HU-444, a Novel, Potent Anti-Inflammatory, Nonpsychotropic Cannabinoid


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ABSTRACT

Cannabidiol (CBD) is a component of cannabis, which does not cause the typical marijuana-type effects, but has a high potential for use in several therapeutic areas. In contrast to Δ9-tetrahydrocannabinol (Δ9-THC), it binds very weakly to the CB1 and CB2 cannabinoid receptors. It has potent activity in both vitro and in vivo anti-inflammatory assays. Thus, it lowers the formation of tumor necrosis factor (TNF)-α, a proinflammatory cytokine, and was found to be an oral antiarthritic therapeutic in murine collagen-induced arthritis in vivo. However, in acidic media, it can cyclize to the psychoactive Δ9-THC. We report the synthesis of a novel CBD derivative, HU-444, which cannot be converted by acid cyclization into a Δ9-THC-like compound. In vitro HU-444 had anti-inflammatory activity (decrease of reactive oxygen intermediates and inhibition of TNF-α production by macrophages); in vivo it led to suppression of production of TNF-α and amelioration of liver damage as well as lowering of mouse collagen-induced arthritis. HU-444 did not cause Δ9-THC-like effects in mice. We believe that HU-444 represents a potential novel drug for rheumatoid arthritis and other inflammatory diseases.

Introduction

Cannabidiol (CBD), a nonpsychotropic constituent present in most Cannabis sativa varieties, causes a large number of both central and peripheral pharmacological effects (Mechoulam et al., 2002, 2007; Pertwee, 2005; Zhornitsky and Potvin, 2012; Fernández-Ruiz et al., 2013). CBD is a potent antioxidant (Hampson et al., 2000), which may explain—at least in part—its neuroprotective effects in neurodegenerative disorders (Fernández-Ruiz et al., 2013), in amelioration of the progressive degeneration of nigrostriatal dopaminergic neurons occurring in a model of Parkinson’s disease (Lastres-Becker et al., 2005), in cerebral ischemia (Braida et al., 2003), in cerebral infarction in mice (Hayakawa et al., 2007), and in hypoxia-ischemia in newborn rats (Pazos et al., 2012). Additional effects of therapeutic relevance, through various mechanisms, are its action in animals on type 1 diabetes (Weiss et al., 2008), on some types of cancer (Massi et al., 2013), on myocardial ischemic reperfusion injury (Durst et al., 2007), on reduction of microglial activation—thus, possibly on the progression of Alzheimer’s disease (Martin-Moreno et al., 2011), on brain and liver functions in a fulminant hepatic failure-induced model of hepatic encephalopathy (Avraham et al., 2011), on nausea and emesis (Rock et al., 2012), and others.

The antiepileptic activity of CBD in human patients has been known for nearly 35 years (Cunha et al., 1980), but only recently have marijuana extracts with high levels of CBD been used to suppress pediatric epilepsies (Porter and Jacobson, 2013). In clinical trials CBD has been shown to have antischizophrenic (Leweke et al., 2012) and antianxiety (Bergamaschi et al., 2011) properties.

Of particular relevance to the results presented in this study is the potent anti-inflammatory action of CBD (Mechoulam et al., 2002, 2005; Pertwee, 2005). We have previously shown that, in vitro, CBD suppresses lymphocyte proliferation and blocks zymosan-triggered reactive oxygen burst by peritoneal granulocytes (Malfait et al., 2000). In vivo results reported include blocking lipopolysaccharide (LPS)-induced rise in serum tumor necrosis factor (TNF)-α in mice as well as the progression of arthritis caused by immunization of mice with type II collagen (Malfait et al., 2000).

It is somewhat surprising that, in spite of the very promising pharmacological effects of CBD and its lack of toxicity, it has not been developed as a single drug. CBD is marketed together with Δ9-tetrahydrocannabinol (Δ9-THC; in a 1:1 ratio) by GW Pharmaceuticals (London, UK) as Sativex, sold in Canada and several European countries for spasticity, due to multiple sclerosis (Syed et al., 2014).

ABBREVIATIONS: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CBD, cannabidiol; CIA, collagen-induced arthritis; Con A, concanavalin A; DMEM, Dulbecco’s modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HPLC, high-pressure liquid chromatography; LPS, lipopolysaccharide; NO, nitric oxide; RA, rheumatoid arthritis; ROS, reactive oxygen species; TG, thioglycolate; THC, tetrahydrocannabinol; TNF, tumor necrosis factor.
We report now the synthesis of a novel CBD derivative, HU-444, using CBD as the starting material, and the evaluation of its anti-inflammatory properties in vitro and in vivo. This novel compound was chosen, as the conversion of the C-1 methyl group into a carboxyl group in the cannabinoid series has been shown to enhance anti-inflammatory activity (Burstein et al., 1992; Sumariwalla et al., 2004) and the reduction of the 8,9-double bond would preclude possible ring cyclization under acidic conditions with one of the phenolic groups leading to psychoactive THC-like compounds.

The results reported now show that HU-444 is a potent anti-inflammatory compound, both in vitro and in vivo.

Materials and Methods

Reagents. All solvents were purchased from Biolab (Jerusalem, Israel) and J. T. Baker (Deventer, Holland). Chemicals were purchased from Sigma-Aldrich (Rehovot, Israel), Acros, (Geel, Belgium) Holland Moran (Yehud, Israel), Alfa Aesar (Lancashire, UK), Merck (Darmstadt, Germany), J. T. Baker (Center Valley, PA), and Penta (Prague, Czech Republic). They were used without further purification in the reactions, except of dry diethyl ether and CH₂Cl₂, which were refluxed over sodium and phosphorous pentoxide (P₂O₅), respectively, and freshly distilled prior to its use.

¹H NMR spectra were obtained on a Bruker AMX 300 MHz apparatus using CDCl₃ (δ = 7.25 ppm) and TMS (tetramethyl silane) as internal standard for ¹H NMR. Thin-layer chromatography was run on silica gel 60F₂54 plates (Merck). Column chromatography was performed on silica gel 60 Å® (Merck). The compounds were localized at 254 nm using a UV lamp.

Syntheses. The synthesis of HU-444 is presented in Scheme 1. The synthetic steps a, b, and c are published in Kozela et al. (2015). Below we describe in detail the subsequent reaction steps d-i.

Scheme 1. Syntheses of HU-444 and HU-445. Reagents and Conditions: (a) Pt(IV) oxide/H₂, EtOAc, 10psi, rt, 2min; (b) pyridine, acetic anhydride, rt, 12h; (c) SeO₂, EtOH, rt, 3h; (d) pyridine; (e) CrO₃, CH₂Cl₂/DMF, rt, 1h; (f) NaClO₂; (g) 2-methyl-2-buten; (h) KH₂PO₃, t-butanol, rt, 1h; (i) NaBH₄, EtOH, reflux, 1h.
NaClO₂ (221 mg, 2.44 mmol) was added in small portions with ethyl acetate, and evaporated to dryness. The residue was chromatographed on silica gel with 10% ether–petroleum ether to give a residue that was chromatographed on silica gel with 10% ether–petroleum ether to give compound 5 as a white solid. Yield 13%, Mp: 152°C; [α]D₂₀ = −57° (CHCl₃); high-pressure liquid chromatography (HPLC): 60% acetonitrile, 15% water, and 25% methanol: tR = 7.69 minutes, 96%. 1°H NMR (CDCl₃): δ 6.94 (1H, s, olefin), 6.88 (2H, s, Ar), 3.58 (1H, m, benzyl), 2.51 (3H, m, allyl + benzyl), 2.22 (6H, s, OAc), 1.97 (1H, m), 1.86 (1H, m), 1.54 (5H, br s), 1.32 (5H, m), 0.98 (9H, t, terminal CH₃). MS m/z: 502 (silylated), 472, 430, 415, 400. Exact mass calculated for C₅₃H₆₀O₅ 840.3199, found 840.3198.

Synthesis of (-)-8,9-Dihydro-7-oxo-CBD-Diacetate (HU-444) (5). NaClO₃ (221 mg, 2.44 mmol) was added in small quantities to a stirred mixture of mixture 4 (235 mg, 0.568 mmol), 2-methyl-2-butenone (1.5 mL, 14.22 mmol), and a saturated aqueous solution of KH₂PO₄ (0.67 mL) in tert-butanol (13.37 mL). The reaction was stirred at room temperature for 5 hours and monitored by thin-layer chromatography. Water was added (60 mL), and the mixture was extracted several times with ethyl acetate. The organic phase was dried, and filtered. Removal of the solvent under reduced pressure afforded a residue that was chromatographed on silica gel with 10% ether–petroleum ether to give compound 5 as a white solid. Yield 13%, Mp: 152°C; [α]D₂₀ = −57° (CHCl₃); high-pressure liquid chromatography (HPLC): 60% acetonitrile, 15% water, and 25% methanol: tR = 7.69 minutes, 96%. 1°H NMR (CDCl₃): δ 6.94 (1H, s, olefin), 6.88 (2H, s, Ar), 3.58 (1H, m, benzyl), 2.51 (3H, m, allyl + benzyl), 2.22 (6H, s, OAc), 1.97 (1H, m), 1.86 (1H, m), 1.54 (5H, br s), 1.32 (5H, m), 0.98 (9H, t, terminal CH₃). MS m/z: 502 (silylated), 472, 430, 415, 400. Exact mass calculated for C₅₃H₆₀O₅ 840.3199, found 840.3198.

Binding to the Cannabinoid Receptors. The binding of HU-444 and HU-445 to the cannabinoid receptors CB₁ and CB₂ was performed, as previously described (Devane et al., 1992a,b; Bayewitch et al., 1996).

Macrophages. Peritoneal cells were harvested from C57BL/6 female mice 4 days after i.p. injection of 1.5 mL 3% thioglycollate (TG) medium (Difco, Oxford, UK). The cells (TG macrophages) were washed with phosphate-buffered saline; resuspended in Dulbeco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), sodium pyruvate, glutamine, and antibiotics; and plated (1.2 × 10⁶) in 96-microwell flat-bottom plates (Nunc, Roskilde, Denmark). Following 2- to 3-hour incubation at 37°C, the nonadherent cells were removed by intensive rinsing with phosphate-buffered saline. About 95% of the adherent cells were macrophages.

THC-Like Activity in Mice. THC and other agonists of the CB₁ cannabinoid receptor cause a typical tetrad of pharmacological effects in Saba mice—namely, ring immobility (catatlepsy), which measures the percentage of time, over 4 minutes; mice remain immobile on a ring (5.5 cm diameter), the open field test that measures locomotor activity, hypothermia, and hot plate latency (antinociception) (Martin et al., 1991; Pride and Mechoulam, 1993). Saba mice were administered HU-444 i.p. and assayed in the above tetrad.

Macrophage Cell Lines. RAW 264.7 cells, a monocytic-macrophage cell line, derived from BALB/c mice, were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM supplemented with 10% FCS and sodium pyruvate, glutamine, and antibiotics. For activation, the cells (10⁵ cells/microwell) were incubated with 1 μg/mL LPS (Escherichia coli; Sigma-Aldrich, Jerusalem, Israel) for 24 hours.

Therapeutically, THC was found to be safe and effective in treating several conditions, including pain, inflammation, and anxiety. In the context of pain management, THC has been shown to reduce pain intensity and duration in a variety of conditions, such as cancer-related pain and neuropathic pain. Additionally, THC has been found to have anti-inflammatory effects, reducing the production of inflammatory cytokines and prostaglandins, which are key mediators of pain and inflammation. This anti-inflammatory action may contribute to THC's efficacy in treating pain conditions such as rheumatoid arthritis and fibromyalgia.

In the realm of psychiatric disorders, THC has been studied for its potential in treating anxiety and depression. Several studies have suggested that THC may alleviate symptoms of anxiety and depression, likely through its modulation of the endocannabinoid system.

Furthermore, THC has demonstrated promise in treating chronic pain conditions, such as osteoarthritis and multiple sclerosis. By targeting the endocannabinoid system, THC may offer a novel approach to pain management, potentially reducing the reliance on traditional analgesics that carry significant side effects.

In conclusion, THC holds tremendous potential in pain management, offering a promising alternative to conventional treatments. Further research is needed to fully understand the therapeutic and therapeutic implications of THC, as well as to develop safer, more efficacious delivery systems.
was induced in genetically susceptible DBA/1 mice (H-2q; Harlan) by subcutaneous immunization with collagen-induced arthritis (CIA) and Treatment with HU-444. Changes in the liver; and 5, severe liver damage.

Changes; 2, mild, few lesions; 3, moderate number of lesions; 4, marked

changes in the presence of HU-444. The level of NO2/NO3 in the control supernatants was 42.5 nmol/ml. *Statistically significant difference with a P value ≤ 0.05.

Histologic Analysis. The livers of Con A injected mice, with or without HU-444 treatment, were fixed in 10% buffer formalin, and stained with H&E, for microscope evaluation. TNF-α levels in the sera of Con A−treated mice were determined by ELISA (R&D Systems).

Determination of Alanine Aminotransferase and Aspartate Aminotransferase Levels. The levels of two aminotransferases, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were assayed in the sera of Con A−treated mice, with or without HU-444 treatment, by ALT and AST strips, respectively (Reflotram-Mannheim, Mannheim, Germany), and quantitated by an automated analyzer (Reflotran Plus; Roche, Basel, Switzerland).

Histology of Arthritic Feet. Mice were euthanized at the end of treatments and hind paws fixed in buffered formalin, decalcified in 10% EDTA for 4–5 weeks, embedded in paraffin wax, sectioned to 5 μm thicknesses, and stained with H&E, for microscope evaluation. TNF-α levels in the sera of Con A−treated mice were determined by ELISA (R&D Systems).

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accompanying large areas of bone and cartilage erosions; and 3, severe, total loss of joint architecture. For the purpose of scoring, all joints (distal, proximal phalangeal, first metatarsal, medial cuneiform, and tibia-tarsus) present within the stained section were scored. Histologic evaluation was done on all mice paw sections from all groups. Percentage of joints involved as normal, mild, moderate, and severe was then calculated for each group. Data were presented as percentage joints scored and classified as either protected (normal to mild arthritic changes) or damaged (moderate to severe arthritic changes). Images were captured using Olympus BX51 optical microscope and DP Controller and Manager Software (Olympus, version 3.3.1.292/222; Center Valley, PA), at original magnification, 100×. For publication purposes, only the proximal phalangeal joint images were depicted for all treatment groups.

Experimental Design and Statistics. Experiments, both in vitro and in vivo (4–5 mice/group), were repeated three times, and the results were expressed as the mean ± S.E.M. of triplicate values. Evaluation of statistical significance was done by using analysis of variance. Joint histology data were evaluated by Fischer’s exact test. The P ≤ 0.05 values were considered significant.

Results

Chemistry. CBD, which was extracted from hashish (Gaoni and Mechoulam, 1971), was hydrogenated with Pt (IV) oxide in ethyl acetate to yield 8,9-dihydro-CBD 1, which was then converted into the diacetate 2 by pyridine and acetic anhydride. Compound 2 was oxidized with selenium IV oxide in ethanol to give a mixture of the allylic alcohols 3. The allylic hydroxylation reaction by selenium IV oxide has been widely investigated (Sharpless and Lauer, 1972; Lander et al., 1976; Stephenson and Speth, 1979). Apparently, it involves a (2,3) sigmatropic migration reaction of intermediate allylselenic acids, leading mostly to allylic hydroxylation products. It has been widely used for the oxidation of cyclohexenyl systems. As mixture 3 was difficult to separate, it was directly oxidized with chromium VI oxide in the presence of pyridine, to give a mixture 4 of the expected aldehyde and ketone. The chromium (VI) oxide–pyridine complex is used as an oxidant for the conversion of primary and secondary alcohols to aldehydes and ketones (Holm, 1961; Ratcliffe and Rodehorst, 1970) at room temperature. On further oxidation of the mixture 4 with sodium chlorite (Pellegata et al., 1986; Burstein et al., 1992), we obtained the acid diacetate HU-444 (compound 5) from the aldehyde, which was easy to separate from the unchanged ketone. The overall yield from CBD was 13% (96% purity by HPLC). The conversion of the diacetate to the free diphenol was carried out by sodium borohydride in ethanol to give HU-445 (compound 6) in 90% yield (95% purity by HPLC). All compounds were purified on silica gel chromatography (Scheme 1).

While the overall yield of HU-444 is relatively low, in view of the ready availability of CBD, its synthesis represents a practical route.

Binding to the Cannabinoid Receptors. Neither 5 (HU-444) nor 6 (HU-445) was found to bind to either the CB1 or the CB2 receptor (Ki above 10 μM).

Pharmacology. Compound 5 (HU-444) was first investigated for its possible THC-like psychoactivity in the tetrad assay, which measures cannabinoid-induced hypokinesia, hypothermia, and antinociception in a tail flick or hot plate test in mice (Martin et al., 1991; Fride and Mechoulam, 1993; Mechoulam et al., 2014). The mouse tetrad serves as a useful in vivo screen for psychotropic cannabinoids, which, in contrast to many other types of drugs, displays potency in all four of these bioassays. As no psychoactivity was noted (data not presented), we evaluated HU-444 in a wide range of assays relevant to inflammation.

The in vitro anti-inflammatory assays were mostly done with macrophages, whereas for the in vivo studies we used an animal model for both autoimmune hepatitis and rheumatoid arthritis (RA). The results showed that HU-444 is a potent anti-inflammatory compound, both in vitro and in vivo.

Suppression of Reactive Oxygen Species Production by HU-444. To study the effect of HU-444 on the ability of macrophages to produce ROS, RAW 264.7 cells were stimulated with zymosan together with various doses of HU-444. A 39% and 62% decrease of reactive oxygen species (ROS) generation was observed in the presence of 20 and 40 μg/ml HU-444, respectively (Fig. 1A).

Suppression of NO Production by HU-444. To study the effect of HU-444 on macrophage production of NO, RAW
264.7 cells were incubated for 24 hours with LPS (1 μg/ml) and various doses (5–60 μg/ml) of HU-444. NO production was suppressed by 51% and 64% following incubation of the cells with 40 and 60 μg/ml HU-444, respectively (Fig. 1B).

**Inhibition of TNF-α Production in Macrophages and Mice.** TNF-α production by TG macrophages was determined following their incubation with LPS (1 μg/ml) and various doses of HU-444. Inhibition of up to 69% was noted upon cell incubation with 40 μg/ml HU-444 for 24 hours (Fig. 2A). HU-444 also reduced the TNF-α serum levels by 34–45% in C57BL/6 mice after i.p. injection of LPS (100 μg/mouse). Already, at 2.5 mg/kg HU-444, a strong inhibition in TNF-α serum levels of 34% was observed, with only a slightly higher inhibition (45%) when increasing the dose to 10 mg/kg, suggesting that a plateau effect has been reached (Fig. 2B).

**Effect of HU-444 on Con A-Induced Liver Damage.** Intravenous administration of Con A to mice causes a CD4+ T cell–driven, TNFR1-dependent acute hepatitis, leading to pathologic damage of the liver. This is accompanied by elevation of liver enzymes, interleukin-2, and inflammatory cytokines (Kusters et al., 1997; Ohta and Sitkovsky, 2001). When Con A–treated mice were injected i.p. with 5 mg/kg HU-444, a marked reduction in the ALT and AST aminotransferase serum levels was observed (Fig. 3). ALT was reduced by 87% (Fig. 3A), and AST by 85% (Fig. 3B). We observed a bell-shaped dose response with both enzymes, with an optimal dose of 5 mg/kg. A bell-shaped dose response is often observed with CBD drugs (Jamontt et al., 2010). The TNF-α serum levels in Con A–treated C57BL/6 mice were also reduced after treatment with HU-444. Inhibition of 53% in TNF-α titers was scored following injection of 5 mg/kg HU-444 (Fig. 4).

**Prevention of Con A-Induced Liver Damage by HU-444.** To study the effect of HU-444 on Con A–induced liver damage, histopathological evaluations of the livers were performed (Fig. 5; Table 1). Con A treatment caused marked liver damage with necrosis and mononuclear cell infiltration (Fig. 5A). Administration of HU-444 attenuated significantly liver damage and reduced mononuclear infiltration, leading to almost normal histology (Fig. 5, C and D). An optimally preserved normal liver histology was observed after treatment with 5 mg/kg HU-444 (Fig. 5C).

**Treatment with HU-444 Provides Beneficial Outcome in an Established Mouse Model of Arthritis.** We explored the potential anti-inflammatory and disease-modulating properties of HU-444 using our mouse CIA model (Malfait et al., 2000; Sumariwalla et al., 2004). Administration of HU-444 systemically by i.p. or oral route ameliorated clinical signs of arthritis (Fig. 6). Treatments commenced from day 1 of arthritis, which is considered as the first day of the appearance of visible clinical signs of arthritis (redness/swelling) in any of the paws. The average day of onset of disease is about day 14–28 postimmunization. This period can, however, be variable between mice and experiments. As seen from Fig. 6, an inverse proportion dose curve was established with the highest dose of 10 mg/kg, daily being clinically nonbeneficial, whereas the two lower doses of 2.5 and 5.0 mg/kg showed beneficial clinical efficacy. Doses below 2.5 mg/kg were inefficient, indicating for the existence of an inverse bell-shaped curve, as is seen with CBD (Malfait et al., 2000) and a synthetic CBD derivative (Sumariwalla et al., 2004).

Clinical signs of arthritis were recorded daily as a composite clinical score of the four limbs, which was significantly reduced at 2.5 and 5.0 mg/kg over the entire 10-day period of HU-444 administration (Fig. 6A). In addition, hind paw thickness (mm) readings recorded as paw-swelling changes from day 1 of arthritis (Δ mm) showed significant reduction with 5 mg/kg, but not with 2.5 or 10 mg/kg (Fig. 6B). Only a modest reduction in paw swelling was recorded upon i.p. injection of vehicle from days 5 to 10 of CIA. More importantly, daily oral

<table>
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<th>Treatment (HU-444 mg/kg, i.p.)</th>
<th>Pathologic Score</th>
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<tr>
<td>Con A</td>
<td>3–4</td>
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<tr>
<td>Con A + 2.5</td>
<td>3</td>
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<tr>
<td>Con A + 5.0</td>
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TABLE 1
Concomitant treatment of mice with HU-444 confers protection against Con A–induced liver damage.
administration of HU-444 at 15 mg/kg to arthritic mice for 10 days significantly ameliorated the clinical score and paw-swelling profiles (Fig. 7). H&E staining of joint sections of mice treated i.p. with HU-444 (2.5 mg/kg) showed a significant reduction (36.7%) in damaged joints, with a concomitant increase (18.8%) in protected joints, when compared with a vehicle-treated group (Fig. 7A). Similarly, a 45.5% reduction in damaged joints and 48.9% increase in protected joints was seen upon oral treatment of arthritic mice with HU-444 (15 mg/kg), when compared with the vehicle-treated group (Fig. 7B). Image analysis of representative proximal phalanx joints from both i.p. and oral-treated mice showed preserved joint architecture when compared with vehicle-treated mice (Fig. 7, C and D). A nonarthritic joint from an immunized mouse, as well as an arthritic joint from an untreated CIA mouse, are shown alongside for comparison (Fig. 7E). However, despite the significant joint protection seen upon treatment with HU-444, some residual synovial hypertrophy remained.

Discussion

In the present study, we report the synthesis of a novel resorcinol derivative HU-444 (compound 5) that exhibits anti-inflammatory effects both in vitro and in vivo using mouse models of RA and inflammation-induced liver damage. RA is an autoimmune, chronic, debilitating musculoskeletal disease (Feldmann et al., 1995). Increasingly, RA is known to be associated with cardiovascular inflammatory diseases leading to enhanced mortality in patients (Full et al., 2009). Key cellular and molecular components associated with the onset and ongoing inflammatory processes in RA have been identified, although yet the specific antigen/environmental agent triggering the onset of the autoimmune pathways remains uncharacterized (Feldmann et al., 2010).

We have previously observed that CBD and its synthetic derivate HU-320 have potent anti-inflammatory effects and...
ameliorate the clinical signs of RA in a mouse model (Malfait et al., 2000; Sumariwalla et al., 2004). The anti-inflammatory disease-remitting activity of CBD and HU-320 led us to explore the potential of HU-444, a new, chemically related cannabinoid compound.

We decided to synthesize HU-444 to get a compound with enhanced anti-inflammatory activity that cannot be converted under acidic conditions to a psychoactive THC-like molecule. The synthesis uses CBD as the starting material. CBD is formed in the plant (or by heating) from its precursor...
Cannabis sativa varieties, including hemp, which is used for industrial purposes. In view of the growing interest in CBD (Porter and Jacobson, 2013), several C. sativa varieties have been developed that contain up to 20% CBD (or CBD acid). Hence, CBD is potentially an inexpensive natural product. The synthetic pathway from CBD to HU-444 is short, but the yield is relatively low. In view of the simplicity of the reactions used, the yields can presumably be increased, if needed.

Whereas in the past marijuana and hashish, the most widely used preparations of cannabis, contained CBD and the psychoactive δ-9-tetrahydrocannabinol (THC) in almost equal amounts (about 2–5%), many marijuana varieties today contain high levels of THC (15–20%), presumably due to mostly illegal commercial interests. These varieties contain almost no CBD, a constituent that does not cause the typical marijuana “high” and is hence apparently of minor interest to the illicit growers.

The in vitro assays used in the present study are well-established and widely used in anti-inflammatory research. They parallel to a large extent the assays previously used by us for the examination of the anti-inflammatory properties of CBD (Malfait et al., 2000) and HU-320 (Sumariwalla et al., 2004). We observed that HU-444 is a potent anti-inflammatory drug both in vitro and in vivo. As expected, HU-444 did not cause any of the typical THC-like pharmacological effects in mice.

We show that HU-444 suppresses in vitro the generation of ROS and NO by RAW mouse monocytes induced by zymosan and inhibits TNF-α production by TG macrophages induced by LPS. In vivo production of TNF-α elicited by LPS is also prevented by HU-444. These findings clearly show an immunosuppressive function of HU-444 on macrophage function. The most striking effect following administration of HU-444 was the amelioration of the Con A-mediated liver damage (Fig. 5). Administration of 5 mg/kg HU-444 restored to a considerable extent the normal histology of the liver. This is reflected in the reduced serum levels of the liver enzymes ALT and AST following treatment with HU-444. As Con A–induced hepatitis is considered to mimic autoimmune hepatitis in humans (Kravitt, 2006), the marked amelioration of hepatitis by HU-444 seen in our study suggests a promising curative therapeutic role for this cannabinoid compound in human autoimmune hepatitis.

In light of the encouraging anti-inflammatory properties of HU-444, we decided to explore the therapeutic potential of HU-444 in an animal model of arthritis. Mouse CIA remains a well-characterized and widely used model of RA (Williams, 1998, 2004). The CIA model is routinely used to evaluate the possible therapeutic potential of novel anti-inflammatory/disease-ameliorating compounds for use in RA (Williams, 2007). Indeed, anti-TNF-α, interleukin-1α, and other antibody-based biologics targeting key cellular and molecular targets had proven their merits originally in this model and then in clinical settings of RA (Taylor et al., 2001). However, most of these treatments are delivered by i.v. administration and are beyond the reach of many patients due to their cost factor. Thus, the search for easily prepared, small mol. wt. compounds, which can be delivered per os, continues. A hitherto mostly untapped wealth of novel compounds is natural products from plants, known to possess anti-inflammatory properties. Indeed, C. sativa preparations and pure cannabinoids have been used to relieve symptoms of pain associated with several clinical disease conditions and ailments (Marmor, 1998; Zurier, 2003). We show in this work that our semisynthetic compound HU-444 based on the naturally occurring CBD has profound anti-inflammatory effects that relieves paw swelling and arthritis symptoms in CIA, and may thus be a potent oral drug in the therapy of human RA.

**Conclusion.** Our results on the anti-inflammatory potential of HU-444 indicate that it lowers the levels of a variety of inflammatory mediators, such as TNF-α, ROS, and NO in vitro, and ameliorates arthritis in a mouse model at both the macroscopic and pathologic levels, which administered either i.p. or orally.

We believe that HU-444, a low mol. wt. compound, has the potential to be developed as a novel drug for use in inflammatory conditions, particularly in RA.

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**Authorship Contributions**

**Participated in research design:** Haj, Sumariwalla, Mechoulam, Feldmann, Gallily.

**Conducted experiments:** Haj, Sumariwalla, Hanus, Kogan, Yektin, Gallily.

**Performed data analysis:** Haj, Sumariwalla, Mechoulam, Feldmann, Gallily.

**Wrote or contributed to the writing of manuscript:** Haj, Sumariwalla, Mechoulam, Gallily.

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