells having many of the biochemical and morphological characteristics of blood monocytes (Harris and Ralph, 1985). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, non-replicative cells with characteristics of tissue macrophages, including isoenzyme patterns, 17 CR3 expressions, 18 and other phenotypic markers (Pearlman et al., 1988). The purpose of this study was to investigate antiinflammatory activities of cannabinoids using U937 cell lines (\textit{in-vitro}).

4.2. Materials and Methods

4.2.1. Chemicals and reagents

Fetal bovine serum (FBS), penicillin, streptomycin and RPMI1640 were purchased from GIBCO (Grand Island, NY) and U937 cell lines from ATCC (CRL-1593.2). Lipopolysaccharide (Escherichia coli O111:B4) and phorbol 12-myristate 13-acetate (PMA) were from Sigma–Aldrich (St. Louis, MO, USA). The Human TNF-\(\alpha\) ELISA kit was purchased from BioSource International.
Inc. (Camarillo, CA, USA). DMEM, fetal bovine serum (FBS), penicillin and streptomycin solution, phosphate buffered saline, were supplied by GIBCO Netherlands BV (Breda, The Netherlands). DMSO was purchased from Biosolve BV (Valkenswaard, The Netherlands).

4.2.2. Plant Cannabinoids

The cannabinoids used in this study were kindly provided by Dr Arno hazekamp.

4.2.2. Cell culture

Human monocyte-like histiocytic lymphoma cells U937 were cultured as described in chapter 3.

4.2.4. TNF-α ELISA

TNF-α in culture supernatants were performed as described in chapter 3.

4.2.5. Cell viability assay

Cell viability assay was performed as described in chapter 3.

4.2.6. Data analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. One way ANOVA was performed. Posthoc comparison between means and controls were made using Dunnett’s test. Value of $P \leq 0.05$ was considered statistically significant.

4.3. Results and Discussions

Cannabis has a long history as a medicinal preparation, mainly for properties such as analgesia, antiemesis, ocular hypotension, and anticonvulsion
Evaluation of anti-TNF-α activity of eight major cannabinoids isolated from *Cannabis sativa* (Mechoulam et al., 1998). Recent research *in-vitro* and in animal models has led to increasing evidence that cannabinoids are also important modulators of the immune system (Klein et al., 1998) and thus could cannabinoids have a role in the treatment of chronic inflammatory diseases. It is therefore important to find out whether nonpsychoactive cannabinoids are suitable for treating chronic inflammatory diseases.

![Figure 1: Structures of cannabinoids studied in this chapter.](image-url)

Δ⁹-Tetrahydrocannabinol (Δ⁹-THC)  Cannabinol (CBN)  Cannabigerol (CBG)

Cannabichromene (CBC)  Cannabidiol (CBD)  Cannabidiolic acid (CBDA)

Δ⁹-Tetrahydrocannabinolic acid (Δ⁹-THCA)  Cannabigerolic acid (CBGA)
Evaluation of anti-TNF-α activity of eight major cannabinoids isolated from Cannabis sativa

The inhibitory effects of eight major cannabinoids and Cannabis sativa extract on TNF-α inhibition was evaluated in U937 cell line stimulated by LPS. Crude extract, Δ⁹-THC, Δ⁹-THCA, CBD, CBDA, CBG, CBGA, CBC and CBN were evaluated for their ability to inhibit TNF-α at a concentration of 10 µgmL⁻¹, 1 µgmL⁻¹ and 0.01 µgmL⁻¹ respectively. In this study, it was found that phytocannabinoids show highly significant inhibition of TNF-α at a concentration of 1µg/ml. Maximum inhibition was observed in Δ⁹-THCA followed by CBDA > CBGA > CBD >Δ⁹-THC > CBG > CBN and CBC. They all show activity in a concentration-dependent manner. It was also found that all these compounds show toxicity towards cell lines at concentration of 10µg/ml.

TNF-α inhibition and cell viability of cannabinoids after stimulated with LPS has shown in Figure 2-4.

**Figure 2:** TNF-α inhibition and cell viability of LPS stimulated U937 cell lines treated with different cannabinoids. One way ANOVA was performed. Posthoc comparison between means and controls (LPS+DMSO) were made using Dunnett’s test. Each error bar represents ±SEM of three replicates. * = the value shows a significant difference (P ≤ 0.05).
Delta-9-tetrahydrocannabinol (THC) is one of more than 80-100 cannabinoids in the marijuana plant and has been recognized as the major psychoactive component of this plant. THC being psychoactive and most studied component of marijuana is widely acknowledged because of its therapeutic effects including relief of nausea and vomiting associated with cancer and its treatments; stimulation of appetite in AIDS patients and patients with anorexia and wasting syndrome; analgesia; and muscle relaxation. In this study, we show that Δ⁹-THC inhibits TNF-α release in LPS stimulated U937 cell line. THC shows toxicity towards cells at highest concentration while at low concentration (1µg/ml), it shows significant inhibition of TNF-α. Various in-vitro studies have shown that THC exhibits a variety of inhibitory effects on immune functions. THC has been used with success in controlling severe cachexia seen in patients with cancer or AIDS (Kusher et al., 1994; Razdan, 1986). THC has been shown to inhibit TNF-α production in various models of cell lines (Fischer-Stenger et al., 1993; Zheng and Specter, 1996; Zhi-Ming et al., 1992). The mechanism by which THC inhibits TNF-α production is not yet clear but there are several possibilities proposed (Specter et al., 1990). There are reports saying THC as a lipophilic compound, can be incorporated into the cell membranes and cell membrane alteration could be the reason of inhibitory action. There is also a report regarding THC acid and its potential to inhibit TNF-α release in LPS stimulated U937 cell lines. Moreover, it was also found that the inhibitory effect on TNF-α production by THC and THCA are not mediated via CB1 and CB2 receptors instead via TLR4 and IFN receptors (Figure 3) (Kozela et al., 2010; Verhoeckx et al., 2004).

Cannabidiol (CBD; Fig. 3), the most abundant nonpsychoactive cannabinoid in the plant has been studied more extensively in recent years. CBD is well-known for its immunosuppressive, antiinflammatory and antioxidant properties both in-vitro and in various preclinical models (Fernández-Ruiz et al., 2005; Mechoulam et al., 2007). We here report that CBD significantly suppresses the level of TNF-α associated with LPS in U937 cell lines. At higher dose (10µg/ml) CBD shows significant toxicity towards cell lines but at lower dose (1µg/ml), it strongly inhibits the release of TNF-α in LPS stimulated cell lines.
Figure 3: TNF-α inhibition and cell viability of LPS stimulated U937 cell lines treated with different cannabinoids. One way ANOVA was performed. Posthoc comparison between means and controls (LPS+DMSO) were made using Dunnett’s test. Each error bar represents ±SEM of three replicates. * = the value shows a significant difference ($P \leq 0.05$).

There are several reports which support our results regarding the inhibition of TNF-α. Costa et al., (2007) reported that in synovial cells isolated from mice, CBD treatment inhibits the release of TNF-α. In addition, oral administration of CBD (2.5–20 mg/kg) reduces neuropathic and inflammatory pain in rats. In another study it has been shown that a low dose of CBD suppresses TNF-α production induced by lipopolysaccharide (LPS) in mice (Carrier et al., 2006). CBD has been shown to reduce joint inflammation in collagen-induced arthritis (CIA) in mice (Sumariwalla et al., 2004) and carrageenan paw edema in rats (Costa et al., 2004). Though CBD did not reduce inducible nitric oxide synthase (iNOS) in these studies, others (Esposito et al., 2006; Esposito et al., 2007) have reported that CBD does inhibit iNOS in a beta-amyloid induced murine model of neuroinflammation. CBD also reduces intestinal inflammation in mice (Capasso et al., 2008). In addition to its ability to suppress production of the inflammatory cytokine TNF-α, CBD appears to
exert antiinflammatory activity by suppressing fatty acid amidohydrolase (FAAH) activity, thereby increasing concentrations of the antiinflammatory endocannabinoid anandamide (Ben-Shabat et al., 2006).

The complex mechanisms whereby these compounds exert their effects is illustrated by the fact that hydrogenation at different double bonds has different effects on bioactivities, none of which appear dependent on CB1 activation. Further, insight into mechanisms whereby CBD exerts therapeutic effects is provided by experiments which indicate that CBD attenuates inflammation induced by high glucose in diabetic mice (Rajesh et al., 2007). Specifically, CBD treatment reduces mitochondrial superoxide, iNOS, nuclear factor kappa B (NF-kB) activation, and transendothelial migration of monocytes. Another potential therapeutic use of CBD may lie in its ability to counter some undesirable effects of THC (sedation, psychotropic effects, tachycardia), thus suggesting that if given together with THC, it may allow higher doses of THC (Russo and Guy, 2006). Several studies pointed out that cannabinoids could have CB1/CB2 receptor-independent mechanisms of action. CBD exhibits very low affinity towards CB1 and CB2 and thus shows immunosuppressive effects through non-CB1 and non-CB2 mechanisms (Kaplan et al., 2003). There was reported that CBD inhibits production of pro-inflammatory cytokines by decreased activity of NF-κB (Kozela et al., 2010).

Cannabichromene (CBC) is, together with Δ⁹-tetrahydrocannabinol, cannabidiol and cannabinol, the most abundant naturally occurring cannabinoid (Brown and Harvey, 1990; Holley et al., 1975). It is particularly abundant in freshly harvested dry-type cannabis material and it is the second most abundant cannabinoid in some strains of marijuana growing in the USA (Brown et al., 1990). It is reported that in USA during period 1993–2008, CBC represented 0.7 and 0.9% of the constituents from hashish or hash oil, respectively (Mehmedic et al., 2010). Despite the relative abundance of this compound in cannabis preparations, very little is known about its pharmacology. Cannabichromene was reported to have anti-inflammatory activity in the carrageenan paw edema assay (DeLong et al., 2010; Wirth et al., 1980) and has analgesic effects (Davis and Hatoum, 1983). CBC inhibits prostaglandin synthesis in vitro, but less potently than CBD or THC (Burstein et al., 1973). CBC exhibits strong antibacterial activity and mild antifungal activity, superior to THC and CBD in most instances (Eisohly et al., 1982). The mechanism by which CBC exerts its antiinflammatory effects is not known but it is confirmed.
Figure 4: TNF-α inhibition and cell viability of LPS stimulated U937 cell lines treated with different cannabinoids. One way ANOVA was performed. Posthoc comparison between means and controls (LPS+DMSO) were made using Dunnett’s test. Each error bar represents ±SEM of three replicates. * = the value shows a significant difference ($P \leq 0.05$).

that these effects are mediated through a non cannabinoid receptor mechanism of action (DeLong et al., 2010).

Cannabigerol (CBG; Fig. 4) is the biosynthetic precursor of CBC, CBD, and THC, and is present only in minor amounts. CBG has being shown less affinity towards CB1 receptors as compared to THC, approximately the same as CBD (Devane et al., 1988). CBG is also reported to inhibit the uptake of serotonin and norepinephrine in rat brains, less effectively than CBD and THC, but CBG inhibits GABA uptake more effectively than CBD and THC (Banerjee et al., 1975). CBG acts as an analgesic (more potently than THC), it inhibits erythema (much more than THC), and it blocks lipoxygenase, again more
effectively than THC (Evans, 1991). CBG has antibacterial (Appendino et al., 2008; Mechoulam and Gaoni, 1965) and antitumoural activities (Baek et al., 1998b).

Its activity against gram-positive bacteria, mycobacteria, and fungi is superior to that of THC, CBD, and CBC (Eisohly et al., 1982). CBG inhibits the growth of human oral epitheloid carcinoma cells (Baek et al., 1998a). CBG has been found to activate alpha (2)-adrenoceptors, to block 5-HT1A and CB1 receptors and bind to CB2 receptors (Cascio et al., 2010) and may serve as a treatment for glaucoma (Colastani, 1990). It has been recently reported that CBG strongly inhibits the synthesis of IL-1β, IL-6, PGE2 and TNF-α in a dose-

Figure 5: Possible antiinflammatory mechanism exhibited by THC & CBD. Pointed arrows represent activation while blunt arrows show suppression.
dependent manner. PPARγ receptors have been shown to be involved in the modulation of inflammation, as PPARγ agonists downregulate the expression of several proinflammatory cytokines. Activation of PPARγ receptors might explain the TNF-α inhibitory action of CBG (Granja et al., 2012; Jiang et al., 1998).

Cannabinol (CBN; Fig. 4) is the degradation product of THC, and is found most often in aged cannabis products. CBN has been reported for its anticonvulsant and antiinflammatory activities (Evans, 1991; Turner et al., 1980). CBN shows greater affinity for CB2 receptors thus it may affect cells of the immune system more than the central nervous system. Furthermore, it is also reported that CBN modulates thymocytes by attenuating the activity of the c-AMP response element-binding protein (CREB), nuclear factor κB (NF-κB), and interleukin-2 (IL-2). CBN inhibits the expression of these proteins in splenocytes, via decreased activation of ERK MAP kinases (Faubert and Kaminski, 2000).

4.4. Conclusion

Cannabis sativa has been used throughout the history not only for its fiber, but also as a medicinal plant. Here, we have demonstrated that acidic forms of different cannabinoids are more active and strongly inhibit the release of TNF-α. Maximum activity was found in ∆9-THCA followed by CBDA and CBGA. These acidic forms also showed strong toxicity towards U937 cells at highest concentration of 10μg/ml. Our studies support earlier findings that cannabinoids are potent antiinflammatory agents and they exert their effects through suppression of cytokine production.

References

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