Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
Molecular Pharmacology Fast Forward. Published on June 5, 2006 as doi:10.1124/mol.106.023937

MOL 23937

Cannabidiol-induced apoptosis in human leukemia cells: A novel role of cannabidiol in the regulation of p22 *phox* and Nox4 expression.

Robert J. McKallip, Wentao Jia, Jerome Schlomer, James W. Warren, Prakash S. Nagarkatti, and Mitzi Nagarkatti

The Department of Pathology, Microbiology, and Immunology, The University of South Carolina School of Medicine [RJM, JWW,PSN,MN]. The Department of Microbiology and Immunology, Virginia Commonwealth University [WJ, JS].

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Copyright 2006 by the American Society for Pharmacology and Experimental Therapeutics.

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 23937

Running Title: Cannabidiol-induced apoptosis in human leukemia cells

Address correspondence and reprint requests to Dr. Robert J. McKallip. Department of Pathology, Microbiology and Immunology. University of South Carolina School of Medicine, 6439 Garner's Ferry Road, Columbia, SC 29209. Phone number (803) 253-5837. Fax number (803) 733-1515. E-mail address: rmckallip@gw.med.sc.edu

Text pages = 19

Number of Tables = 0

Number of Figures = 8

Number of References = 37

Number of word in the abstract = 214

Number of words in the introduction = 703

Number of words in the Discussion = 1499

Abbreviations: Activation induced cell death (AICD), activated T cell autonomous cell death (ACAD), 3,3′-dihexylcarbocyanine iodide ($DiOC_6$), Delta-9-tetrahydrocannabinol (THC).

Recommended section: Cellular and Molecular

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract:

In the current study, we examined the effects of the non-psychoactive cannabinoid, cannabidiol, on the induction of apoptosis in leukemia cells. Exposure of leukemia cells to cannabidiol led to CB2-mediated reduction in cell viability and induction in apoptosis. Furthermore, cannabidiol treatment led to a significant decrease in tumor burden and an increase in apoptotic tumors *in vivo*. Mechanistically, cannabidiol exposure resulted in activation of caspase-8, caspase-9, and caspase-3, cleavage of PARP and a decrease in full-length Bid, suggesting possible cross-talk between the intrinsic and extrinsic apoptotic pathways. The role of the mitochondria was further suggested as exposure to cannabidiol led to loss of mitochondrial membrane potential and release of cytochrome C. Interestingly, cannabidiol exposure led to an increase in reactive oxygen species (ROS) production as well as an increase in the expression of the NAD(P)H oxidases, NOX4 and $p22^{phox}$. Furthermore, cannabidiol-induced apoptosis and ROS levels could be blocked by treatment with the ROS scavengers, or the NAD(P)H oxidase inhibitors. Finally, cannabidiol exposure led to a decrease in the levels of p-p38 MAPK, which could be blocked by treatment with a CB2-selective antagonist or ROS scavenger. Together, the results from this study reveal that cannabidiol, acting through CB2 and regulation of NOX4 and $p22^{phox}$ expression, may be a novel and highly selective treatment for leukemia.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction:

Medicinally, marijuana has been implicated as a potent therapeutic agent alleviating such complications as intraocular pressure in glaucoma, and cachexia, nausea, and pain in AIDS and cancer patients. A number of recent studies now suggest the possible use of these compounds for the treatment of cannabinoid receptor-expressing tumors. For example, anandamide was shown to inhibit the proliferation of the human breast cancer cell lines, MCF-7 and EFM-19 *in vitro* (De Petrocellis et al., 1998). Also, THC was shown to induce apoptosis in human prostate PC-3 cells and in C6 glioma cells in culture (Galve-Roperh et al., 2000; Ruiz et al., 1999; Sanchez et al., 1998). THC-induced apoptosis involved cannabinoid receptor-dependent (Galve-Roperh et al., 2000; Sanchez et al., 1998) or independent pathways (Ruiz et al., 1999). Such studies have triggered interest in targeting cannabinoid receptors *in vivo* to induce apoptosis in transformed cells. To this end, cannabinoids were shown to inhibit the growth of C6 glioma cells *in vivo* (Sanchez et al., 2001). Furthermore, recent studies from our laboratory demonstrated that targeting cannabinoid receptors may be a novel approach to treating lymphoblastic disease (McKallip et al., 2002).

A significant limitation to the use of a number of these compounds is their unwanted psychotropic activity. Cannabidiol (CBD) is a nonpsychoactive derivative of marijuana that is currently being examined for its use in the treatment of cancer. For example, Massi et al. demonstrated that cannabidiol was capable of suppressing the proliferation of human glioma cell lines (Massi et al., 2004). Additionally, the HL-60 myeloblastic cell line was shown to be sensitive to CBD-induced apoptosis while monocytes from normal individuals were relatively resistant to CBD-induced apoptosis, suggesting that CBD may be effective at treating AML (Gallily et al., 2003).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Although a number of reports demonstrate the ability of CBD to induce apoptosis in tumor cells, little work has been done demonstrating CBD mechanism of action. Massi et al. found that apoptosis in human glioma cell lines following exposure to CBD was mediated through CB2 receptor and the generation of reactive oxygen species (ROS).The generation of ROS can play an important role in the induction of apoptosis in T cells undergoing either activation induced cell death (AICD) or activated T cell autonomous cell death (ACAD) (Hildeman et al., 2003). Furthermore, the regulation of ROS generation can be significantly impacted by NAD(P)H oxidases (Lee et al., 2000; Suzuki et al., 1998). Numerous studies have been carried out examining the ability of compounds to induce apoptosis in tumor cells by increasing ROS production (Chang et al., 2005; Hu and Brindle, 2005; Kang et al., 2004; Kim et al., 2004; Lebedeva et al., 2005). However, little is known about the ability of cannabinoids or signaling through cannabinoid receptors to regulate the expression or activity of NAD(P)H oxidases and/or to control of ROS generation in leukemia. However in a recent study, the NAD(P)H oxidase NOX 5, which plays a significant role in mediating Ca^{2+}-dependent ROS generation, was shown to be expressed in lymph nodes and the spleen, suggesting a possible role of NAD(P)H oxidases in the regulation of ROS production in cells of the immune system (Banfi et al., 2001).

The observation that CBD can mediate apoptosis through cannabinoid receptor 2 (CB2) combined with results from our previous study, where we demonstrated that when compared to a human glioma cell line, a number of human leukemias and lymphomas expressed significantly higher levels of CB2, suggests the possibility that human leukemias and lymphomas may be highly sensitive to the CB2-mediated effects of cannabidiol (Massi et al., 2004; McKallip et al., 2002). Therefore, in the current study we examined the potential use of cannabidiol in the

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

treatment of lymphoblastic disease. Using the murine EL-4 leukemia and the human Jurkat and Molt-4 leukemia cell lines, we demonstrated that exposure to cannabidiol led to tumor killing by induction of apoptosis. Examination of the mechanism of cannabidiol-induced apoptosis revealed that cannabidiol was acting through CB2. Furthermore, we demonstrated the involvement of the NAD(P)H oxidases; $p22^{phox}$ and NOX4 and the subsequent generation of reactive oxygen species in CBD-induced apoptosis. Together, these studies suggest that cannabidiol, acting through CB2, may be a novel regulator of NAD(P)H oxidase expression and that CBD may prove to be a potent non-psychoactive and specific treatment of leukemia.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods:

Mice. Adult female (6-8 weeks of age) C57BL/6 mice were purchased from the National Institutes of Health (NIH). The mice were housed in polyethylene cages and given rodent chow and water *ad libitum*. Mice were housed in rooms maintaining a temperature of 74 $\pm$ 2^0F and on a 12 h light/dark cycle.

Reagents. Cannabidiol initially dissolved in EtOH was obtained from Tocris Cookson Inc. (Ellisville, MO). Cannabidiol was further diluted with tissue culture medium for *in vitro* studies and PBS for *in vivo* studies. SR141716A (cannabinoid receptor 1 (CB1)-selective antagonist) and SR144528 (CB2-selective antagonist), were obtained from Sanofi Recherche (Montpellier, France). α-Tocopherol, diphenylene iodinium (DPI), capsazepine (CPZ, vanilloid receptor 1 (VR1)-selective antagonist), apocynin, and N-acetylcysteine (NAC), were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies specific for caspase-2, -3, -8, -9, -10, Bid, cytochrome c, and PARP were purchased from Cell Signaling Technology (Danvers, MA). Antibodies specific for $p22^{pnox}$ and NOX4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines. The murine lymphoma, EL-4 and the human leukemia cell lines, Jurkat, and MOLT-4 were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 1mM glutamine, 40 μg/ml gentamicin sulfate, and 50 μM 2-mercaptoethanol. Serum-free medium was used in assays examining the effect of cannabidiol on tumor cell viability and apoptosis.

Measurement of the effect of cannabidiol on tumor cell viability *in vitro*. Tumor cells were adjusted to 0.5 x 10^6 cells/ml in serum-free medium. Next, the cells (0.5 x 10^6) were cultured for 24 hours in 24-well plates in 2 ml serum-free medium in the presence or absence of various

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

concentrations of cannabidiol. Finally, the cells were harvested, washed twice in PBS and the viable cell count was determined by trypan blue dye exclusion.

Detection of cannabidiol-induced apoptosis *in vitro*. Tumor cells (0.5 x 10^6 cells/well) were cultured in 24-well plates in the presence or absence of various concentrations of cannabidiol and/or cannabinoid receptor antagonists for 24 h. Next, the cells were harvested, washed twice in PBS and analyzed for the induction of apoptosis using the TUNEL or Wright-Giemsa method. To detect apoptosis using the TUNEL method the cells were washed twice with PBS and fixed with 4% p-formaldehyde for 30 minutes at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 minutes and incubated with FITC-dUTP and TdT (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 37^0C and 5% CO_2. Five thousand cells were analyzed per sample. To detect apoptosis using the Wright-Giemsa method centrifuged preparations were stained with Wright-Giemsa stain and observed by light microscopy for signs of apoptosis including nuclear condensation, cell shrinkage, and formation of apoptotic bodies (Jia et al., 2003).

Measurement of tumor cell viability and induction of apoptosis *in vivo*. Groups of 5 C57BL/6 mice were injected with 1 x 10^6 EL-4 tumor cells i.p suspended in 0.2 ml PBS. The control mice received PBS alone. Ten days later, the mice were injected with various concentrations of cannabidiol (0, 12.5 or 25 mg/kg) i.p. The mice were sacrificed 24 hours later and the EL-4 tumor cells were harvested from the peritoneal cavity by injecting 5.0 ml PBS, followed by aspiration of the peritoneal fluid from the cavity. The contaminating red blood cells were removed using red blood lysing solution (Sigma, St. Louis, MO) and the tumor cells were

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

washed twice with PBS. The number of viable cells was determined by trypan blue dye exclusion and apoptosis was determined using the TUNEL assay. The presence of tumor cells in the peritoneal cavity was confirmed by the ability of the cells to grow *in vitro* and by the phenotype ($Thy1^+$, $CD4^-$, $CD8^-$).

Analysis of mitochondrial membrane potential ($\Delta\psi_m$). Jurkat cells were treated for 16 h with various concentration of CBD. To assess any loss of mitochondrial membrane potential, 3,3′-dihexylcarbocyanine iodide ($DiOC_6$, Molecular Probes, Inc., Eugene, OR) was added to the cells for a final concentration of 40 nM, 15 min prior to the end of the incubation. The cells were then harvested and analyzed using a flow cytometer.

Determination of Reactive Oxygen Species. Jurkat cells were labeled for 1h with 5 μM carboxy-H_2DCFDA (Molecular Probes, Eugene, OR). Next, excess carboxy-H_2DCFDA was removed by washing the cells and then suspending them in serum-free, phenol red-free RPMI. The labeled cells were then exposed to various concentrations of CBD for 24h and the levels of ROS were determined by flow cytometric analysis and changes in ROS levels were depicted as the percent increase in fluorescence.

Analysis of protein expression by Western blot analysis. Protein was isolated from Jurkat cells by freeze thawing and the protein concentration was determined by Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The protein (15-30 μg) was run on a 12% acrylamide gel. The protein was transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane in 5% blotting grade blocker non-fat dry milk (BioRad, Hercules CA) or

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

BSA for 1h at room temperature. The membrane was rinsed and then washed 3 times for 10 minutes with TBS containing 0.1% Tween 20. The membrane was then probed with the specific primary antibody in 5% blocking solution overnight at 4^0C. The membrane was then rinsed and washed 3 times with TBS containing 0.1% Tween 20 and probed with the appropriate HRP-conjugated secondary antibody for 1 hr at room temperature. The proteins were then visualized following incubation with ECL solution and exposure to X-ray film.

Assessment of cytosolic cytochrome c levels. Jurkat cells were harvested and centrifuged at 600g for 10 minutes. The cells were then lysed using 100 μl lysing buffer (75 mM NaCl, 8mM Na_2HPO_4, 1mM NaH_2PO_4, 1mM EDTA, and 350 μg/ml digitonin). Next, the lysates were centrifuged at 12,000g for 5 minutes. The proteins from resulting fraction (S-100) were quantified, separated by SDS-PAGE electrophoresis and probed using mAb specific for cytochrome C.

Flow cytometric analysis of phosphorylated proteins. Tumor cells (0.5 x 10^6 cells/well) were cultured in 24 well in the presence of vehicle, cannabidiol (5.0 μM) $\pm$ SR144528 (5.0 μM), or cannabidiol $\pm$ α-tocopherol (5.0 μM) for 24 h. The cells were harvested and washed twice in PBS. Nonspecific staining was blocked by culturing the cells with Fc Block (BD Pharmingen, San Jose, CA). Next, the cells were fixed for 30 minutes with 4% paraformaldehyde and then permeabilized by suspending the cells in the BD Perm/Wash solution for 15 minutes. The presence of the various phosoproteins was determined by staining with fluorescently labeled mAbs specific for phosphorylated and non-phosphorylated ERK, p38, and JNK (Cell Signaling Technology, Danvers, MA) followed by flow cytometric analysis.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Statistical Analysis. Student's t-test or Tukey Kramer tests were used to compare vehicle and CBD treated groups. $P<0.05$ was considered to be statistically significant.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Exposure of EL-4 to cannabidiol leads to a reduction in viability and induction of apoptosis *in vitro*. We examined whether cannabidiol-exposure had an effect on the viability of EL-4, tumor cells *in vitro*. To this end, the tumor cells were cultured in serum-free medium and exposed to various concentrations of cannabidiol (0, 1.25, 2.5, 5 and 10 μM) for 24 hours and the viability was determined by trypan blue dye exclusion (Figure 1A). The results showed that exposure to cannabidiol at concentrations of 2.5 μM or greater led to a significant reduction in the number of viable cells. Next, we analyzed the cannabidiol-treated tumor cells for induction of apoptosis by TUNEL staining (Figure 1B) and demonstrated that cannabidiol induced significant apoptosis in EL-4 tumor cells *in vitro*. Finally, we examined the effects of CB1-, CB2-, and VR1-selective antagonist on cannabidiol-induced cytotoxicity. EL-4 tumor cells were exposed to cannabidiol (5 μM) in the presence or absence of CB1- (SR141716A), CB2 (SR144528), or VR1-selective (CPZ) antagonist. EL-4 tumor cells were harvested 24h later and the cell viability was determined by trypan blue dye exclusion (Figure 1C). The results showed that treatment with the CB2-selective, but not the CB1- or VR1-selective antagonist was able to significantly inhibit the cannabidiol-induced reduction in cell viability. Together, these results suggested that exposure of EL-4 cells to $\geq$ 2.5 μM cannabidiol *in vitro* led to CB2-dependent cell killing by induction of apoptosis.

Cannabidiol treatment leads to reduced tumor burden and increased tumor cell apoptosis *in vivo*. We examined whether treatment of tumor-bearing mice with cannabidiol was effective at killing tumor cells *in vivo*. To this end, C57BL/6 mice were injected with EL-4 tumor cells (1 x 10^6). On day 10 of tumor growth, the mice were injected i.p. with various doses of cannabidiol

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

(12.5, or 25 mg/kg) or the vehicle. One day later, the mice were sacrificed and injected with 5 ml PBS into the peritoneal cavity. The peritoneal fluid was aspirated out and analyzed for viable tumor cells as well as for the presence of apoptotic tumors. The data demonstrated that cannabidiol caused a dose-dependent decrease in the viable tumor cell number found in the peritoneal cavity (Figure 2A). Furthermore, there was a significant increase in the percentage of apoptotic tumor cells following CBD treatment (Figure 2B). These data suggested that cannabidiol was effective at killing the EL-4 tumor cells *in vivo* by inducing apoptosis.

Cannabidiol induces apoptosis in human leukemia cells *in vitro*. Next, we examined whether exposure of human leukemia cells to cannabidiol would lead to induction of apoptosis. To this end, Jurkat and MOLT-4 cells were exposed to various concentrations of cannabidiol (2.5, and 5 μM) or the vehicle for 24 h, and the effect on cell viability was determined by trypan blue dye exclusion and the induction of apoptosis was determined using the TUNEL method. The results showed that exposure of the Jurkat or MOLT-4 cells to $\geq$ 2.5 μM cannabidol led to significant decrease in the tumor cell viability (Figure 3A) and an increase in the levels of apoptosis (Figure 3B). Fig 3B shows a representative experiment using TUNEL assay. Next, experiments were set up to determine the earliest time point in which CBD-induced apoptosis could be detected. To this end, Jurkat cells were cultured with CBD (5.0 μM) for 8, 16, and 24h (Figure 3C). The effect of CBD on the induction of apoptosis at the various time points was determined using the TUNEL assay and revealed that significant CBD-induced apoptosis could be detected following 24 h exposure. While at the earlier time points little increase in the percentage of apoptotic cells could be detected. More specifically, following 8h of exposure to CBD the levels of apoptosis increased from 15.7% (vehicle) to 22.1% (CBD) and following 16h of exposure to CBD the

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

levels of apoptosis increased from 10.9% (vehicle) to 19.9% (CBD). However, following 24h of exposure to CBD the levels of apoptosis increased from 6.4% (vehicle) to 48.7% (CBD). Together, these data suggested that cannabidiol exposure can induce apoptosis in human leukemia cells.

The role of CB2 in cannabidiol-induced apoptosis. Next, we tested whether cannabidiol was acting through CB1, CB2 or VR1 receptors to induce apoptosis. To this end, Jurkat cells were incubated with 5 μM cannabidiol in the presence of CB1-, CB2-, or VR1- selective antagonists or the vehicle. After 24 h, the viable cell number was determined by trypan blue dye exclusion (Figure 4A). The results showed that exposure to cannabidiol led to a dramatic reduction in the number of viable tumor cells. However, when the cells were co-cultured with the CB2-selective antagonist, the viable cell numbers increased significantly thereby reversing the effect of cannabidiol. In contrast, co-culture with either the CB1 or VR1 antagonist was unable to inhibit the effects of cannabidiol on Jurkat viability. Next, we examined whether co-culture with the CB2 antagonist would have any effect on cannabidiol-induced apoptosis. To this end, Jurkat cells were incubated with 5 μM cannabidiol in the presence or absence of the CB2-selective antagonist. The presence of apoptotic cells was assayed 24 h later using the TUNEL (Figure 4B) and Wright-Giemsa staining (Figure 4C) methods. The results from the TUNEL assay showed that exposure of Jurkat cells to 5.0 μM CBD led to the induction of apoptosis in 83.5% of the cells compared to 3.5% observed in the vehicle treated cells. However, if the Jurkat cells were preexposed to the CB2-selective antagonist, SR144528, the level of CBD-induced apoptosis was significantly reduced to 18.7%. Similar results were seen using the Wright-Giemsa assay, where it was shown that following exposure to 5.0 μM CBD the majority of the Jurkat cells displayed

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

classical morphological features of apoptosis, including nuclear condensation, cell shrinkage, and formation of apoptotic bodies. In contrast, Jurkat cells pretreated with SR144528 displayed significantly fewer signs of apoptosis following CBD exposure. Treatment with either the CB1 or VR1 antagonist was unable to prevent cannabidiol-induced apoptosis (data not shown). Together, these results suggested that cannabidiol-induced reduction in viable cell number and the induction of apoptosis of Jurkat cells was mediated through CB2.

Cannabidiol induces activation of the caspase cascade through CB2. The activation of the caspase cascade is commonly associated with the induction of apoptosis. Therefore, to further investigate the mechanism of cannabidiol-induced apoptosis, we examined the activation pattern of caspases following CBD exposure. To this end, Jurkat cells were exposed to various concentrations of cannabidiol (2.5 and 5 μM) or the vehicle for 24 hours. Next, the cells were harvested and the presence of the various caspases was determined by Western blot analysis (Figure 5A). The results demonstrate that exposure to CBD at concentrations of 2.5 μM or greater led to activation of the caspase cascade. More specifically, we observed cleavage of caspase-8, and reduction in procaspase-2, -9, and -10, which are thought to be involved in initiating the caspase cascade. In addition, the cleavage of the effector type caspase-3 and PARP were observed following exposure to CBD. Next, the involvement of CB2 was examined by culturing Jurkat cells with the CB2 antagonist, SR144528, in the presence or absence of CBD. The results showed that the CB2 antagonist was able to significantly prevent CBD-mediated induction of the caspase cascade. Interestingly, CBD-induced cleavage of Bid was observed in Jurkat cells suggesting possible cross-talk between the intrinsic and extrinsic apoptotic pathways.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

CBD exposure leads to loss of mitochondrial potential and release of cytochrome C. The role of the mitochondria in CBD-induced apoptosis of Jurkat cells was further investigated by examining the effect of CBD on Jurkat mitochondrial membrane potential as well as the levels of cytosolic cytochrome C. Exposure of Jurkat cells to 2.5 μM or greater CBD for 24 h led to a significant reduction in the mitochondrial membrane potential (Figure 5B). Furthermore, 2.5 μM or greater CBD led to a significant increase in the level of cytosolic cytochrome C (Figure 5C). Together, these results suggest a direct role of the mitochondria in CBD-induced apoptosis.

CBD induces increased generation of ROS. CBD exposure has been associated with increase levels of ROS in glioma cells (Massi et al., 2004). Furthermore, ROS have been associated with activation of the intrinsic apoptotic pathway (Singh et al., 2005; Zorov et al., 2000). Therefore, in the current study we examined the effect of CBD-exposure on Jurkat production of ROS. The results showed that exposure of Jurkat and MOLT-4 cells to 2.5 μM or greater CBD for 24h led to a significant increase in the levels of ROS production (Figure 6A). The involvement of the CBD-induced increase in ROS generation in Jurkat apoptosis was further confirmed using the ROS scavengers, α-tocopherol and NAC. More specifically, Jurakat cells were exposed to CBD ± α-tocopherol or CBD ± NAC and then analyzed for viable cell number (Figure 6B), induction of apoptosis (Figure 6C) and ROS production (Figure 6D). The results demonstrated that treatment with ROS scavengers led to a significant reduction in CBD-induced Jurkat cell killing and apoptosis. Furthermore, the degree to which the ROS scavengers prevented the effects of CBD on tumor cell killing directly correlated with the ability of the scavengers to reduce the level of CBD-induced ROS.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

CBD exposure leads to increased expression of Nox4 and p22^{phox}. NAD(P)H oxidases can play a significant role in the regulation of reactive oxygen species (Lundqvist-Gustafsson and Bengtsson, 1999; Sim et al., 2005; Suzuki et al., 1998). Therefore, we examined whether exposure of Jurkat cells to CBD had an effect on the expression of NAD(P)H oxidases. In initial experiments, the effect of CBD on NAD(P)H oxidases was screened using RT-PCR. These studies revealed that CBD exposure led to a significant increase in the levels of Nox4 and p22^{phox}, while the expression of other NAD(P)H oxidases, including Nox1, Nox2, Nox3, Nox5, p47^{phox}, and p67^{phox} was unaffected (data not shown). The effect of CBD on Nox4 and p22^{phox} were confirmed by Western blot analysis (Fig. 7A). Furthermore, the role of CB2 in CBD-induced alteration in Nox4 and p22^{phox} expression was confirmed. More specifically, treatment with the CB2-selective antagonist inhibited the CBD-induced effects on Nox4 and p22^{phox} protein expression (Fig. 7A). The role of NAD(P)H oxidases in CBD-induced killing of Jurkat cells was further confirmed using the NAD(P)H oxidase inhibitors, diphenylene iodinium (DPI) and apocynin, demonstrating that treatment with NAD(P)H inhibitors led to a significant reduction in both CBD-induced cell toxicity (Fig. 7B) as well as CBD-induced apoptosis (Fig. 7C) in Jurkat cells. Furthermore, the degree to which the NAD(P)H oxidase inhibitors prevented the effects of CBD on tumor cell killing directly correlated with the ability of the inhibitors to reduce the level of CBD-induced ROS (Figure 7D).

The effect of CBD on the levels of p-ERK, p-JNK and p-p38 MAPK in Jurkat cells. The regulation of ERK, JNK, and p38 MAPK have been shown to play an important role in the survival or induction of apoptosis of a number of cell types including leukemias (Dasmahapatra et al., 2005; Yu et al., 2004). Therefore, we examined whether exposure to CBD had any effect

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

on the levels of the phosphorylated forms of these signaling molecules. To this end, Jurkat cells were exposed for 4 or 16h to vehicle or 5 μM CBD. Next, the cells were stained with mAbs specific for ERK, p-ERK, JNK, p-JNK, p38 and p-p38 MAPK and analyzed by flow cytometric analysis (Figure 8A). The results demonstrated that exposure to CBD for 16h led to a significant reduction in the levels of p-p38 MAPK, while p-JNK and p-ERK were unaffected. The effect of CBD on the levels of p-p38 were confirmed by Western blot analysis, where it was shown that following 16h of exposure, CBD led to a significant reduction in the levels of p-p38 (Figure 8B). No significant effects on p-p38 were seen following 4 or 8h exposure to CBD. The involvement of CB2 in the downregulation of these signaling molecules was examined by pre-exposing the Jurkat cells to the CB2-selective antagonist SR144528 and the results showed that the CBD-mediated effects on p-p38 were dependent on CB2 signaling (Figure 8C). Furthermore, the role of ROS generation in the CBD-mediated reduction of p-p38 was examined by treating Jurkat cells with α-tocopherol prior to CBD exposure. The results from this experiment demonstrated that treatment with the ROS scavenger was able to prevent the reduction of p-p38 following CBD exposure, suggesting that the effects on ROS generations were upstream of the effects on p38 (Figure 8C).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion:

In the current study, we demonstrated that cannabidiol can induce apoptosis in murine as well as human leukemia cells. Cannabidiol-induced apoptosis was mediated through CB2, as treatment with a CB2-selective antagonist, but not CB1-selective or VR1-selective antagonist, was able to significantly reduce the levels of apoptosis following cannabidiol exposure. Furthermore, cannabidiol was effective *in vivo* as treatment with cannabidiol led to a significant reduction in tumor burden and an increase in the level of apoptotic tumors in EL-4 bearing mice. Together these data suggest that cannabidiol may be a novel treatment of leukemia.

Previously, we demonstrated that treatment with THC led to significant levels of apoptosis in a number of leukemias (McKallip et al., 2002). Cannabidiol lacks any significant psychotropic activity. Therefore, its use in the treatment of human diseases may be preferable when compared to THC or other psychoactive cannabinoids. Importantly, we show that CBD and other cannabinoids such as THC have similar anticancer activity. For example, we demonstrated significant levels of apoptosis in both human and mouse leukemia cell lines following exposure to $\geq$ 2.5 μM CBD. These levels were similar to reported levels of THC necessary to induce apoptosis in these cell lines (McKallip et al., 2002). Furthermore, evidence suggests that the doses of CBD used *in vitro* in the current study were pharmacologically relevant. This is supported by an earlier study in which rats injected with 50 mg/kg of the cannabinoid, THC were shown to exhibit 10 μM of THC in the serum within 10 hours of administration (Chan et al., 1996).

In a recent study, exposure of glioma cells to 25 μM CBD led to tumor killing by the induction of apoptosis (Massi et al., 2004). In comparison, we found that exposure of leukemia cells to as low as 2.5 μM CBD led to significant levels of apoptosis, suggesting that leukemia

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

cells may be highly sensitive to CBD-induced apoptosis. The current study as well as the report examining the effect of CBD on gliomas (Massi et al., 2004) demonstrated that CB2 is involved in CBD-induced apoptosis, suggesting that the level of CB2 expression may play an important role in the sensitivity of tumor cells to killing by CBD. Therefore, the use of CBD to target CB2 on leukemia cells may prove to be a highly effective and selective strategy for treating this disease.

The role of CB1, CB2 and VR1 in CBD-mediated effects remains controversial. For example, work examining the effects of CBD on glioma cells demonstrated that CB2 was involved in CBD-induced apoptosis (Massi et al., 2004), while the same group of investigators showed that the CBD-mediated suppression of glioma cell migration was through a cannabinoid receptor-independent mechanism (Vaccani et al., 2005). Additionally, studies have implicated VR1 in CBD-mediated effects (Bisogno et al., 2001). Furthermore, due to the lipophilic properties of CBD, nonspecific killing through intercalation into the cell membrane may also influence CBD's activity. However, in the current study, we demonstrated that treatment with the CB2-selective antagonist SR144528 led to significant inhibition of CBD-mediated apoptosis in Jurkat cells. This effect was specific for CB2, as treatment with CB1- or VR1-antagonists was ineffective at preventing CBD-mediated apoptosis (data not shown).

Currently, compounds that can influence the generation of ROS are being examined for their potential use in the treatment of a number of diseases including cancer (Chang et al., 2005; Hu and Brindle, 2005; Kang et al., 2004; Kim et al., 2004; Lebedeva et al., 2005). However, little has been reported regarding the ability of cannabinoids to regulate the generation of ROS. In a study examining the effect of CBD on glioma cells, CBD-induced apoptosis was related to increased ROS generation (Massi et al., 2004). In the current study, we demonstrated a similar

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

role of ROS in CBD-induced apoptosis in Jurkat cells. More specifically, we demonstrated that exposure to CBD led to an increase in ROS production and that treatment with ROS scavengers significantly inhibited CBD-induced apoptosis in Jurkat cells. Importantly, the level by which the ROS scavengers inhibited CBD-induced apoptosis and loss of cell viability correlated with the level of inhibition of CBD-induced increase in ROS levels, further supporting the role of ROS in CBD-induced apoptosis. Together, these data suggest that regulation of ROS generation by cannabidiol may be an effective strategy to treat malignancies of the immune system.

NAD(P)H oxidases are a group of enzymes involved in the regulation of ROS production (Singh et al., 2005; Zorov et al., 2000). Nox4 and p22^{phox} are NAD(P)H oxidases that play a role in a number of processes including cell survival and apoptosis (Martyn et al., 2006; Pedruzzi et al., 2004; Vaquero et al., 2004). However, the specific effects of these enzymes in these processes remain unclear. For example, studies examining pancreatic cancer cell survival suggested that growth factor-induced ROS generation controlled by various NAD(P)H oxidases, including NOX4, promoted tumor cell survival (Edderkaoui et al., 2005; Vaquero et al., 2004). In contrast, it was reported that NOX4 played a significant role in mediating apoptosis in human aortic smooth muscles cells following exposure to 7-ketocholesterol (Pedruzzi et al., 2004). To date, expression of these enzymes and their contribution to the induction of apoptosis in leukemia cells has not been reported. In the current study, we demonstrated that CBD exposure led to increased expression of the NAD(P)H oxidases, Nox4 and p22^{phox}, suggesting the possibility that regulation of these enzymes and the subsequent generation of ROS may play a significant role in the induction of apoptosis in leukemia cells. Therefore, targeting Nox4 and p22^{phox} using CBD may be a novel approach for treating malignancies of the immune system.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

The mechanism by which CBD exposure leads to increased expression of NOX4 and p22^{phox} is not clear. However, initial observations in this study showing that pretreatment with CB2-selective antagonist, but not CB1- or VR1-selective antagonists significantly inhibited the CBD-mediated increase in NOX4 and p22^{phox} expression, suggests that CBD regulation of these enzymes is mediated through CB2 activation. Interestingly, the mechanism of action seems to be distinct from that of THC, which can lead to increased ceramide production and has been associated with G-protein coupled receptor signaling (Galve-Roperh et al., 2000; Rueda et al., 2000). In the current study, we were unable to block CBD-induced apoptosis using various inhibitors of ceramide generation or pertussis toxin, which blocks G-protein coupled receptor signaling (data not shown). Taken together, these data suggest that although both THC and CBD exposure can lead to the induction of apoptosis in leukemia cells, their mechanisms of action may be distinct from one another. Therefore, treatments using the combination of THC and CBD may significantly enhance the efficacy of tumor killing. To date, this approach has not been reported for the treatment of leukemia or lymphoma. However, initial studies demonstrated that the combination of CBD and THC was better tolerated than THC alone (reviewed in Russo, 2005), possibly allowing for the use of increased levels of THC without the unwanted psychoactive side effects. In fact, it was shown that CBD could antagonize some of the undesirable effects of THC, such as its psychoactive properties, by blocking conversion of THC to the more psychoactive 11-hydroxy-THC (Bornheim and Grillo, 1998). On the other hand, as shown in the current study and by other investigators examining the effects on gliomas (Massi et al., 2004), CBD alone has been shown to possess potent antitumor activity. Taken together, these studies suggest that the combination of THC and CBD may produce a powerful combination for the treatment of various cancers, including gliomas and malignancies of the immune system.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

In addition, results from this study demonstrated that CBD exposure led to the reduction of p-p38 levels, while p-ERK and p-JNK levels were unaffected. Support for the role of p38 in CBD-induced apoptosis come from reports that demonstrate that CBD exposure led to reduced levels of p-p38 in PC12 neuronal cells (Esposito et al., 2006). Furthermore, in a separate study it was shown that reduction in p-p38 levels in Jurkat cells led to the induction of apoptosis (Nemoto et al., 1998). Taken together, these results suggest that CBD-induced apoptosis is regulated, at least in part, through alterations in p38 signaling.

In summary, the current study demonstrates that CBD-induced apoptosis may constitute a novel approach to treat malignancies of the immune system. CBD has been reported to be non-psychoactive, making it more attractive than its psychoactive counterpart, THC, as a therapeutic agent. Here we found that the CBD-induced apoptosis was dependent on CB2 receptor signaling. CB2 is expressed almost exclusively on immune cells. Therefore use of CB2 receptor agonists, such as CBD, may be significantly less toxic to non-immune cells. Furthermore, we demonstrated, for the first time, that exposure of leukemia cells to CBD led to increased production of ROS which was mediated through regulation of NOX4 and $p22^{phox}$, suggesting a novel role of CBD in the regulation of ROS production in leukemia cells. Thus, further research on the use of CBD to target transformed immune cells could lead to a new and highly selective anti-cancer agent.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

References

Banfi B, Molnar G, Maturana A, Steger K, Hegedus B, Demaurex N and Krause KH (2001) A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* **276**(40):37594-37601.

Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R and Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br J Pharmacol* **134**(4):845-852.

Bornheim LM and Grillo MP (1998) Characterization of cytochrome P450 3A inactivation by cannabidiol: possible involvement of cannabidiol-hydroxyquinone as a P450 inactivator. *Chem Res Toxicol* **11**(10):1209-1216.

Chan PC, Sills RC, Braun AG, Haseman JK and Bucher JR (1996) Toxicity and carcinogenicity of delta 9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. *Fundam Appl Toxicol* **30**(1):109-117.

Chang YF, Chi CW, Chern YT and Wang JJ (2005) Effects of 1, 6-Bis[4-(4-amino-3-hydroxyphenoxy)phenyl]diamantane (DPD), a reactive oxygen species and apoptosis inducing agent, on human leukemia cells in vitro and in vivo. *Toxicol Appl Pharmacol* **202**(1):1-12.

Dasmahapatra G, Rahmani M, Dent P and Grant S (2005) The tyrphostin adaphostin interacts synergistically with proteasome inhibitors to induce apoptosis in human leukemia cells through a reactive oxygen species (ROS)-dependent mechanism. *Blood*.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

De Petrocellis L, Melck D, Palmisano A, Bisogno T, Laezza C, Bifulco M and Di Marzo V (1998) The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc Natl Acad Sci U S A* **95**(14):8375-8380.

Edderkaoui M, Hong P, Vaquero EC, Lee JK, Fischer L, Friess H, Buchler MW, Lerch MM, Pandol SJ and Gukovskaya AS (2005) Extracellular Matrix Stimulates Reactive Oxygen Species Production And Increases Pancreatic Cancer Cell Survival Through 5-Lipoxygenase And Nadph Oxidase. *Am J Physiol Gastrointest Liver Physiol*.

Esposito G, De Filippis D, Maiuri MC, De Stefano D, Carnuccio R and Iuvone T (2006) Cannabidiol inhibits inducible nitric oxide synthase protein expression and nitric oxide production in beta-amyloid stimulated PC12 neurons through p38 MAP kinase and NF-kappaB involvement. *Neurosci Lett* **399**(1-2):91-95.

Gallily R, Even-Chena T, Katzavian G, Lehmann D, Dagan A and Mechoulam R (2003) Gamma-irradiation enhances apoptosis induced by cannabidiol, a non-psychotropic cannabinoid, in cultured HL-60 myeloblastic leukemia cells. *Leuk Lymphoma* **44**(10):1767-1773.

Galve-Roperh I, Sanchez C, Cortes ML, del Pulgar TG, Izquierdo M and Guzman M (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med* **6**(3):313-319.

Hildeman DA, Mitchell T, Kappler J and Marrack P (2003) T cell apoptosis and reactive oxygen species. *J Clin Invest* **111**(5):575-581.

Hu DE and Brindle KM (2005) Immune cell-induced synthesis of NO and reactive oxygen species in lymphoma cells causes their death by apoptosis. *FEBS Lett* **579**(13):2833-2841.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Jia W, Yu C, Rahmani M, Krystal G, Sausville EA, Dent P and Grant S (2003) Synergistic antileukemic interactions between 17-AAG and UCN-01 involve interruption of RAF/MEK- and AKT-related pathways. *Blood* **102**(5):1824-1832.

Kang YH, Yi MJ, Kim MJ, Park MT, Bae S, Kang CM, Cho CK, Park IC, Park MJ, Rhee CH, Hong SI, Chung HY, Lee YS and Lee SJ (2004) Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. *Cancer Res* **64**(24):8960-8967.

Kim WH, Park WB, Gao B and Jung MH (2004) Critical role of reactive oxygen species and mitochondrial membrane potential in Korean mistletoe lectin-induced apoptosis in human hepatocarcinoma cells. *Mol Pharmacol* **66**(6):1383-1396.

Lebedeva IV, Su ZZ, Sarkar D, Gopalkrishnan RV, Waxman S, Yacoub A, Dent P and Fisher PB (2005) Induction of reactive oxygen species renders mutant and wild-type K-ras pancreatic carcinoma cells susceptible to Ad.mda-7-induced apoptosis. *Oncogene* **24**(4):585-596.

Lee YS, Kang YS, Lee SH and Kim JA (2000) Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in HepG2 human hepatoblastoma cells. *Cell Death Differ* **7**(10):925-932.

Lombard C, Nagarkatti M and Nagarkatti PS (2005) Targeting cannabinoid receptors to treat leukemia: role of cross-talk between extrinsic and intrinsic pathways in Delta9-tetrahydrocannabinol (THC)-induced apoptosis of Jurkat cells. *Leuk Res* **29**(8):915-922.

Lundqvist-Gustafsson H and Bengtsson T (1999) Activation of the granule pool of the NADPH oxidase accelerates apoptosis in human neutrophils. *J Leukoc Biol* **65**(2):196-204.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC and Knaus UG (2006) Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* **18**(1):69-82.

Massi P, Vaccani A, Ceruti S, Colombo A, Abbracchio MP and Parolaro D (2004) Antitumor effects of cannabidiol, a nonpsychoactive cannabinoid, on human glioma cell lines. *J Pharmacol Exp Ther* **308**(3):838-845.

McKallip RJ, Lombard C, Fisher M, Martin BR, Ryu S, Grant S, Nagarkatti PS and Nagarkatti M (2002) Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood* **100**(2):627-634.

Nemoto S, Xiang J, Huang S and Lin A (1998) Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase. *J Biol Chem* **273**(26):16415-16420.

Pedruzzi E, Guichard C, Ollivier V, Driss F, Fay M, Prunet C, Marie JC, Pouzet C, Samadi M, Elbim C, O'Dowd Y, Bens M, Vandewalle A, Gougerot-Pocidalo MA, Lizard G and Ogier-Denis E (2004) NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. *Mol Cell Biol* **24**(24):10703-10717.

Rueda D, Galve-Roperh I, Haro A and Guzman M (2000) The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N- terminal kinase. *Mol Pharmacol* **58**(4):814-820.

Ruiz L, Miguel A and Diaz-Laviada I (1999) Delta9-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism. *FEBS Lett* **458**(3):400-404.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Russo E (2005) A tale of two cannabinoids: The therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. *Med Hypotheses.*

Sanchez C, de Ceballos ML, del Pulgar TG, Rueda D, Corbacho C, Velasco G, Galve-Roperh I, Huffman JW, Ramon YCS and Guzman M (2001) Inhibition of glioma growth in vivo by selective activation of the cb(2) cannabinoid receptor. *Cancer Res* **61**(15):5784-5789.

Sanchez C, Galve-Roperh I, Canova C, Brachet P and Guzman M (1998) Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett* **436**(1):6-10.

Sim S, Yong TS, Park SJ, Im KI, Kong Y, Ryu JS, Min DY and Shin MH (2005) NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by Entamoeba histolytica. *J Immunol* **174**(7):4279-4288.

Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, Zeng Y, Watkins SC, Johnson CS, Trump DL, Lee YJ, Xiao H and Herman-Antosiewicz A (2005) Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. *J Biol Chem* **280**(20):19911-19924.

Suzuki Y, Ono Y and Hirabayashi Y (1998) Rapid and specific reactive oxygen species generation via NADPH oxidase activation during Fas-mediated apoptosis. *FEBS Lett* **425**(2):209-212.

Vaccani A, Massi P, Colombo A, Rubino T and Parolaro D (2005) Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism. *Br J Pharmacol* **144**(8):1032-1036.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Vaquero EC, Edderkaoui M, Pandol SJ, Gukovsky I and Gukovskaya AS (2004) Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. *J Biol Chem* **279**(33):34643-34654.

Yu C, Rahmani M, Almenara J, Sausville EA, Dent P and Grant S (2004) Induction of apoptosis in human leukemia cells by the tyrosine kinase inhibitor adaphostin proceeds through a RAF-1/MEK/ERK- and AKT-dependent process. *Oncogene* **23**(7):1364-1376.

Zorov DB, Filburn CR, Klotz LO, Zweier JL and Sollott SJ (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med* **192**(7):1001-1014.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes:

This work was supported in part by grants from National Institutes of Health (RO1DA016545, R21DA014885, K12DA14041 and P50DA05274), The American Cancer Society (IRG-100036) and The Jeffress Memorial Trust Fund (J-741).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Exposure of EL-4 to cannabidiol leads to a reduction in viability and induction of apoptosis *in vitro*.. (A) The effect of THC on tumor cell viability was determined by culturing EL-4, in the presence of various concentrations of CBD (1.25, 2.5, 5, or 10 μM) or the vehicle. The viable cell number was determined by trypan blue dye exclusion. (B) The effect of CBD on the induction of apoptosis in EL-4 tumor cells was determined by culturing the tumor cells for 24 hours in the presence of 2.5, 5, or 10 μM CBD or the vehicle. Apoptosis was quantified using the TUNEL method and the cells were analyzed using a flow cytometer. (C) The role of CB1, CB2, and VR1 in mediating the effects of CBD on EL-4 cell viability was determined by culturing EL-4 tumor cells with 5 μM CBD in the presence or absence of CB1 (SR141716A, 5μM), CB2 (SR144528, 5μM), or VR1 (CPZ, 5μM) antagonists. The viable cell number was determined by trypan blue dye exclusion. Asterisk indicates statistically significant difference when compared to the vehicle treated groups, $p < 0.05$. Double asterisk indicates significant difference when compared to CBD treated groups, $p < 0.05$.

Figure 2. Cannabidiol treatment leads to reduced tumor burden and apoptosis *in vivo*. C57BL/6 mice were injected on day 0 with 1 x 10^6 EL-4 tumor cells i.p. On day 10, the mice were treated with various doses of CBD (12.5 or 25 mg/kg i.p.) or the vehicle. One day later, the peritoneal cavity was flushed with 5 ml PBS and the tumor cells were collected by aspiration. (A) The cell number was determined by trypan blue dye exclusion. (B) The tumor cells recovered from the peritoneal cavity were tested for apoptosis using the TUNEL method. The data represent the mean $\pm$ SEM from groups of 4 mice. Asterisk indicates statistically significant difference when compared to the vehicle treated groups, $p < 0.05$.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Cannabidiol induces apoptosis in the Jurkat and MOLT-4 human leukemia cell lines *in vitro*. Human tumors, Jurkat and Molt-4, in serum free medium, were cultured in the presence of various concentrations of CBD (2.5 and 5 μM), or the vehicle for 24 h. (A) The viable cell number was determined by trypan blue dye exclusion. The data represent the mean ± SEM from a representative experiment. (B) The induction of apoptosis was determined by TUNEL method. The results from a representative experiment in which human tumor cells cultured with 2.5 or 5 μM of CBD or the vehicle is shown. Asterisk indicates statistically significant difference when compared to the vehicle treated groups, $p < 0.05$. (C) Jurkat cells were exposed to CBD (5 μM) for 8, 16, or 24 h. The cells were harvested and analyzed for the induction of apoptosis using the TUNEL assay. The results depicted indicate the increase in the percentage of TUNEL positive cells when compared to vehicle treated cells.

Figure 4. Cannabidiol-induced apoptosis is mediated through CB2. (A) The role of CB1, CB2, and VR1 in mediating the effects of CBD on Jurkat cell viability was determined by culturing Jurkat tumor cells with 5 μM CBD in the presence or absence of the CB1-selective antagonist, (SR141716A, 5μM), the CB2-selective antagonist, (SR144528, 5μM), or the VR1-selective antagonist, capsazepine (CPZ, 5μM). The viable cell number was determined by trypan blue dye exclusion and expressed as percentage of control viability. The role of CB2 in mediating CBD-induced apoptosis was determined by culturing Jurkat tumor cells for 24h with 5 μM CBD in the presence or absence of CB2 antagonist (SR144528, 5μM). Apoptosis was assayed using the (B) TUNEL and (C) Wright-Giemsa staining methods. Asterisk indicates statistically significant difference when compared to the vehicle treated groups, $p < 0.05$. Double asterisk indicates significant difference when compared to CBD treated groups, $p < 0.05$.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5. Cannabidiol induces activation of the caspase cascade, loss of mitochondrial membrane potential, and release of cytochrome c. (A) Jurkat tumor cells were exposed to various concentrations of CBD (2.5 or 5.0 μM) or the vehicle or for 24 h, In addition, the role of CB2 in the CBD-induced changes in caspase activity was monitored by culturing Jurkat cells with CBD (2.5 or 5.0 μM) as well as the CB2-selective antagonist, SR144528. Next, the cells were lysed, the cellular proteins were isolated and Western analysis was performed. The levels of the procaspases as well as the presence of the cleaved form (CF) of various caspases were examined. (B) Jurkat tumor cells were exposed to various concentrations of CBD (2.5 and 5.0 μM) or the vehicle for 24 h. Fifteen minutes prior to the end of the incubations, $DiOC_6$ was added for a final concentration of 40 nM. The cells were harvested and analyzed by flow cytometry. The percentage of cells with loss of mitochondrial membrane potential is depicted. (C) The effect of CBD exposure on the level of cytosolic cytochrome c in Jurkat cells was determined by culturing the cells with CBD (2.5 and 5.0 μM) $\pm$ CB2-selective antagonist, SR144528 (5.0 μM) for 24 h. The cells were harvested, washed and cytosolic proteins were analyzed for cytochrome c by Western blot analysis.

Figure 6. **The effect of CBD on the production of reactive oxygen species in human leukemia cells.** (A) Jurkat and MOLT-4 tumor cells were exposed to various concentration of CBD (2.5, 5.0 and 10.0 μM) or the vehicle for 24 h and ROS production was determined as described in the *Materials and Methods* section and depicted as % increase in fluorescence. (B) The effect of ROS scavengers on CBD-induced cell toxicity was determined. Jurkat cells were cultured for 24 h with vehicle (control) or CBD (5 μM) $\pm$ α-tocopherol (20 μM) or CBD (5 μM) $\pm$ NAC (10 mM). The viable cell number was determined by trypan blue dye exclusion. The

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

data represent the mean ± SEM of triplicate cultures. (C) The effect of α-tocopherol on CBD-induced apoptosis was determined. Jurkat cells were cultured for 24 h with vehicle (control) or CBD (5 μM) ± α-tocopherol (5 μM, 10 μM, or 20 μM). Apoptosis was determined using the TUNEL method. (D) The effect of ROS scavengers of CBD-altered ROS levels was determined. Jurkat cells were cultured for 24 h with vehicle (control) or CBD (5 μM) ± α-tocopherol (20 μM) or CBD (5 μM) ± NAC (10 mM). ROS production was determined as described in the *Materials and Methods* section and depicted as % increase in fluorescence. Asterisk indicates statistically significant difference when compared to the vehicle treated groups, $p < 0.05$.

Figure 7. CBD exposure leads to increased expression of Nox4 and p22^{phox}. (A) Jurkat cells were cultured for various time points with vehicle (control) or CBD (2.5 or 5.0 μM) ± CB2 selective antagonist, SR144528 (5.0 μM). Next, the expression of Nox4 and p22^{phox} in Jurkat cells was determined by Western blot analysis. The effect of DPI and apocynin on CBD-induced toxicity and apoptosis was determined by culturing the cells for 24h with vehicle (control), CBD (5 μM) ± DPI (5 μM), or CBD (5 μM) ± apocynin (5 μM). The viable cell number was determined by trypan blue dye exclusion (B) and apoptosis was quantified using the TUNEL method (C). (D) The effect of NAD(P)H oxidase inhibitors on CBD-altered ROS levels was determined. Jurkat cells were cultured for 24 h with vehicle (control) or CBD (5 μM) ± DPI (5 μM) or CBD (5 μM) ± apocynin (5 μM). ROS production was determined as described in the *Materials and Methods* section and depicted as % increase in fluorescence. Asterisk indicates statistically significant difference when compared to the vehicle treated groups, $p < 0.05$.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

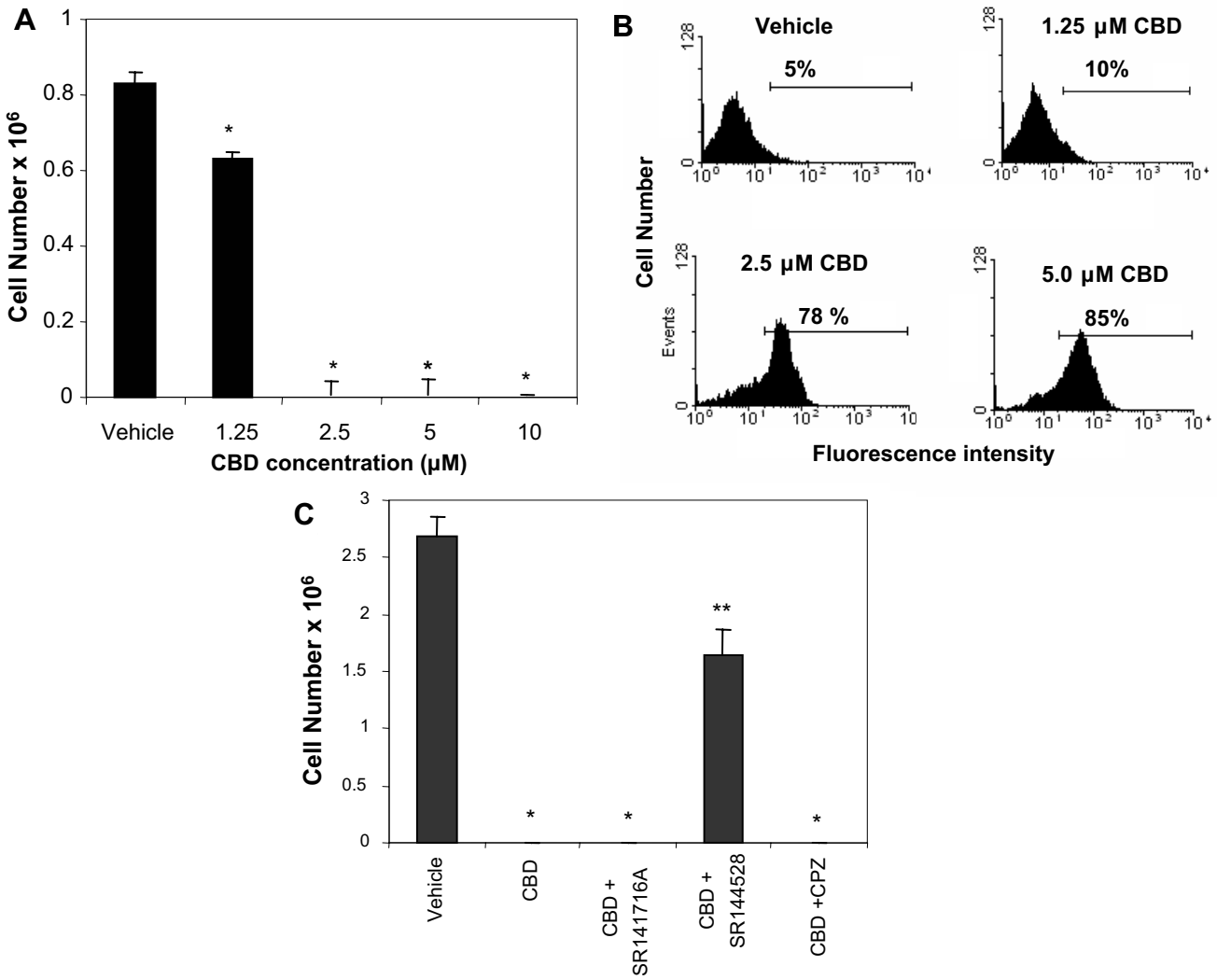
Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 8. The effect of CBD on the expression of p-ERK, p-JNK and p-p38 in Jurkat cells. (A) Jurkat cells were exposed to CBD (5 μM) or vehicle for 4 or 16h , after which the cells were harvested and the levels of ERK, p-ERK, JNK, p-JNK, p38, and p-p38 were determined by flow cytometric analysis . (B) The effect of CBD exposure (4, 8, and 16h) on the levels of p38 and p-p38 were analyzed by Western blot analysis. (C) The role of CB2 and ROS in CBD-induced alterations in p-p38 levels was examined by treating Jurkat cells with CBD (5 μM), CBD (5 μM) + SR144528 (5.0 μM) or CBD (5 μM) + α-Tocopherol (5.0 μM) or vehicle for 16h, after which the cells were harvested and the levels of p-p38 were determined by flow cytometric analysis.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

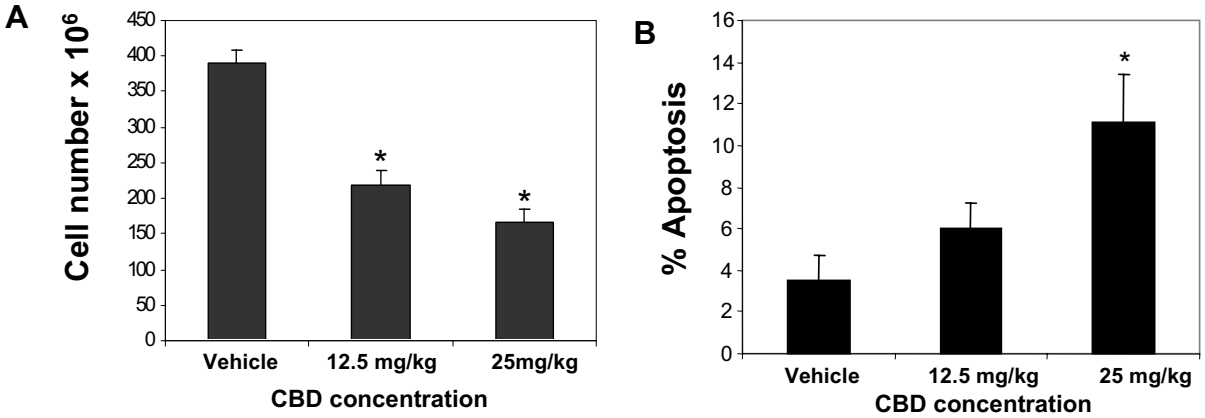
Figure 1



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

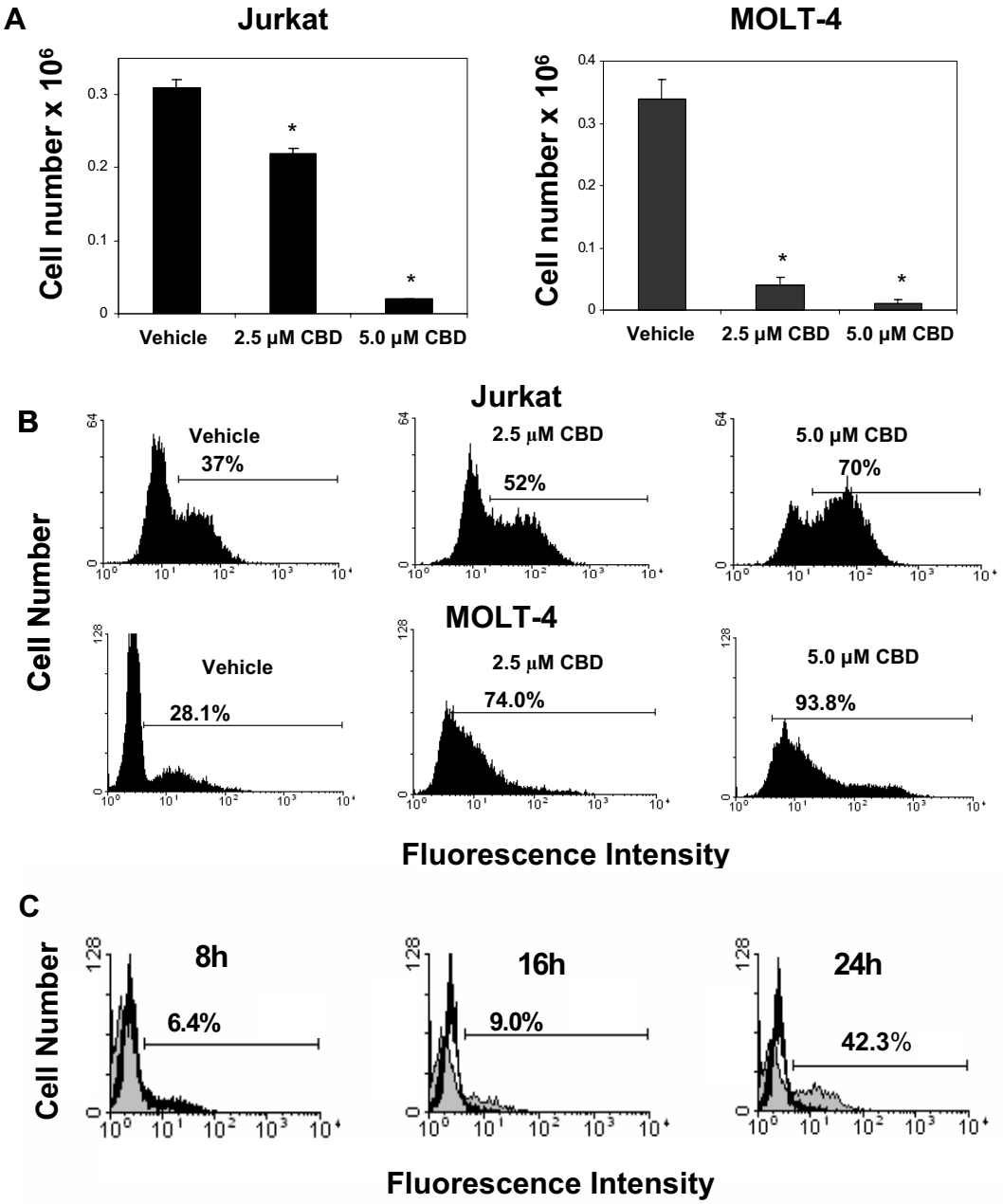
Figure 2



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

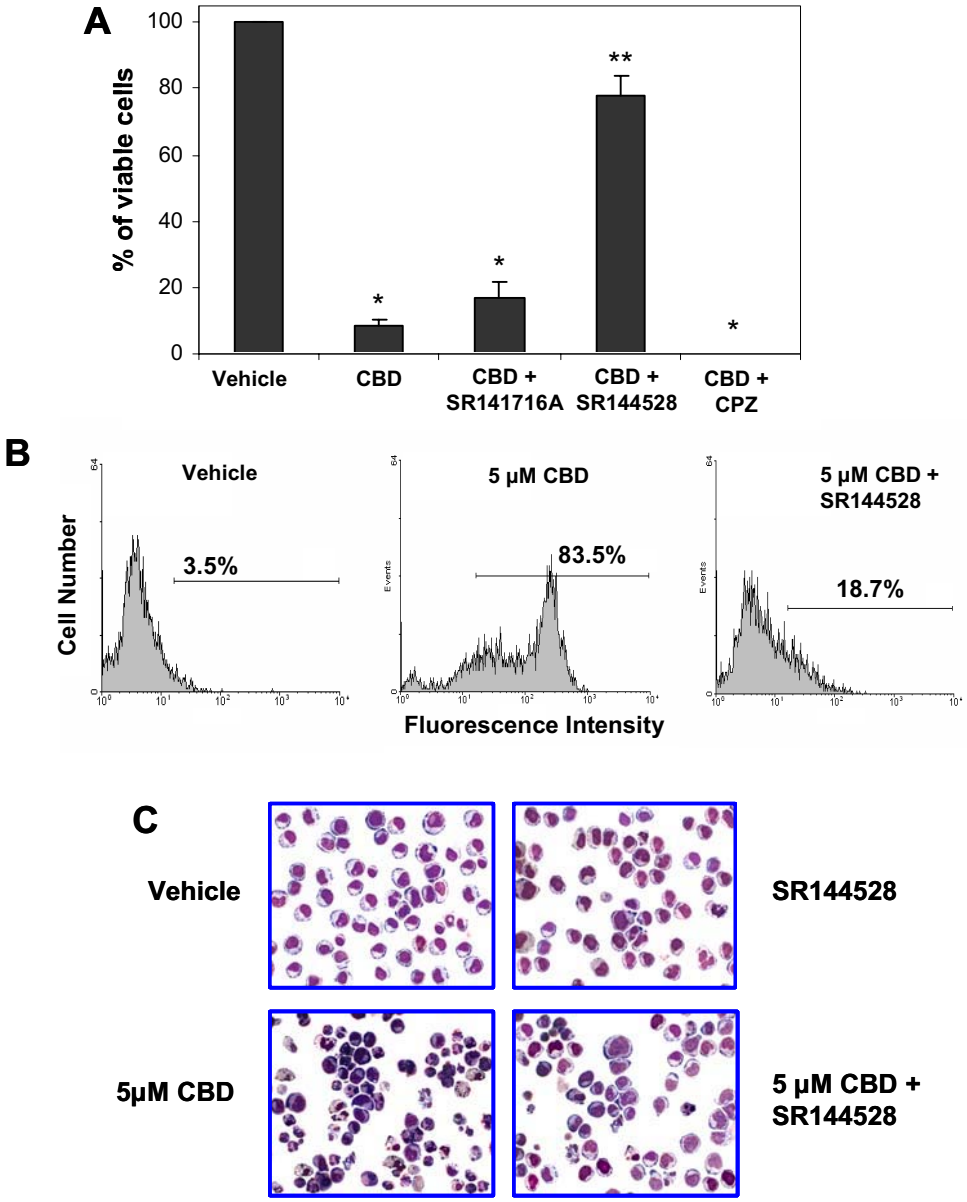
Figure 3



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

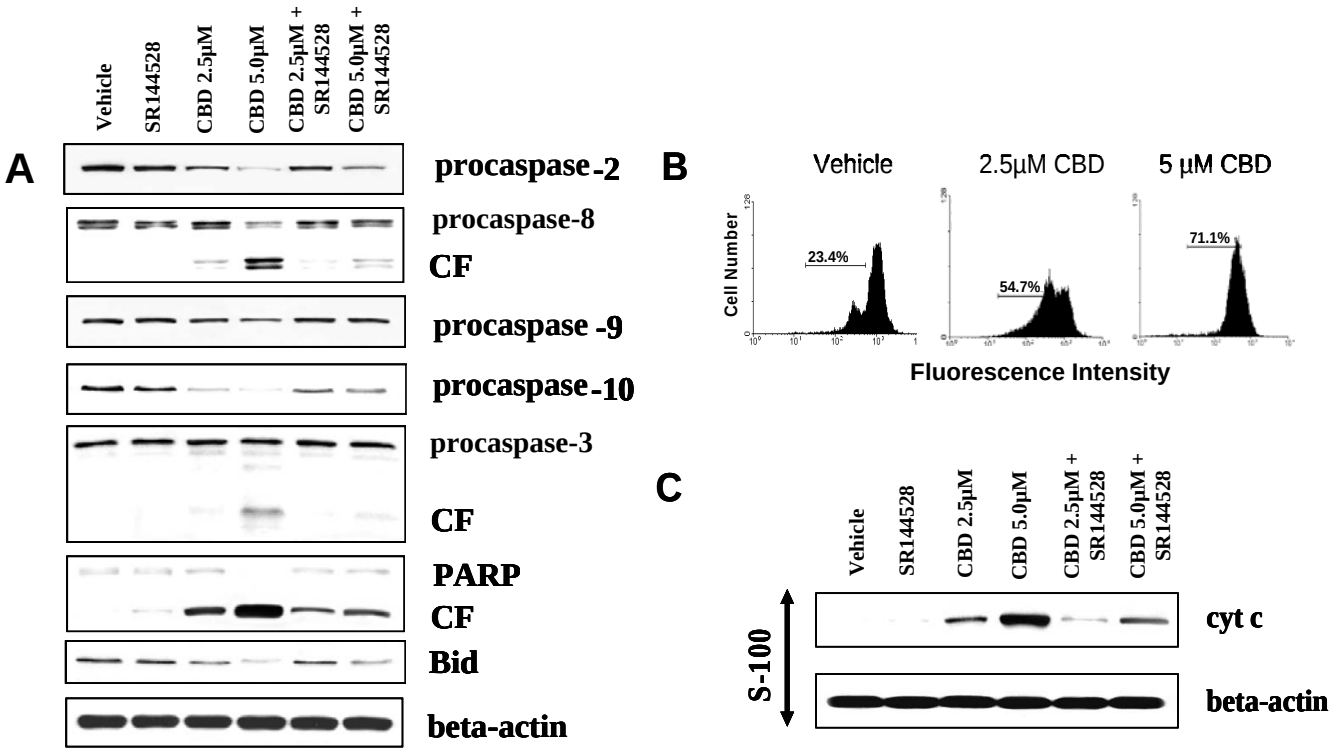
Figure 4



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

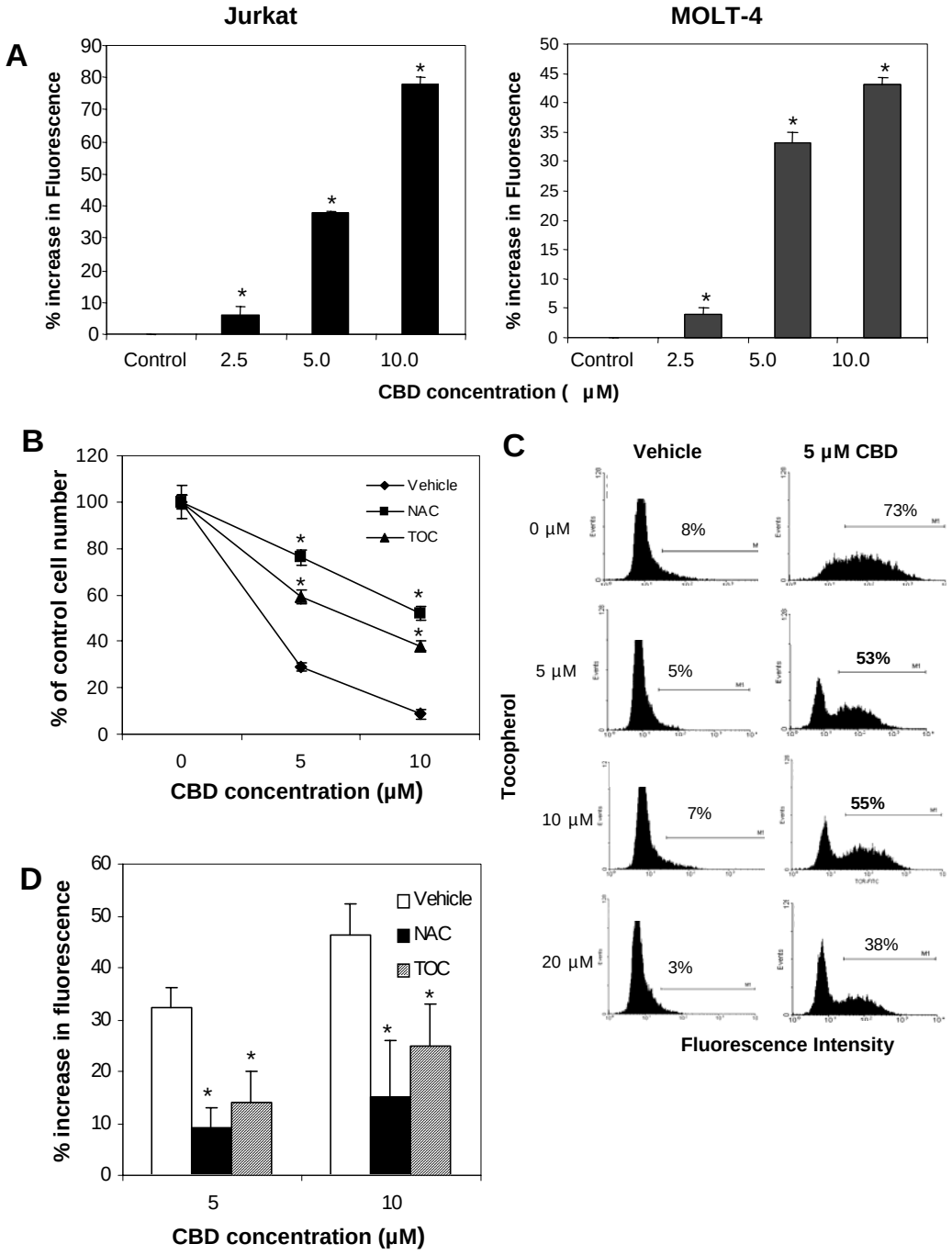
Figure 5



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

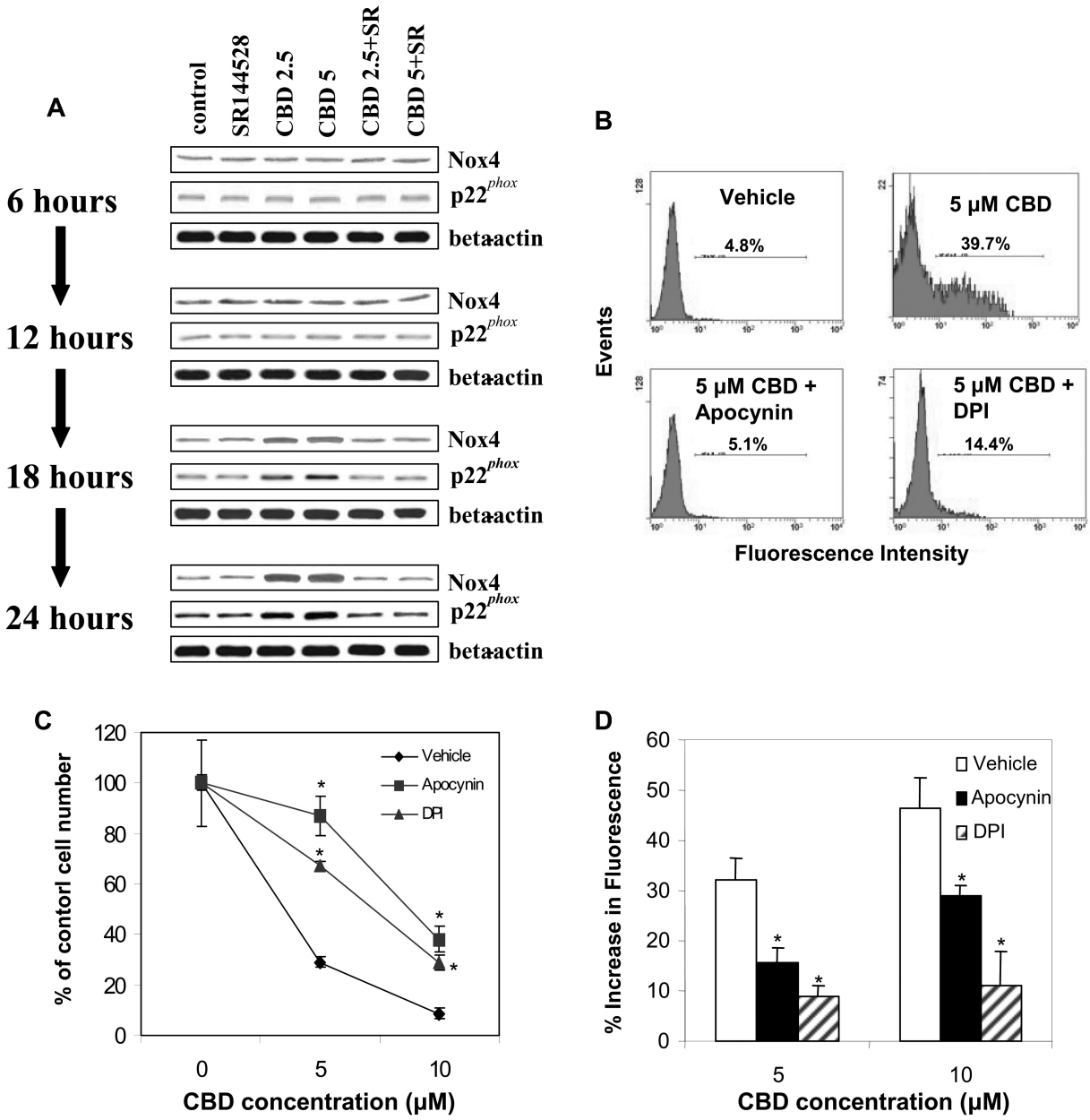
Figure 6



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

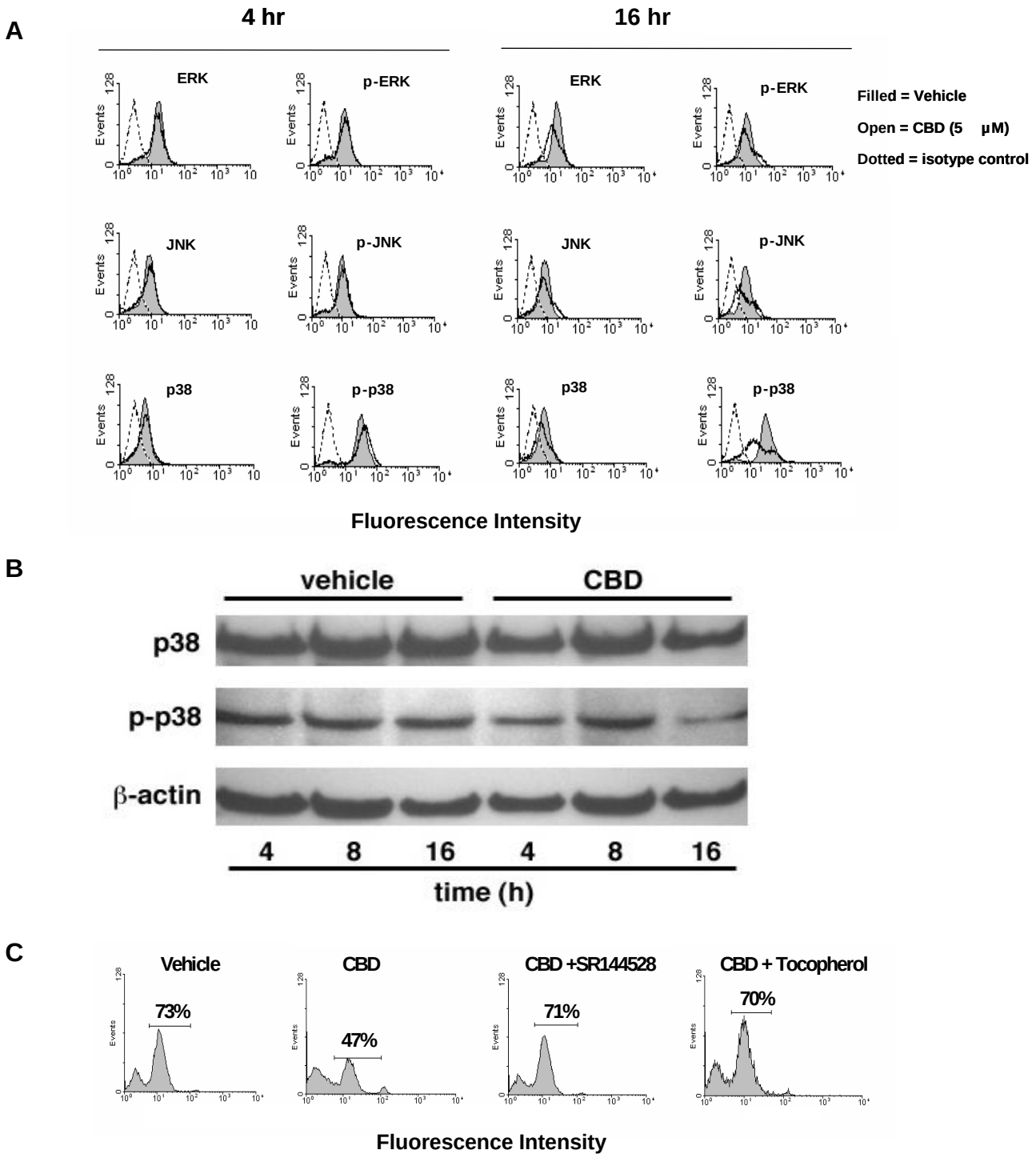
Figure 7



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on June 5, 2006 as DOI: 10.1124/mol.106.023937
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 8



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024