

Ana Juknat, Ewa Kozela, Nathali Kaushansky, Raphael Mechoulam and Zvi Vogel*

Anti-inflammatory effects of the cannabidiol derivative dimethylheptyl-cannabidiol – studies in BV-2 microglia and encephalitogenic T cells

DOI 10.1515/jbcpp-2015-0071

Received June 18, 2015; accepted September 13, 2015; previously published online November 5, 2015

Abstract

Background: Dimethylheptyl-cannabidiol (DMH-CBD), a non-psychoactive, synthetic derivative of the phytocannabinoid cannabidiol (CBD), has been reported to be anti-inflammatory in RAW macrophages. Here, we evaluated the effects of DMH-CBD at the transcriptional level in BV-2 microglial cells as well as on the proliferation of encephalitogenic T cells.

Methods: BV-2 cells were pretreated with DMH-CBD, followed by stimulation with the endotoxin lipopolysaccharide (LPS). The expression levels of selected genes involved in stress regulation and inflammation were determined by quantitative real-time PCR. In addition, MOG_{35–55}-reactive T cells (T_{MOG}) were cultured with antigen-presenting cells in the presence of DMH-CBD and MOG_{35–55} peptide, and cell proliferation was determined by measuring [³H]thymidine incorporation.

Results: DMH-CBD treatment downregulated in a dose-dependent manner the mRNA expression of LPS-upregulated pro-inflammatory genes (*Il1b*, *Il6*, and *Tnf*) in BV-2 microglial cells. The expression of these genes was also downregulated by DMH-CBD in unstimulated cells. In parallel, DMH-CBD upregulated the expression of genes related to oxidative stress and glutathione homeostasis such as *Trb3*, *Slc7a11/xCT*, *Hmox1*, *Atf4*, *Chop*, and *p8* in both stimulated and unstimulated microglial

cells. In addition, DMH-CBD dose-dependently inhibited MOG_{35–55}-induced T_{MOG} proliferation.

Conclusions: The results show that DMH-CBD has similar anti-inflammatory properties to those of CBD. DMH-CBD downregulates the expression of inflammatory cytokines and protects the microglial cells by inducing an adaptive cellular response against inflammatory stimuli and oxidative injury. In addition, DMH-CBD decreases the proliferation of pathogenic activated T_{MOG} cells.

Keywords: anti-inflammation; cannabidiol; cannabinoids; cystine/glutamate transporter; dimethylheptyl-cannabidiol; gene expression; glutathione; microglia; oxidative stress; proliferation; T cells.

To the memory of Prof. Itai Bab.

Introduction

Preparations derived from *Cannabis sativa* (marijuana and hashish) have become widespread since ancient times, both as therapeutic agents and in recreational smoking [1–3]. Among the more than 60 phytocannabinoids identified in *Cannabis* extracts, the two most abundant are Δ^9 -tetrahydrocannabinol (THC), the major psychotropic constituent, and cannabidiol (CBD), the major non-psychoactive component. Cannabinoids were shown to exert a wide range of therapeutic effects, and many of the cannabinoids, especially CBD, were shown to possess potent anti-inflammatory and immunomodulatory activities [4–15]. In addition, it was shown that several cannabinoids have pro-apoptotic, neuroprotective, and antitumor properties [16, 17].

The biological activities of cannabinoids are mostly mediated through cannabinoid receptors, the CB₁ (primarily, but not exclusively, expressed in the central nervous system) and the CB₂ (mainly expressed in immune cells) [18, 19, and references therein]. However, cannabinoids were also shown to act via mechanisms independent of CB₁ and CB₂. For example, CBD has very low affinity for both CB₁ and CB₂ receptors and is therefore devoid of the psychotropic effects produced by *Cannabis* or THC that are

*Corresponding author: Prof. Zvi Vogel, The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, Phone: +972 54 550 8893, Fax: +972 8 934 4131, E-mail: zvi.vogel@weizmann.ac.il

Ana Juknat and Ewa Kozela: The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; and Weizmann Institute of Science, Neurobiology Department, Rehovot, Israel

Nathali Kaushansky: Weizmann Institute of Science, Neurobiology Department, Rehovot, Israel

Raphael Mechoulam: Institute for Drug Research, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem, Israel

mediated via CB₁ [4, 20]. Therefore, the anti-inflammatory activities of CBD do not seem to be mediated via either CB₁ or CB₂ [21 and references therein].

Several groups including ours have shown that various microglial cell functions are affected by cannabinoids, including CBD. For example, our group reported that THC and CBD differentially inhibit the lipopolysaccharide (LPS)-activated nuclear factor κ B (NF- κ B) and interferon β /signal transducers and activators of transcription (IFN- β /STAT) pro-inflammatory pathways in a CB₁/CB₂-independent manner [7] and that CBD affects the expression of genes related to oxidative stress and glutathione (GSH) deprivation via the general control non-derepressible 2 (GCN2)/eukaryotic initiation factor 2 α (eIF2 α)/p8/ATF4/CHOP-tribbles homologue 3 (Trb3) pathway [11, 12]. In addition, CBD-stimulated genes were shown to be controlled by nuclear factors known to be involved in the regulation of stress response and inflammation, mainly through the [electrophile response element (EpRE)/antioxidant response element (ARE)]-nuclear factor-erythroid 2-related factor 2 (Nrf2)/ATF4 system and the Nrf2/Hmox1 axis [11, 12].

CBD was also shown to alleviate the pathological symptoms in a demyelinating disease, the experimental autoimmune encephalomyelitis (EAE), commonly used as a model of multiple sclerosis (MS). Our group reported that CBD ameliorated the severity of EAE in myelin oligodendrocyte glycoprotein 35–55 (MOG_{35–55})-immunized mice and attenuated microglial activation and T-cell recruitment in these CBD-treated, MOG_{35–55}-induced EAE mice [8]. Moreover, we were able to show that CBD inhibited the *in vitro* MOG-induced proliferation of MOG_{35–55}-reactive T cells (T_{MOG}) cells and markedly reduced their Th17 inflammatory phenotype [8, 9].

Several CBD derivatives were also shown to have anti-inflammatory and anti-proliferative properties [22, 23]. In view of the therapeutic potential of CBD, we were interested in looking into the activity of its synthetic non-psychoactive derivative, the dimethylheptyl-cannabidiol (DMH-CBD). DMH-CBD has been reported to induce apoptosis of human acute myeloid leukemia HL-60 cells [24] and was reported to inhibit the production of reactive oxygen intermediates, nitric oxide, and tumor necrosis factor alpha (TNF α) in LPS-activated RAW macrophages [25]. In the present work, we studied the transcriptional effects mediated by DMH-CBD within genes involved in the regulation of stress response and inflammation in LPS-activated BV-2 microglial cells. In addition, we studied the effects of DMH-CBD on the proliferation of MOG_{35–55}-activated T_{MOG} cells. The results show that DMH-CBD induces similar anti-inflammatory, anti-proliferative, and stress response effects to those previously observed for CBD.

Materials and methods

Reagents

LPS (*Escherichia coli* serotype O55:B5) was obtained from Sigma (St. Louis, MO, USA). LPS was dissolved in water, stock solutions (1 mg/mL) were stored in aliquots at -20°C and diluted in culture medium before experiments. Lyophilized MOG_{35–55} peptide (MEVGVYRSPFSRVVHLYRNGK) purchased from GenScript (Piscataway, NJ, USA) was reconstituted in sterile phosphate buffered solution (PBS) and the stock solution stored in aliquots at -20°C . (–)-5'-DMH-CBD was synthesized as previously described [26]. Stock solution of DMH-CBD was prepared in ethanol and diluted into culture medium before experiments. The final concentrations of ethanol in the experiments did not exceed 0.1%–0.2% and had no effect on the results. Fetal calf serum and other tissue culture medium and reagents were obtained from Biological Industries (Kibbutz Beit Haemek, Israel).

Microglial cell culture

The BV-2 mouse microglial cell line was kindly provided by Prof. E. J. Choi from Korea University (Seoul, South Korea). Cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose and 1 mM sodium pyruvate and supplemented with 5% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL) under a humidified 5% CO₂ atmosphere at 37 $^{\circ}\text{C}$. Cells (1×10^6 cells in 100-mm plates) were pretreated for 2 h with DMH-CBD, at the doses indicated in the text, followed by addition of LPS (100 ng/mL) for another 4 h.

RNA extraction and quantitative real-time PCR (qPCR)

Extraction, quantification, and quality of extracted RNA were performed and analyzed as described previously [11]. copy DNA (cDNA) was synthesized using the QuantiTect Reverse Transcription kit according to the manufacturer's instructions (Qiagen AG, Basel, Switzerland). qPCR was carried out as detailed previously [11]. Expression levels of genes were normalized to the reference gene β_2 -microglobulin (*B2m*), whose expression has been found not to be affected by the treatments and are expressed as fold change as reported earlier [11]. Primer sets were designed using the PrimerQuest, an online tool provided by Integrated DNA Technologies (<http://eu.idtdna.com/Primerquest/Home/Index>) and synthesized by Metabion International (Planegg-Steinkirchen, Germany). GenBank™ accession numbers for the different genes and the primer sequences used for amplification were reported earlier [9, 11]. The qPCR runnings were repeated three times using mRNA preparations from three independent experiments.

Encephalitogenic T-cell proliferation

T_{MOG} raised from MOG_{35–55} immunized mice was obtained and maintained as previously described [8, 9, 27]. To assay the effect of DMH-CBD on T_{MOG} proliferation, T_{MOG} cells were cultured in 96-well plates (1.25×10^4 cells/well) together with splenic antigen-presenting

cells (APCs; 5×10^5 cells/well). APCs were irradiated (25 Gy) before co-culturing to prevent them from proliferation. The co-cultured cells ($T_{MOG}/APCs$) were maintained in 0.2 mL of RPMI medium containing 2.5% fetal calf serum, supplemented with streptomycin (100 $\mu\text{g}/\text{mL}$), penicillin (100 U/mL), 2 mM L-glutamine, and 50 μM β -mercaptoethanol. DMH-CBD at final doses of 0.1, 1, 5, and 10 μM was added to the cells together with the MOG₃₅₋₅₅ peptide at either 1 or 2.5 $\mu\text{g}/\text{mL}$. The cells were then incubated for 72 h at 37 °C in 5% CO₂-humidified air. Cell proliferation was measured by pulsing the cells with [³H]thymidine (0.5 $\mu\text{Ci}/\text{well}$; Perkin Elmer, Boston, MA, USA) for the last 16 h of the incubation period, and the cells were then harvested and counted using a Matrix 96 Direct β -counter (Packard Instruments, Meriden, CT, USA). The proliferative responses of the T_{MOG} cells in each well were converted to percentage values with MOG₃₅₋₅₅ effect expressed as 100%. Percentage values are calculated from the stimulation index data, which are the fold changes in mean counts per minutes (cpm) of MOG₃₅₋₅₅ cultures over mean cpm of cultures without MOG (spontaneous proliferation). Statistical analysis was performed on percentage values [8, 27].

Statistical analysis

Data are expressed as the mean \pm SEM of three independent experiments and analyzed for statistical significance using Student's t-test or one-way analysis of variance (ANOVA), followed by Dunnett's or Newman-Keul's post hoc test. $p < 0.05$ was considered significant. GraphPad Prism 5 software (La Jolla, CA, USA) was used for statistical analysis of the data.

Results

DMH-CBD treatment downregulates mRNA expression of LPS-upregulated pro-inflammatory genes in BV-2 cells

BV-2 cells were pretreated for 2 h with DMH-CBD (1, 5, and 10 μM) and then stimulated with LPS (100 ng/mL) for an additional 4 h. The control treatment with DMH-CBD alone lasted for 6 h and with LPS alone for 4 h. These treatment time points were chosen following our previous reports [7, 11]. The cells were then collected, mRNA extracted, and the expression of several genes determined by qPCR. qPCR analysis showed strong upregulation in the mRNA levels of *Il1b*, *Il6*, and *Tnf* following LPS stimulation of microglial cells (Figure 1A–C). Treatment with DMH-CBD significantly decreased, in a dose-dependent manner, the LPS-upregulated expression of *Il1b* mRNA [by 88% at 10 μM DMH-CBD; ANOVA $F(4,10)=187.9$, $p < 0.001$] and of *Il6* [by 82% at 10 μM DMH-CBD; ANOVA $F(4,10)=9.97$, $p < 0.01$] (Figure 1A,B). The level of LPS-upregulated *Tnf* mRNA was also decreased by DMH-CBD, reaching a 48% reduction at

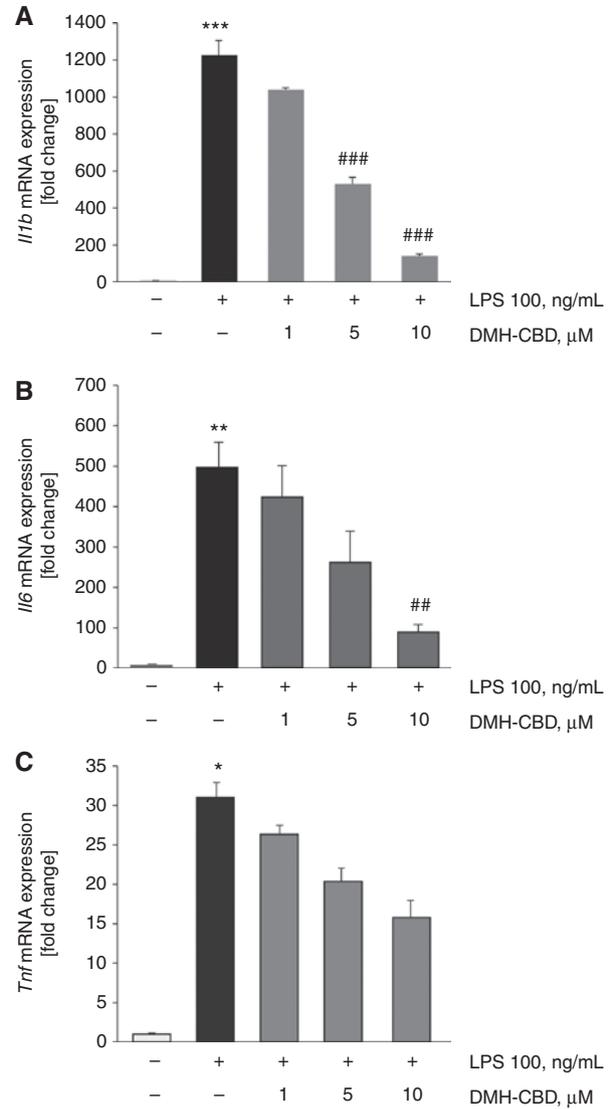


Figure 1: DMH-CBD downregulates the levels of LPS-upregulated *Il1b*, *Il6*, and *Tnf* mRNAs in BV-2 microglial cells.

Cells were treated for 2 h with the indicated concentrations of DMH-CBD and then activated for 4 h with 100 ng/mL LPS. Cells were harvested and mRNA was extracted for qPCR analysis. qPCR data were plotted as the mean \pm SEM of three to four independent experiments. Statistical significance was assessed using one-way ANOVA followed by Newman-Keul's post hoc test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control; ## $p < 0.01$ and ### $p < 0.001$ vs. LPS.

10 μM DMH-CBD, a result that unexpectedly was found to be not statistically significant [ANOVA $F(4,10)=4.6$, $p < 0.05$] (Figure 1C). As shown in Figure 2, DMH-CBD by itself (without LPS) also reduced the basal transcript levels of *Il1b*, *Il6*, and *Tnf* in a dose-dependent manner. At a dose of 10 μM , DMH-CBD decreased the expression of *Il1b* mRNA by 82% [ANOVA $F(3,18)=27.2$, $p < 0.001$], *Il6* by 67% [ANOVA $F(3,18)=14.4$, $p < 0.01$, and *Tnf* by 66% [ANOVA $F(3,18)=43.4$, $p < 0.001$].

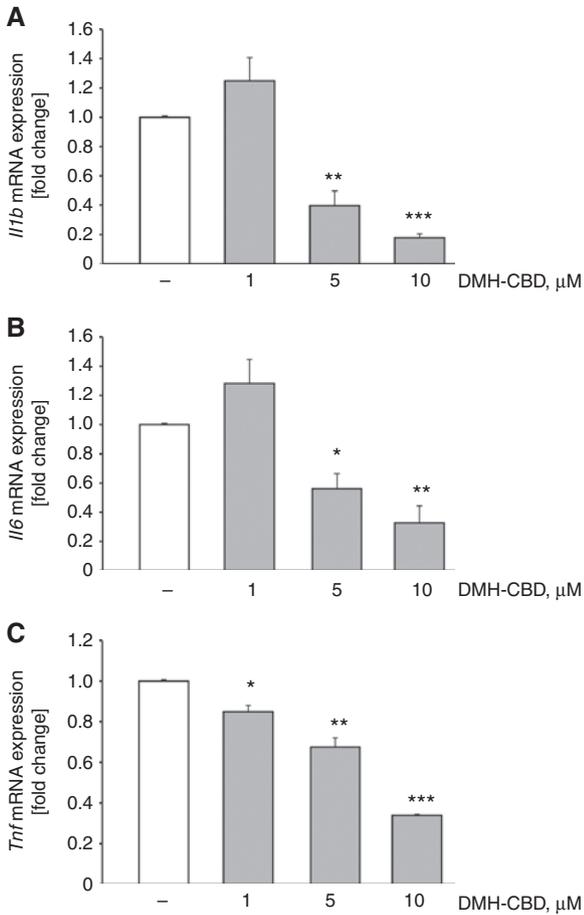


Figure 2: DMH-CBD downregulates the basal levels of *Il1b*, *Il6*, and *Tnf* mRNAs.

Conditions were as in Figure 1, except that no LPS was added. qPCR data were plotted as the mean±SEM of three to four independent experiments. Statistical significance was assessed using one-way ANOVA followed by Newman-Keul's post hoc test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control.

DMH-CBD treatment upregulates mRNA expression of genes related to oxidative stress and GSH homeostasis in BV-2 cells

qPCR analysis demonstrated that incubation of BV-2 cells with DMH-CBD (at 10 μM) leads to upregulation of several genes related to stress and GSH homeostasis (Figure 3A–F). These included *tribbles homologue 3* [*Trb3*; by 10-fold; ANOVA $F(3,8)=94.1$, $p < 0.001$], *solute carrier family 7, member 11* [*cystine/glutamate transporter subunit Slc7a11/xCT*; by 5.4-fold; ANOVA $F(3,12)=132.4$, $p < 0.001$], and *heme oxygenase 1* [*Hmox1*; by 4-fold; ANOVA $F(3,8)=49.1$, $p < 0.001$]. DMH-CBD also increased the expression of several other genes, but to a significantly lower extent, such as the *activating transcription*

factor 4 [*Atf4*; by 60%; ANOVA $F(3,8)=60.3$, $p < 0.001$], *C/EBP homologous protein* [*Chop/Ddit3/Gadd153*; by 50%; ANOVA $F(3,8)=49.1$, $p < 0.001$], and *nuclear protein 1* [*Nupr1/p8*; by 36%; ANOVA $F(3,8)=240.1$, $p < 0.001$]. The expressions of *Trb3*, *Hmox1*, and *Atf4* mRNAs were not affected by LPS. Meanwhile, *Slc7a11/xCT* mRNA (Figure 3B) was highly upregulated by LPS (by 8.2-fold, $p < 0.05$) and synergistically upregulated by the combined DMH-CBD+LPS treatment (by 34-fold vs. control, $p < 0.001$, and by 3.7-fold vs. LPS, $p < 0.001$). *p8* expression was upregulated by LPS by 3.4-fold ($p < 0.01$) and by the combination of DMH-CBD+LPS by 4.8-fold vs. control ($p < 0.01$) and by 40% vs. LPS ($p < 0.01$) (Figure 3F). In contrast, *Chop* mRNA was slightly downregulated by LPS (by 30%, $p < 0.01$) (Figure 3E).

DMH-CBD inhibits MOG_{35-55} -induced T_{MOG} cell proliferation

We previously showed that CBD ameliorates clinical symptoms of MOG_{35-55} -induced EAE [8]. Moreover, we showed that CBD inhibits the MOG_{35-55} -induced proliferation of T_{MOG} cells co-cultured with irradiated APCs in the presence of MOG_{35-55} [8]. Therefore, we have analyzed the effect of DMH-CBD in this in vitro model of MOG_{35-55} -stimulated encephalitogenic T cells. T_{MOG} /APCs were co-cultured in the presence of MOG_{35-55} at a dose of 1 or 2.5 $\mu\text{g}/\text{mL}$. These concentrations were chosen following our previous reports [8, 9]. T-cell proliferation was analyzed using [^3H]thymidine incorporation as previously reported [8]. Incubation with 1 $\mu\text{g}/\text{mL}$ MOG_{35-55} resulted in a 24 ± 11 -fold increase ($p < 0.05$, Student's t-test) in T_{MOG} proliferation and incubation with 2.5 $\mu\text{g}/\text{mL}$ MOG_{35-55} produced a 42 ± 10 -fold increase ($p < 0.01$, Student's t-test) in T_{MOG} proliferation vs. non-stimulated cells (Figure 4A). Results presented in Figure 4B,C shows the inhibitory effect of DMH-CBD at various doses (0.1–10 μM) on the MOG_{35-55} -induced proliferation of T_{MOG} cells stimulated by either 1 or 2.5 $\mu\text{g}/\text{mL}$ of MOG_{35-55} . As shown in Figure 4B, addition of 5 μM DMH-CBD to 1 $\mu\text{g}/\text{mL}$ MOG_{35-55} -stimulated T_{MOG} /APCs resulted in inhibition of T_{MOG} proliferation by 51%, whereas 10 μM DMH-CBD inhibited T_{MOG} proliferation by 97% ($p < 0.01$). Similar results were observed when T_{MOG} cells were stimulated with 2.5 $\mu\text{g}/\text{mL}$ of MOG_{35-55} (Figure 4C). In this case, 5 μM DMH-CBD inhibited the proliferation by 62% ($p < 0.01$) and 10 μM DMH-CBD by 97% ($p < 0.001$). Lower doses of DMH-CBD (e.g. 0.1 and 1 μM) had no significant effect on T_{MOG} proliferation induced by MOG_{35-55} at either 1 or 2.5 $\mu\text{g}/\text{mL}$.

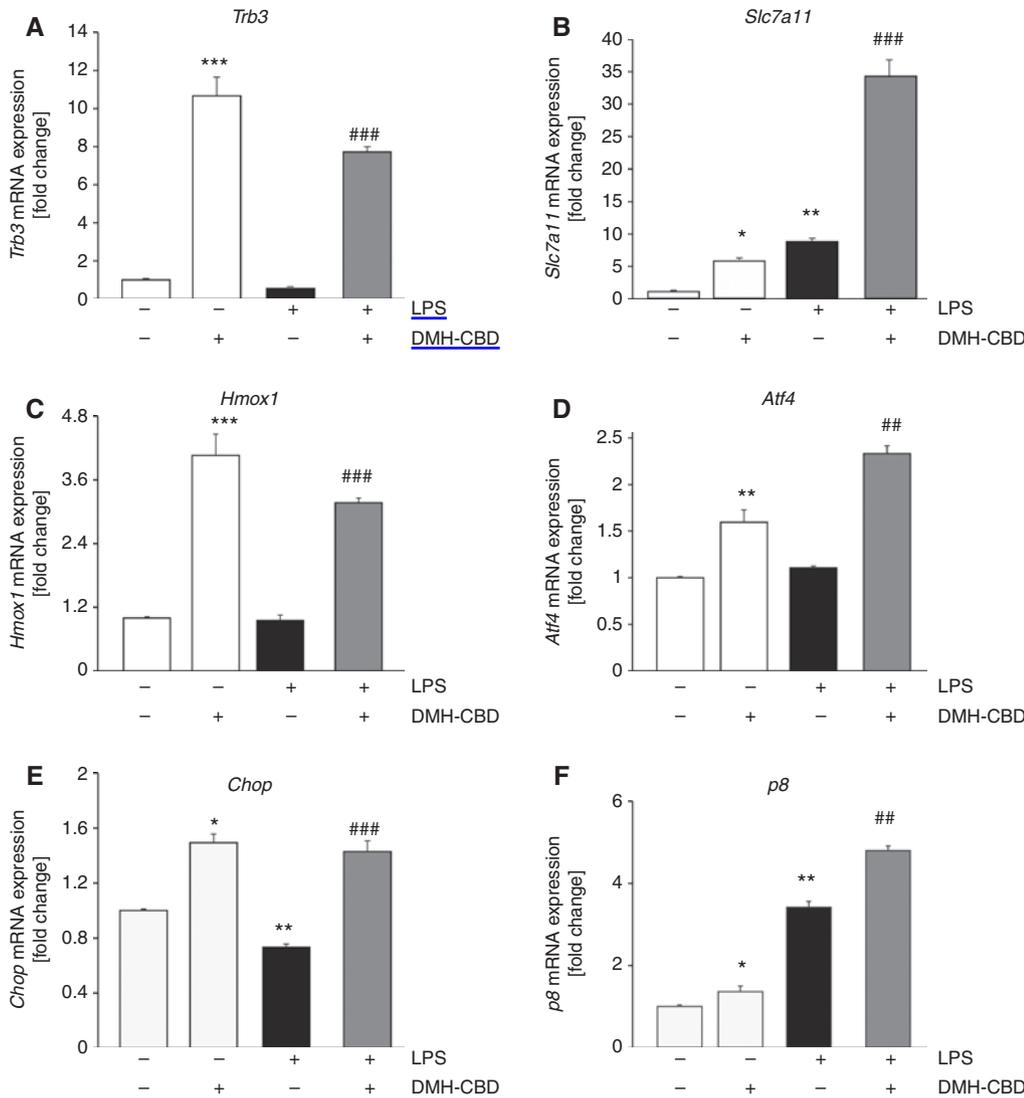


Figure 3: DMH-CBD treatment upregulates mRNA expression of genes related to oxidative stress and GSH homeostasis in BV-2 microglial cells.

Cells were incubated with 10 μ M DMH-CBD and 100 ng/mL LPS as indicated. qPCR data were plotted as the mean \pm SEM of three to four independent experiments. Statistical significance was assessed using one-way ANOVA followed by Newman-Keul's post hoc test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control; ## $p < 0.01$ and ### $p < 0.001$ vs. LPS.

Discussion

The present study describes the effects of DMH-CBD on inflammatory and stress-related genes in BV-2 microglial cells and on the proliferation of MOG₃₅₋₅₅-activated T_{MOG} cells.

We found that DMH-CBD downregulates in a dose-dependent manner the mRNA expression of LPS-upregulated *Il1b* and *Il6* transcripts. Moreover, DMH-CBD dose dependently downregulates the expression of these transcripts in unstimulated BV-2 cells. These findings show that DMH-CBD possesses anti-inflammatory and protective properties. DMH-CBD effects are similar to those

found for CBD in our previous work [7, 11, 13] where we reported that CBD decreases the production and release of the pro-inflammatory cytokines IL-1 β and IL-6, but not of TNF α in LPS-stimulated BV-2 microglial cells [7, 13].

In addition, we show here that DMH-CBD strongly upregulates the expression of the genes *Trb3*, *Slc7a11/xCT*, and *Hmox1* and, to a lesser extent, of the genes *Atf4*, *Chop/Ddit3/Gadd153*, and *p8/Nupr1*. Previous work from our laboratory showed that these genes were also significantly upregulated by CBD [11]. Most of these genes (*Trb3*, *Slc7a11/xCT*, *Atf4*, *Chop/Ddit3/Gadd153*, and *p8/Nupr1*) are known to contain amino acid response elements and to be regulated in response to amino acid deprivation [28]. The

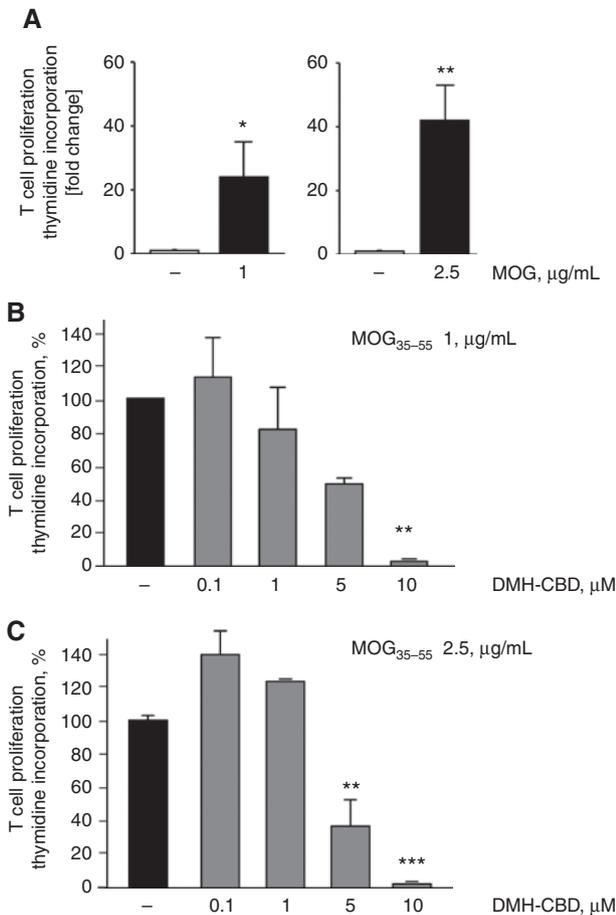


Figure 4: DMH-CBD inhibits MOG₃₅₋₅₅-induced T_{MOG} cell proliferation. T_{MOG}/APC co-cultures were exposed to MOG₃₅₋₅₅ (1 or 2.5 µg/mL), and T_{MOG} proliferation was determined by [³H]thymidine incorporation assay in the presence or absence of DMH-CBD at the indicated concentrations. Each experiment (n=2) was carried out in triplicate. (A) Effect of MOG₃₅₋₅₅ stimulation on T_{MOG} proliferation expressed as fold change of mean values±SEM, vs. non-stimulated cells (*p<0.05 and **p<0.01, Student's t-test). (B, C) Effect of various concentrations of DMH-CBD on T_{MOG} proliferation expressed as mean percentage values±SEM. The MOG₃₅₋₅₅ effect (in the absence of DMH-CBD) is expressed as 100%. (B) One-way ANOVA F(4,5)=18.2, p<0.01; (C) one-way ANOVA F(4,5)=52.2, p<0.001; Dunnett's post hoc test: **p<0.01 and ***p<0.001 vs. MOG₃₅₋₅₅-treated cells.

upregulation of these amino acid-response genes suggests an effect of DMH-CBD on GCN2/eIF2 α /ATF4-mediated stress pathway [29–31]. These results demonstrate that DMH-CBD is inducing a metabolic adaptation program typical for nutrient limiting conditions, characterized by repression of protein synthesis and upregulation of amino acid biosynthesis and transporter genes [32].

Another gene upregulated by DMH-CBD is *Nupr1/p8* (36%). *Nupr1/p8* was found to be induced by ATF4 in response to various cellular stressors, and its expression was associated with enhanced transcriptional activation

of genes downstream of ATF4 (such as *Chop* and *Trb3*). This suggests that *p8* promotes the transcription of stress-regulated genes via a positive feedback on ATF4 pathway [33]. Other cannabinoids like CBD, THC, and the synthetic WIN 55,212-2 were also shown to induce *p8* expression [11, 34, 35]. In addition, our results show upregulation of *p8* mRNA expression by LPS, an effect also shown by others [36], and by the combined treatment of DMH-CBD+LPS.

As described above, DMH-CBD highly upregulated the expression of the mRNA of the transporter *Slc7a11/xCT* and of the antioxidant inducible enzyme *Hmox1*, both known to be modulated in response to oxidative stress via the ARE/EpRE-Nrf2 system [37]. The *Slc7a11/xCT* constitutes the stress inducible subunit of the cystine/glutamate transport system x_c⁻ [38, 39] whose activation will produce an increase in the levels of GSH in the cells [40]. In addition, we show that LPS strongly upregulates *Slc7a11/xCT* mRNA expression and that the effect of LPS and DMH-CBD applied together on *Slc7a11/xCT* expression is synergistic. This result is in agreement with Sato et al. [41], who reported that LPS induces the activity of the cystine/glutamate transporter in macrophages, increasing GSH levels in the cells. Moreover, they showed partial synergism when macrophages were incubated with LPS together with TNF α .

Redox homeostasis was reported to modulate the microglia and macrophages M1/M2 polarization [42]. In this regard, it was shown that *Hmox1* induction drives a shift to M2 macrophage phenotype, characterized by its intracellular redox status, consisting of high expression of scavenging molecules and GSH [42, 43]. Thus, upregulation of *Slc7a11/xCT* and *Hmox1* by DMH-CBD may serve to protect the cells by inducing an adaptive cellular response against inflammatory stimuli and oxidative injury, utilizing GSH as antioxidant or conjugating/detoxifying agent.

In the present study, we also report that DMH-CBD significantly decreases MOG₃₅₋₅₅-induced T_{MOG} cell proliferation. This result is in agreement with our previous work, which shows that CBD inhibits the MOG₃₅₋₅₅-induced proliferation of T_{MOG} cells stimulated with MOG₃₅₋₅₅ [8]. Cannabinoids, including CBD, have been shown to possess anti-proliferative properties in highly proliferative cell lines, including transformed T-cell lines [44–46]. For example, CBD inhibits proliferation by inducing cell death in cancer cell lines via the transient receptor potential vanilloid channel 2 [47, 48] and THC reduces proliferation and leads to cell death of C6 glioma cells via CB₁ receptor [49]. As for DMH-CBD, it was shown that DMH-CBD (as well as CBD) in combination with γ -irradiation induces apoptotic cell death of HL-60 myeloblastic leukemia cells [24]. Here, we show that DMH-CBD in similarity to

previous results with CBD [8, 9, 50] can prevent the proliferation of primary T cells.

Altogether, our results show that DMH-CBD, a non-psychoactive, synthetic CBD derivative, has anti-inflammatory and anti-proliferative properties. DMH-CBD induces the expression of genes related to oxidative stress and amino acid deprivation, genes that are controlled by Nrf2 and ATF4 transcription factors. In addition, DMH-CBD inhibits the proliferation of MOG_{35–55}-activated T_{MOG} cells. In similarity to CBD, DMH-CBD could be of high therapeutic value in neuro-inflammatory diseases and related syndromes.

Acknowledgments: A.J. is supported by the Israeli Ministry for Absorption in Science.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported by the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

- Kogan NM, Mechoulam R. Cannabinoids in health and disease. *Dialogues Clin Neurosci* 2007;9:413–30.
- Pertwee RG. Emerging strategies for exploiting cannabinoid receptor agonists as medicines. *Br J Pharmacol* 2009;156:397–411.
- Mechoulam R, Hanuš LO, Pertwee R, Howlett AC. Early phytocannabinoid chemistry to endocannabinoids and beyond. *Nat Rev Neurosci* 2014;15:757–64.
- Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. [Non-psychoactive plant cannabinoids: new therapeutic opportunities from an ancient herb.](#) *Trends Pharmacol Sci* 2009;30:515–27.
- Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. *Future Med Chem* 2009;1:1333–49.
- Tanasescu R, Constantinescu CS. Cannabinoids and the immune system: an overview. *Immunobiology* 2010;215:588–97.
- Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z. Cannabinoids Δ^9 -tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF- κ B and interferon- β /STAT proinflammatory pathways in BV-2 microglial cells. *J Biol Chem* 2010;285:1616–26.
- Kozela E, Lev N, Kaushansky N, Eilam R, Rimmerman N, Levy R, et al. Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice. *Br J Pharmacol* 2011;163:1507–19.
- Kozela E, Juknat A, Kaushansky N, Rimmerman N, Ben-Nun A, Vogel Z. Cannabinoids decrease the Th17 inflammatory autoimmune phenotype. *J Neuroimmune Pharmacol* 2013;8:1265–76.
- Kozela E, Juknat A, Kaushansky N, Ben-Nun A, Coppola G, Vogel Z. Cannabidiol, a non-psychoactive cannabinoid, leads to EGR2-dependent anergy in activated encephalitogenic T cells. *J Neuroinflammation* 2015;12:52.
- Juknat A, Pietr M, Kozela E, Rimmerman N, Levy R, Coppola G, et al. Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ^9 -tetrahydrocannabinol in BV-2 microglial cells. *Br J Pharmacol* 2012;165:2512–28.
- Juknat A, Rimmerman N, Levy R, Vogel Z, Kozela E. Cannabidiol affects the expression of genes involved in zinc homeostasis in BV-2 microglial cells. *Neurochem Int* 2012;61:923–30.
- Juknat A, Pietr M, Kozela E, Rimmerman N, Levy R, Gao F, et al. Microarray and pathway analysis reveal distinct mechanisms underlying cannabinoid-mediated modulation of LPS-induced activation of BV-2 microglial cells. *PLoS One* 2013;8:e61462.
- Cabral GA, Jamerson M. Marijuana use and brain immune mechanisms. *Int Rev Neurobiol* 2014;118:199–230.
- Chiurchiù V, Leuti A, Maccarrone M. Cannabinoid signaling and neuroinflammatory diseases: a melting pot for the regulation of brain immune responses. *J Neuroimmune Pharmacol* 2015;10:268–80.
- Massi P, Valenti M, Solinas M, Parolaro D. Molecular mechanisms involved in the antitumor activity of cannabinoids on gliomas: role for oxidative stress. *Cancers* 2010;2:1013–26.
- Sánchez AJ, García-Merino A. Neuroprotective agents: cannabinoids. *Clin Immunol* 2012;142:57–67.
- Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, et al. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB₁ and CB₂. *Pharmacol Rev* 2010;62:588–631.
- Basu S, Dittel BN. Unraveling the complexities of cannabinoid receptor 2 immune regulation in health and disease. *Immunol Res* 2011;51:26–38.
- Pertwee RG. Pharmacological actions of cannabinoids. *Handb Exp Pharmacol* 2005;168:1–51.
- Rimmerman N, Kozela E, Levy R, Vogel Z, Juknat A. Cannabinoid signaling through non-CB₁R/non-CB₂R targets in microglia. In: Abood ME, Sorensen RG, Stella N, editors. *Endocannabinoids: actions at non-CB₁/CB₂ cannabinoid receptors*. New York: Springer, 2013:143–71.
- Xiong W, Cui T, Cheng K, Yang F, Chen SR, Willenbring D, et al. Cannabinoids suppress inflammatory and neuropathic pain by targeting $\alpha 3$ glycine receptors. *J Exp Med* 2012;209:1121–34.
- Kozela E, Haj C, Hanuš L, Shurki A, Chourasia M, Juknat A, et al. HU-446 and HU-465, derivatives of the non-psychoactive cannabinoid cannabidiol, decrease the activation of encephalitogenic T cells. *Chem Biol Drug Des* 2015. DOI: 10.1111/cbdd.12637 [Epub ahead of print].
- Gallily R, Even-Chen T, Katzavian G, Lehmann D, Dagan A, Mechoulam R. γ -Irradiation enhances apoptosis induced by cannabidiol, a non-psychoactive cannabinoid, in cultured HL-60 myeloblastic leukemia cells. *Leuk Lymphoma* 2003;44:1767–73.
- Ben-Shabat S, Hanuš LO, Katzavian G, Gallily R. New cannabidiol derivatives: synthesis, binding to cannabinoid receptor

- and evaluation of their anti-inflammatory activity. *J Med Chem* 2006;49:1113–7.
26. Baek SH, Srebnik M, Mechoulam R. Borontrifluoride on alumina – a modified Lewis acid reagent. An improved synthesis of cannabidiol. *Tetrahedron Lett* 1985;26:1083–86.
 27. Kaushansky N, Zhong MC, Kerlero de Rosbo N, Hoeflberger R, Lassmann H, Ben-Nun A. Epitope specificity of autoreactive T and B cells associated with experimental autoimmune encephalomyelitis and optic neuritis induced by oligodendrocyte-specific protein in SJL/J mice. *J Immunol* 2006;15:7364–76.
 28. Lee J-I, Dominy JE Jr, Sikalidis AK, Hirschberger LL, Wang W, Stipanuk MH. HepG2/C3A cells respond to cysteine deprivation by induction of the amino acid deprivation/integrated stress response pathway. *Physiol Genomics* 2008;33:218–29.
 29. Chaveroux C, Lambert-Langlais S, Cherasse Y, Averous J, Parry L, Carraro V, et al. Molecular mechanisms involved in the adaptation to amino acid limitation in mammals. *Biochimie* 2010;92:736–45.
 30. Averous J, Lambert-Langlais S, Cherasse Y, Carraro V, Parry L, B'chir W, et al. Amino acid deprivation regulates the stress-inducible gene p8 via the GCN2/ATF4 pathway. *Biochem Biophys Res Commun* 2011;413:24–29.
 31. Kilberg MS, Balasubramanian M, Fu L, Shan J. The transcription factor network associated with the amino acid response in mammalian cells. *Adv Nutr* 2012;3:295–306.
 32. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. *Annu Rev Nutr* 2005;25:59–85.
 33. Jin H-O, Seo S-K, Woo S-H, Choe T-B, Hong S-I, Kim J-I, et al. Nuclear protein 1 induced ATF4 in response to various stressors acts as a positive regulator on the transcriptional activation of ATF4. *IUBMB Life* 2009;61:1153–8.
 34. Carracedo A, Lorente M, Egia A, Blázquez C, García S, Giroux V, et al. The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. *Cancer Cell* 2006;9:301–12.
 35. Pellerito O, Calvaruso G, Portanova P, De Blasio A, Santulli A, Vento R, et al. The synthetic cannabinoid WIN 55,212-2 sensitizes hepatocellular carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by activating p8/CCAAT/enhancer binding protein homologous protein (CHOP)/death receptor 5 (DR5) axis. *Mol Pharmacol* 2010;77:854–63.
 36. Jiang Y-F, Vaccaro MI, Fiedler F, Calvo EL, Iovanna JL. Lipopolysaccharides induce p8 mRNA expression in vivo and in vitro. *Biochem Biophys Res Commun* 1999;260:686–90.
 37. Sasaki H, Sato H, Kuriyama-Matsumura K, Sato K, Maebara K, Wang H, et al. Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J Biol Chem* 2002;277:44765–71.
 38. Lewerenz J, Hewet SJ, Huang Y, Kambros M, Gout PW, Kalivas PW, et al. The cystine/glutamate antiporter system x_c⁻ in health and disease: from molecular mechanisms to novel therapeutic opportunities. *Antioxid Redox Signal* 2013;18:522–55.
 39. Ishii T, Mann GE. Redox status in mammalian cells and stem cells during culture in vitro: critical roles of Nrf2 and cystine transporter activity in the maintenance of redox balance. *Redox Biology* 2014;2:786–94.
 40. Lu SC. Glutathione synthesis. *Biochim Biophys Acta* 2013;1830:3143–53.
 41. Sato H, Fujiwara K, Sagara J, Bannai S. Induction of cystine transport activity in mouse peritoneal macrophages by bacterial lipopolysaccharide. *Biochem J* 1995;310:547–51.
 42. Rojo AI, McBean G, Cindric M, Egea J, Lopez MG, Rada P, et al. Redox control of microglial function: molecular mechanisms and functional significance. *Antioxid Redox Signal* 2014;21:1766–801.
 43. Naito Y, Takagi T, Higashimura Y. Heme oxygenase-1 and anti-inflammatory M2 macrophages. *Arch Biochem Biophys* 2014;564:83–8.
 44. McKallip RJ, Jia W, Schlomer J, Warren JW, Nagarkatti PS, Nagarkatti M. Cannabidiol-induced apoptosis in human leukemia cells: a novel role of cannabidiol in the regulation of p22phox and Nox4 expression. *Mol Pharmacol* 2006;70:897–908.
 45. Lee CY, Wey SP, Liao MH, Hsu WL, Wu HY, Jan TR. A comparative study on cannabidiol-induced apoptosis in murine thymocytes and EL-4 thymoma cells. *Int Immunopharmacol* 2008;8:732–40.
 46. McAllister SD, Soroceanu L, Desprez PY. The antitumor activity of plant-derived non-psychoactive cannabinoids. *J Neuroimmune Pharmacol* 2015;10:255–67.
 47. Morelli MB, Offidani M, Alesiani F, Discepoli G, Liberati S, Olivieri A, et al. The effects of cannabidiol and its synergism with bortezomib in multiple myeloma cell lines. A role for transient receptor potential vanilloid type-2. *Int J Cancer* 2014;134:2534–46.
 48. Nabissi M, Morelli MB, Amantini C, Liberati S, Santoni M, Ricci-Vitiani L, et al. Cannabidiol stimulates Aml-1a-dependent glial differentiation and inhibits glioma stem-like cells proliferation by inducing autophagy in a TRPV2-dependent manner. *Int J Cancer* 2015;137:1855–69.
 49. Goncharov I, Weiner L, Vogel Z. Delta9-tetrahydrocannabinol increases C6 glioma cell death produced by oxidative stress. *Neuroscience* 2005;134:567–74.
 50. Wu HY, Chu RM, Wang CC, Lee CY, Lin SH, Jan TR, et al. Cannabidiol-induced apoptosis in primary lymphocytes is associated with oxidative stress-dependent activation of caspase-8. *Toxicol Appl Pharmacol* 2008;226:260–70.