Cell. Mol. Life Sci. 63 (2006) 2057–2066
1420-682X/06/172057-10
DOI 10.1007/s00018-006-6156-x

Cellular and Molecular Life Sciences

Research Article

The non-psychoactive cannabidiol triggers caspase activation and oxidative stress in human glioma cells

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Received 6 April 2006; received after revision 31 May 2006; accepted 22 June 2006
Online First 10 August 2006

Abstract. Recently, we have shown that the non-psychoactive cannabinoid compound cannabidiol (CBD) induces apoptosis of glioma cells *in vitro* and tumor regression *in vivo*. The present study investigated a possible involvement of caspase activation and reactive oxygen species (ROS) induction in the apoptotic effect of CBD. CBD produced a gradual, time-dependent activation of caspase-3, which preceded the appearance of apoptotic death. In addiction, release of cytochrome c and caspase-9 and caspase-8 activation were detected. The exposure to CBD caused in glioma cells an early production of ROS, depletion of intracellular glutathione and increase activity of glutathione reductase and glutathione peroxidase enzymes. Under the same experimental condition, CBD did not impair primary glia. Thus, we found a different sensitivity to the anti-proliferative effect of CBD in human glioma cells and non-transformed cells that appears closely related to a selective ability of CBD in inducing ROS production and caspase activation in tumor cells.

Keywords. Cannabidiol, glioma cells, caspases, reactive oxygen species, oxidative stress, primary glial culture.

Introduction

Apoptosis, also called programmed cell death, is a conserved program of cellular suicide with specific biochemical and morphological features. All apoptotic pathways converge on a family of cysteine aspartases, the caspases, whose activity drives the biochemical events leading to cellular disassembly and death. Two main pathways promote the activation of initiator caspases: the 'mitochondrial' and the 'death-receptor' pathways of cell death, involving caspase-9 and caspase-8, respectively. In different experimental models, these biochemical pathways converge on the activation of the most important executioner caspase, caspase-3, that cleaves several substrate proteins including poly (ADP ribose) polymerase (PARP), resulting in self-destruction of the cells. Apoptosis is required for the development of the nervous system but also occurs during pathophysiological states. Additionally, several anticancer drugs are therapeutically effective by triggering the apoptotic death of malignant cells via activation of different (in many cases yet-to-be identified) mechanisms [1].

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Recently, a role for cannabinoids in the regulation of cell proliferation has been demonstrated, and these findings open new therapeutic possibilities for their use as potential antitumor agents [2, 3]. Cannabinoids are reported to reduce cell proliferation in various cancer cell lines, including breast and prostate cancer cell lines, PC12 pheochromocytoma and malignant glioma cells [2, 3]. The mechanism of this anti-neoplastic activity is not well understood. Some reports correlate the anti-proliferative effects of cannabinoids with their ability to trigger apoptosis in tumor cells. Δ^9-Tetrahydrocannabinol (THC) induced apoptosis in glioma cells in a cannabinoid receptor-dependent mechanism associated with increased levels of intracellular ceramide [4]. However, Sarafian et al. [5, 6] noted that THC promoted necrotic rather than apoptotic cell death. The endocannabinoid anandamide (AEA) was reported to induce apoptosis in neuroblastoma and lymphoma cells [7], but the inhibition of breast cancer MCF-7 cell growth appears unrelated to this mechanism [8]. Thus, there is evidence that cannabinoids possess anticancer action overriding the resistance displayed by the tumors *per se* and can cause the death of tumor cells with different mechanisms depending on the cannabinoid compounds utilized and the tumor cells investigated.

Recently, our laboratory reported that the non-psychoactive cannabinoid compound cannabidiol inhibited the growth of U87 and U373 human glioma cells both *in vivo* and *in vitro*, triggering apoptosis [9]. Since at present the biochemical basis of the action of CBD is still poorly understood, the aim of the present work was to investigate the biochemical pathways activated by CBD in glioma cells. We now show that CBD elicits caspase cascade activation in transformed cells, together with an early increase of reactive oxygen species (ROS) and glutathione (GSH) depletion without affecting primary glial cells. These findings shed light on the cellular antitumor action of the non-psychoactive cannabidiol and might contribute to potential new strategies for its pharmacological approach.

Materials and methods

Materials. CBD was a generous gift from GW Pharmaceuticals (Salisbury, UK). It was initially dissolved in ethanol to a concentration of 100 mM and stored at –20 °C. CBD was further diluted with tissue culture medium for *in vitro* studies to the desired concentration, keeping the ethanol concentration below 0.001%.

Cell culture. U87 human glioma cells were used. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Cell were cultured in 75-cm^2 culturing flask in DMEM supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 10% heat-inactivated fetal bovine serum. For *in vitro* studies, cells were seeded in serum-free medium, consisting of DMEM supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite, in multiwell plates or petri dishes according to experimental protocol. After a 24-h incubation, the medium was removed and new culture medium, containing the compounds to be tested, was added.

Detection of caspase activity. Caspase activity was measured by means of a spectrophotometric assay kit (CaspACE Assay System Colorimetric, Promega, Italy, for caspase-3; Calbiochem, Italy, for caspase-8 and -9) following manufacturer's instructions with some minor modifications. Briefly, at the end of the incubation period, cells were collected in Cell Lysis Buffer (as supplied by the manufacturer) exposed to repeated freeze/thawing cycles, and incubated for 15 min on ice. Insoluble fraction was discarded by centrifugation (5 min at 13 000 rpm) and the protein content in the supernatant was determined according to the method of Bradford [10], and subsequently adjusted to desired concentration in caspase assay buffer (as supplied by the manufacturer). The determination of caspase activity was carried out in a 96-well plate in the presence of the corresponding tetrapeptide conjugated to paranitroaniline (DEVD-pNA for caspase-3, LEHD-pNA for caspase-9, IETD-pNA for caspase-8). Extracts were incubated for 4 h at 37 °C. At the end of the incubation period, released pNA was measured in a spectrophotometer at 405 nm. The specificity of the caspase-3 induction was determined by adding the reversible caspase-3 inhibitor z-DEVD-fmk (50 µM, Calbiochem, Italy) to the cells at the beginning of the experiment, 10 min prior to addition of CBD (25 µM).

Evaluation of cytochrome c release. The release of cytochrome c from the mitochondria to the cytoplasm was evaluated by an enzyme-linked immunosorbent assay (ELISA) for quantitative detection of human cytochrome c (Bender, Italy) in cell culture lysates, following the manufacturer's instructions. Briefly, at the end of the incubation period, cells were collected in Cell Lysis Buffer (1.5×10^6 cells/ml) and incubated 1 h on ice with gentle shaking. Insoluble fraction was discarded by a centrifugation at 1000 g for 15 min and then the supernatant was diluted in Assay Buffer at least 50-fold for the assay. Samples were added to designated wells in a microwell plate coated with monoclonal antibody to human cytochrome c, and biotin-conjugate was then added to all wells. After 2 h at room temperature the wells were washed and a streptavidin-HRP solution was added; the plate was then incubated 1 h at room temperature. After the incubation, the wells were washed, the tetramethylbenzidine (TMB) substrate solution was added, and the plates were incu-

bated for about 15 min at room temperature. After adding the Stop Solution, the color intensity was measured at 450 nm in a microwell reader. The absorbance values of the unknown samples were within the linearity range of the ELISA test, assessed by calibration curves with known amounts of cytochrome c.

Western blot analysis. Cells were scraped and collected by centrifugation, lysed with 20 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100 in the presence of a protease inhibitor cocktail (Sigma-Aldrich, Italy), at 4 °C for 30 min. Extracted proteins (40 µg/well) were separated on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto nitrocellulose membrane. The membrane were then probed overnight with polyclonal anti-caspase-3 antibody (Alexis, Italy, 1 : 1000 in 3% nonfat dry milk in TBS), followed by specific IgG antibody conjugated to horseradish peroxidase (Santacruz, Italy). Detection of proteins was then performed by ECL system. The protein content was determined with BCA protein assay using bovine serum albumin as a standard.

Glial primary cultures. Glial cells were cultured as described in Goetze et al. [11]. Briefly, glial cells were prepared from E17 embryo hemispheres, collected in HBSS and then incubated for 20 min in trypsin EDTA at 37 °C. The hemispheres were resuspended in DMEM-HS (Invitrogen, Italy) and triturated with a 5ml pipette. The cells were then transferred to a 75-ml flask, and, after 24 h, were washed with PBS and maintained in DMEM-HS medium. At 8 days after culture the cells were split before plating in 6-cm petri dishes (20 000 cells/dish). Viability of the cells was evaluated by MTT colorimetric assay, as previously described [9].

Evaluation of ROS production. The formation of intracellular ROS was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Viable cells can deacetylate DCFH-DA to a non-fluorescent product that can quickly react quantitatively with oxygen species within the cell to produce 2′,7′-dichlorofluorescein (DCF), which is fluorescent and trapped inside the cell. The cytofluorimetric measurement of the DCF produced can provide an index of intracellular oxidation. The cells, plated in 6-well plates, were incubated for 20 min with DCFH-DA (20 µM final concentration), then washed, resuspended in fresh medium and treated with CBD at different time of exposure. The fluorescence intensity was measured with a FACSCalibur flow cytometer (Becton Dickinson) equipped with an excitation laser line at 488 nm and CellQuest software (Becton Dickinson).

Determination of GSH content and GSH-related enzymes. After being washed with PBS, cells were resuspended in phosphate buffer 0.1 M (pH 8) containing 5 mM EDTA (1 ml for 10^7 cells), sonicated and centrifuged (30 500 rpm) at 4 °C for 1 h. The cytosolic supernatant was used to measure the GSH content and the activity of GSH-related enzymes, *i.e.* reductase (GRed) and peroxidase (GPox). The GSH content was analyzed fluorimetrically with 350 nm and 420 nm as excitation and emission wavelengths, accordingly to the method of Hissin and Hilf [12] using ophthalaldehyde (OPT) as fluorescent reagent. The GSH concentration was calculated using a standard curve with known amounts of GSH, and expressed as ng/mg protein. The specific activities of GRed and GPox were assayed spectrophotometrically as previously described [13, 14]. Briefly, GRed activity was assayed monitoring, at 340 nm, the decrease in NADPH that is consumed in the conversion of GSSG to GSH. GPox activity was measured as selenium-dependent activity using H_2O_2 as peroxide and the oxidation of NADPH was recorded at 366 nm. The specific activities are expressed as nmol/min/mg protein using the corresponding extinction coefficient.

Statistical analysis. Results are given as mean ± SEM. The significance of differences was evaluated by one-way analysis of variance (ANOVA) for unpaired data followed by *post-hoc* analysis Dunnett's *t*-test.

Results

Caspase-3, -8 and -9 activation after CBD exposure. In a previous study we found that CBD induced apoptosis in human glioma cells after 24 h exposure [9]. To evaluate the involvement of caspases in CBD-induced cell death, we started our analysis from the most important 'effector' caspase, *i.e.* caspase-3.

Because caspase activation is a relatively early event in the apoptotic program, U87 cells were exposed for various periods of time (6–24 h) to a concentration of CBD that was inhibitory (25 µM) or non-inhibitory (10 µM) for glioma growth, as previously evaluated in cell proliferation assays [9]. As shown in Figure 1a, 25 µM CBD significantly activated caspase-3 starting from 14 h after exposure, as evaluated by spectrophotometric analysis of yellow pNA release caused by the cleavage of the synthetic tetrapeptide substrate DEVD-pNA. Maximal caspase-3 activation was present at 17–24 h, when a fivefold increase of enzyme activity with respect to the basal levels was detected. In contrast, the non-inhibitory concentration (10 µM) of CBD did not activate caspase-3 at any time point. Activation of caspase-3 was also confirmed by immunoblotting analysis (Fig. 1b), with an antibody recognizing both the inactive proenzyme (detected as a 32-kDa band) and its proteolytic fragments (detected as 17–20-kDa protein bands). To further confirm the cas-

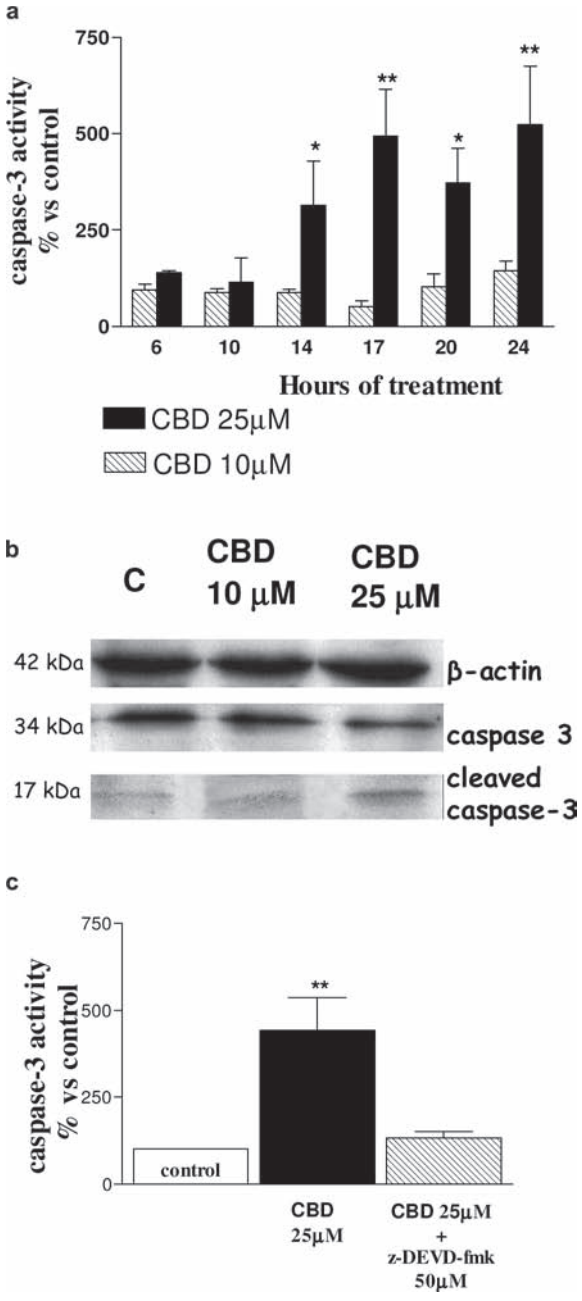

Figure 1. (*a*) Time-dependent induction of caspase-3 activation by CBD. U87 human glioma cells were exposed for various times (6–24 h) to 10 or 25 μM CBD or its vehicle (control). At the end of the incubation with the drug, the caspase-3 activity was determined, evaluating cleavage of the specific caspase-3 substrate DEVD-pNA and release of pNA in a spectrophotometer at 405 nm. Results are expressed as increased percentage of enzyme activity compared with the control cells (100%). Data represent the mean ±SEM of at least four experiments. *p < 0.05, **p < 0.01 *versus* control. Dunnett's *t*-test. (*b*) Western blot analysis of the activation of caspase-3 by 25 μM CBD as evaluated at 17 h peak time. C= control. (*c*) The specificity of the caspase-3 induction was determined after 17 h from CBD exposure by adding the reversible caspase-3 inhibitor z-DEVD-fmk to the cells at the beginning of the experiment. Results are expressed as increased percentage of enzyme activity compared with the control cells (100%). Data represent the mean ±SEM of at least four experiments. **p < 0.01 *versus* control. Dunnett's *t*-test.

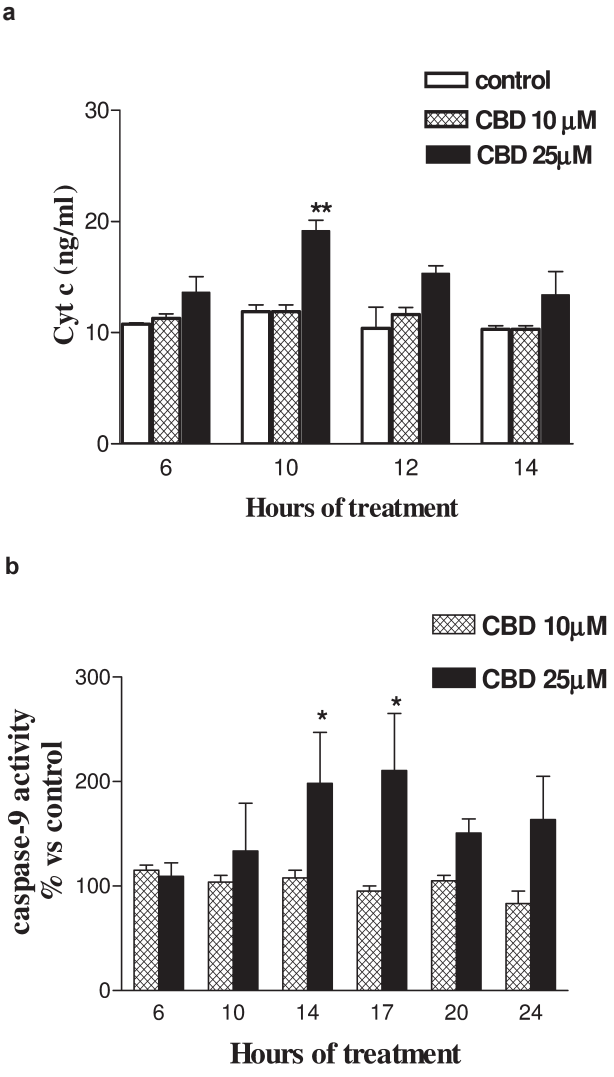

Figure 2. (*a*) Effects of CBD on cytochrome c release from U87 cells. Cells were exposed for various (6–14 h) times to CBD (10 μM or 25 μM) or its vehicle, and the amount of cytochrome c released by mitochondria was determined by ELISA at 405 nm. Results are expressed as ng/ml of cytochrome c calculated from a calibration curve. Data represent the mean ±SEM of at least four experiments. **p < 0.01 *versus* control. Dunnett's *t*-test. (*b*) Time-dependent induction of caspase-9 activation by CBD. U87 human glioma cells were exposed for various periods (6–24 h) to 10 μM or 25 μM CBD or its vehicle (control). At the end of the incubation the caspase-9 activity was determined, evaluating cleavage of the specific caspase-9 substrate LEHD-pNA and release of pNA at 405 nm. Results are expressed as increased percentage of enzyme activity compared with the control cells. Data represent the mean ±SEM of at least four experiments. *p < 0.05 *versus* control. Dunnett's *t*-test.

pase-3 activation, cells were exposed *in vitro* to the specific caspase-3 inhibitor z-DEVD-fmk. z-DEVD-fmk completely prevented either caspase-3 activation (Fig. 1c) or the inhibition of cell viability induced by CBD, as determined by MTT test (data not shown).

To characterize the apoptotic pathways activated by CBD upstream of caspase-3, we focused on the possible role of

the mitochondrial pathway. Activation of this pathway of cell death is coupled, sequentially, to cytochrome c release from mitochondrial membrane, formation of the apoptosome, stimulation of the 'initiator' caspase-9, and activation of effector caspases. As reported in Figure 2a, U87 cells were exposed to CBD for different times (6–14 h), and cytochrome c release was evaluated by ELISA in the cytosolic fraction of untreated cells and cells exposed to 25 μM CBD. The release of cytochrome c was detected starting from 6 h after CBD exposure and reached statistical significance at 10 h. The maximal cytochrome c release preceded the activation of caspase-9 (Fig. 2b), which appeared significantly between 14 and 17 h after the exposure to CBD. As expected, the non-inhibitory concentration of CBD (10 μM) did not cause any release of cytochrome c or activation of caspase-9 at any time point (Fig. 2a, b).

Following a procedure similar to that for caspase-9, we made a detailed (6–24 h) kinetic analysis of the activity of caspase-8, to determine whether the 'death receptor' extrinsic pathway was involved in CBD-induced apoptosis. Cell extracts were incubated with a specific caspase-8 substrate, IETD-pNA, and enzyme activity was evaluated as described in Materials and methods. Figure 3 shows the time course of the specific activation of caspase-8. CBD increased caspase-8 activation 2.5–3.5-fold over basal activity, starting at 10 h after exposure, with an activation occurring before the observed increase of caspase-9 and caspase-3 (see Fig. 2 and Fig. 1, respectively) with a maximal effect seen between 14 and 17 h. In contrast, 10 μM CBD did not affect caspase-8.

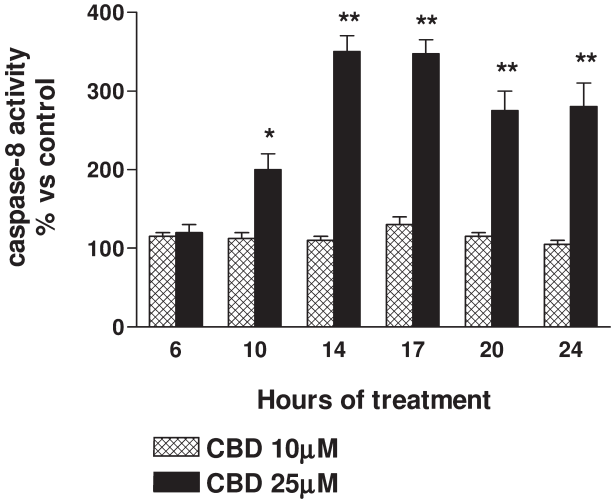

Figure 3. Time-dependent induction of caspase-8 by CBD. U87 human glioma cells were exposed for various periods (6–24 h) to 10 or 25 μM CBD or its vehicle (control). At the end of the incubation, the caspase-8 activity was determined, evaluating cleavage of the specific caspase-8 substrate IETD-pNA and release of pNA at 405 nm. Results are expressed as increased percentage of enzyme activity compared with the control cells (100%). Data represent the mean ± SEM of at least four experiments. *$p < 0.05$, **$p < 0.01$ vs control. Dunnett's *t*-test.

Evaluation of the oxidative stress state induced by CBD. Since in our previous report [9] we demonstrated that the pretreatment of glioma cells with α-tocopherol antagonized the anti-proliferative effect of CBD, we investigated whether CBD acted through the generation of ROS. To detect the production of ROS, we loaded the human glioma cells with the oxidation-sensitive probe DCFH-DA. This dye is nonfluorescent until oxidized to highly fluorescent DCF. As shown in Figure 4a, a kinetic study revealed a time-dependent increase of ROS production in U87 cells treated with 25 μM CBD. ROS formation became significant as early as after 5 h exposure to the drug (Fig. 4a), while 10 μM CBD had no such effect

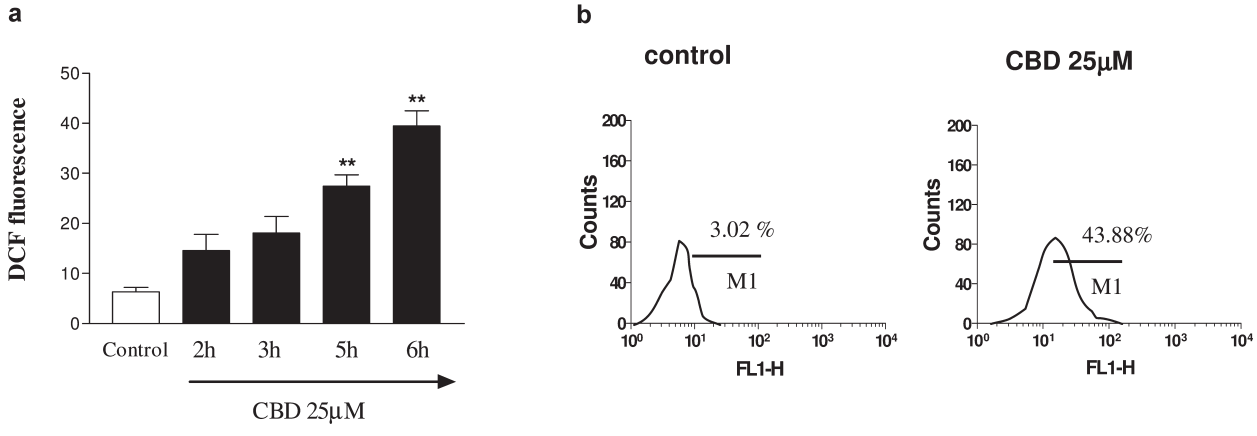

Figure 4. (*a*) Time-dependent production of DCF in glioma cells after exposure to CBD. U87 cells were loaded with DCFH-DA (20 μM final concentration) for 20 min, then exposed to 25 μM CBD for various times (2–6 h). At the end of the incubation, cells were harvested and analyzed with the cytofluorimeter. Data represent the mean ± SEM of at least four experiments. **$p < 0.01$ *versus* control. Dunnett's *t*-test. (*b*) Representative diagrams of the cytofluorimetric analysis performed after 6-h exposure to 25 μM CBD in glioma cells as reported in Materials and methods. The amount of ROS production was quantified as the percentage of cells with increased fluorescence relative to control.

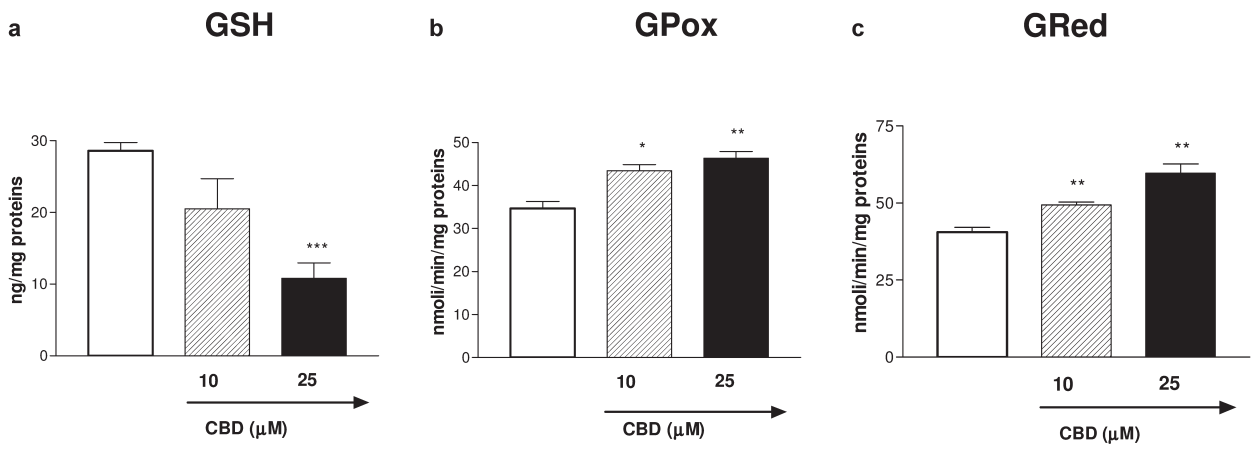

Figure 5. (*a*) Evaluation of the intracellular GSH levels in U87 cells exposed for 6 h to CBD (10 μM or 25 μM); at the end of the incubation cells were lysed and the amount of GSH was measured. (*b*, *c*) Content of GSH-related enzymes GPox and GRed in U87 cells exposed for 6 h to CBD. Data represent the mean ± SEM of at least three experiments. *p < 0.05,**p < 0.01, ***p < 0.001 *versus* control. Dunnett's *t*-test.

at any tested time point (data not shown). To confirm the state of oxidative stress caused by CBD, we also examined the level of intracellular GSH. In U87 cells exposed to CBD for 6 h, GSH was significantly depleted (by about 55%) by 25 μM CBD, while 10 μM concentrations did not cause a significant GSH reduction (Fig. 5a). The activity of GSH-associated enzymes was also tested. GPox and GRed activities were significantly stimulated by the exposure to either 10 μM or 25 μM CBD (Fig. 5b, c).

Effect of CBD in primary cultures. An important step during the development of an anticancer drug consists in the evaluation of its toxicity on non-transformed cells. Therefore, we studied the cytotoxic effect of CBD on glial primary culture (Fig. 6). Glial cells viability was not affected by the treatment with CBD up to a concentration 50 μM, the highest tested in this study; in contrast, as expected, glioma cells viability was inhibited by the drug in a concentration-dependent manner (Fig. 6). This different

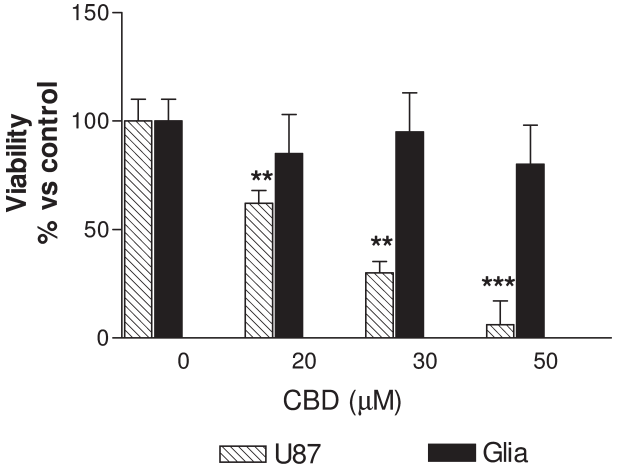

Figure 6. Evaluation of cell viability in primary glial culture and U87 cells after a 24-h exposure to CBD (see Materials and methods). **p < 0.01, ***p < 0.001 *versus* control. Dunnett's *t*-test.

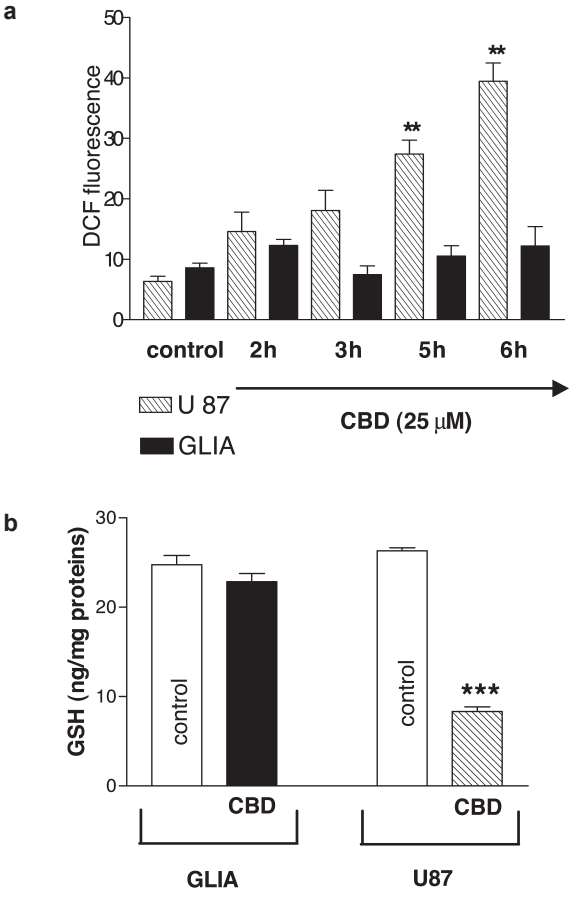

Figure 7. (*a*) Time-dependent production of DCF in U87 and primary glial cells after exposure to CBD. Cells were loaded with DCFH-DA (20 μM final concentration) for 20 min, then exposed to 25 μM CBD for various times (2–6 h). At the end of the incubation, cells were harvested and analyzed with the cytofluorimeter. Data represent the mean ± SEM of at least three experiments. **p < 0.01 *versus* control. Dunnett's *t*-test. (*b*) Evaluation of the intracellular GSH levels in U87 and primary glial cells exposed for 6 h to 25 μM CBD; at the end of the incubation cells were lysed and the amount of GSH was measured. Data represent the mean ± SEM of at least three experiments. ***p < 0.001 *versus* control. Dunnett's *t*-test.

sensitivity of transformed *versus* non-transformed cells was also confirmed by different rates of ROS production. As shown in Figure 7a, CBD induced a time-dependent ROS increase in glioma cells; in contrast, the exposure to the drug did not cause any production of ROS in glial cells. These results were confirmed in GSH content studies: while U87 cells showed a significant reduction in GSH levels after CBD exposure, glial primary cells did not (Fig. 7b).

Discussion

Recently, we have reported that the non-psychoactive cannabinoid compound CBD triggers apoptosis in human glioma cells [9]. Despite the great pharmacological interest in this molecule that is lacks a psychotropic effect and has a high potential of therapeutic use [15], the investigations conducted so far in elucidating the cellular action of CBD are very limited. The present study was undertaken to shed light on the possible molecular mechanism by which CBD induces apoptosis in glioma cells. We demonstrated, for the first time, that CBD induces cell death through a caspase-dependent mechanism triggered by an early production of ROS and marked depletion of GSH content.

The time course of activation of caspases following CBD treatment provides useful information on the mechanism of apoptosis in glioma cells. After 10 h of CBD exposure, the cytoplasmic cytochrome c release was increased; this event preceded the maximal activation of caspase-9, observed between 14 and 17 h. Interestingly, there was also significant activation of caspase-8, which was already evident at 10 h after treatment with the drug. Thus, the time course suggests a concomitant activation of both caspase-8 and -9, and supports the evidence that their activation is the cause, rather than a consequence, of caspase-3 activation, and that both intrinsic and extrinsic pathways of apoptosis are involved in CBD cell death. Although activation of caspase-3 and -9 was expected, the activation of caspase-8, an apical caspase was surprising. There is compelling evidence that caspase-8 is the major initiator caspase recruited by death receptors, mainly the Fas receptor and TNF receptor expressed in different transformed cells, including glioma cells [16]. Even though death receptors and mitochondria represent two distinct apoptotic initiators, the two death pathways are known, in some case, to communicate with each other in the different stages of apoptotic process. It has been reported that a hybrid cannabinoid-vanilloid agonist such as arvanil can induce apoptosis in Jurkat cells, with an unknown mechanism, through a FADD/caspase-8-dependent pathway [17]. An intriguing possibility, supported by a recent paper [18] and also proposed by Sancho et al. [17], suggests a link between ROS generation and Fas-induced cell death. The authors suggest that cisplatin, γ-irradiation or arvanil promote ROS formation in Jurkat cell line, contributing in turn, in an undetermined manner, to Fas receptor aggregation, leading to the activation of caspase-8 and cell death. Also Lombard et al. [19] have described a cross-talk between extrinsic and intrinsic pathways in Δ^9-tetrahydrocannabinol (THC)-induced apoptosis in Jurkat cells, with no clear evidence of the specific cellular mechanism induced by the drug. We still have no experimental evidence of the direct engagement of death receptors in CBD apoptosis, but experiments are now in progress to evaluate this point.

Subsequently, we evaluated the mechanistic basis for the caspase cascade activation by CBD. Since our previous results [9] indicated that the anti-proliferative effect of CBD was receptor independent, but partially prevented only by the antioxidant agent α-tocopherol, we postulated that the effect of CBD might be attributable to ROS production. We therefore investigated in the present work the existence of oxidative stress state in glioma cells after CBD exposure. We report here for the first time that CBD does induce significant ROS production, GSH depletion and increase activity of GPox and GRed enzymes, as early as 5–6 h after CBD exposure, with a time course preceding caspases activation. Cells possess an arsenal of defense and repair mechanism to deal with the potentially dangerous ROS. Major players in these defense mechanisms include antioxidant enzymes and small molecules such as GSH. If the generation of ROS exceeds the scavenging capacity of the cell, and if there is a contemporary decrease in GSH level counteracted by the increased activity of associated anti-oxidant enzymes, the cell could initiate cell death-linked molecular events. Therefore, based on our results, a likely mechanism by which CBD induces apoptosis in human glioma cells might involve the induction of oxidative stress, followed by the activation of caspase-9 and -8 which, in turn, cleave caspase-3.

How does CBD induce ROS accumulation ? It is believed that mitochondria are a major source of ROS, and mitochondria are also the site where many ROS-metabolizing enzymes are situated. Since CBD possesses a very weak affinity for cannabinoid receptors and because we were able to demonstrate a cannabinoid and vanilloid receptor-independent induction of apoptosis induced by CBD [9], we can suppose that CBD, with its lipophilic properties, can act by intercalating into the cell membrane. Alternatively, it has been proposed that CBD can stimulate a putative CB3 receptor or other, as yet unidentified receptors [15, 20–22]. Moreover, since, as recently reported by Drysdale et al. [21], CBD can act as potent modulator of $[Ca^{2+}]_i$ through the modulation of intracellular stores, a role of calcium in driving some aspects of tumor cell death cannot be ruled out. A very intriguing hypothesis, as already demonstrated by Sarker et al. [23] for apoptosis

induced by anandamide, is that the lipophilic CBD signaling could be mediated by a membrane lipid raft domain. Thus, it appears that CBD, a cannabinoid compound with a very low affinity for CB1 and CB2 receptors, is able to cause apoptosis and trigger complete caspase cascade activation in a manner similar to that already described for other cannabinoids. This is not surprising since cell death induced by cannabinoids is sometimes dependent or independent by cannabinoid receptor stimulation [3, 24, 25]. The type of molecule, doses, cells, and system used in the evaluation of cellular proliferation are a crucial issue for debate in the cannabinoid field.

Based on our results, the reported oxidant properties of CBD appear somewhat surprising and in contrast with the antioxidant effects described so far for CBD [26–30] as well as for other cannabinoids. The antioxidant properties of cannabinoids could be related, in some cases, to their chemical structure, which include a phenolic ring typical of many antioxidants isolated from plants. In contrast, a majority of the reports agree with a potent pro-oxidant/cytotoxic action of cannabinoids on tumor cells [22] *versus* a protective effect displayed in normal tissues [31]. On this subject, it has been reported that anandamide induces oxidative stress in PC12 cells by generation of intracellular superoxide anion that triggers caspase activation [32], and that it can enhance the susceptibility of HepG2 cells to oxidative stress [33]. Since the disruption of lipid rafts prevents anandamide-induced apoptosis in many cell lines [23], it has been suggested that anandamide can enter into the cells through cholesterol-rich lipid rafts, inducing an increase in intracellular ROS, which, in turn, triggers the apoptosis [23]. It has also been demonstrated that oxidative stress and excessive intracellular calcium are involved in the anti-proliferative effect of anandamide, as it can be completely blocked by α-tocopherol (an antioxidant) and calpeptin (a calpain inhibitor) [34]. In C6 cells, stearoylethanolamide induces apoptosis through a mechanism involving oxidation of the content of the cells [35]. In addition, Sancho et al. [17] reported that arvanil, a capsaicin-anandamide hybrid molecule, induced ROS production in both wild-type and FADD dominant negative ($FADD_{DN}$) Jurkat cells, suggesting a tight link between ROS generation and cell death. Also THC was reported to be toxic for hippocampal neurons and its action appears related the generation of free radicals by cyclooxygenase [36]. Similarly, Sarafian et al. [37] found the generation of ROS in the endothelial cell line ECV 304 after an exposure to marijuana smoke. Thus, although the precise molecular mechanism by which cannabinoids exert their oxidative action on cells is not yet clear, they are unquestionably able to evoke it. An intriguing hypothesis is that, in experiments *in vitro*, CBD can be partially air oxidized and transformed into cannabidiol quinone, a potent anti-tumor agent with a possible redox cycling activity [38]. Nevertheless, this would not seem to be the case since the CBD treatment of non-transformed glial cells under the same experimental conditions did not induce any oxidative stress state.

Thus, although the antioxidant-pro-oxidant effects of cannabinoids in cells are not completely understood, these properties appear unrelated to the chemical features of the molecule, but it seems to depend from the different biochemical and cellular features of tumor *versus* non-tumor cells rather than from the molecule itself.

A very important finding in the present study was the lack of cytotoxic effect of CBD on non-transformed glial cells, demonstrated by the lack of induction of an oxidative stress state by CBD on primary cultures. Although this very surprising aspect of cannabinoid action has been already documented in other sets of experiments [39–42], there is not at the moment a convincing explanation for this behavior. It may be that this dual property depends on differences in the features of normal *versus* transformed cells. Apparently, an opposite regulation of CB1 and CB2 expression on tumoral *versus* normal cells could justify the different sensitivity to CBD. In our hands, this does not seem to be the case because, beside the demonstration of a receptor-independent effect of CBD in inducing glioma cell death [9], glioma cells (data not shown) and glial cells possess both cannabinoid receptors [43, 44]. Thus, the different sensitivity could be ascribed to differences in signal transduction events downstream on the cell surface and/or in different redox state-cellular features of the tumoral cells *versus* normal cells. This hypothesis is consistent with the finding that oxidative metabolism and associated sensitivity to apoptosis in tumor cells differ from normal cells [45, 46]. This CBD selectivity, if definitively proved for additional tumor cell lines *versus* healthy control cells, might be of considerable therapeutic interest.

In summary, we have demonstrated that CBD triggers apoptosis of human glioma cells by a cellular mechanism that involves an early production of ROS, depletion of GSH, and concomitant activation of initiator caspase-8 and -9, converging in activation of the downstream effector caspase-3, with no effect in non-transformed cells. The present findings may have useful implications for the potential use of CBD in anti-neoplastic therapy, adding new knowledge on its real molecular action on cells. Understanding the precise pathways of induction of apoptosis may help exploit CBD treatment modalities in cancer therapy, either alone or in combination with other chemotherapeutic agents.

Acknowledgements. The authors wish to thank GW Pharmaceuticals for providing CBD and financial support to conduct these studies. We wish to thank Natalia Realini for her technical assistance in primary glial culture and quantitative data analysis.

1 Kaufmann, S. H. and Earnshaw, W. C. (2000) Induction of apoptosis by cancer chemotherapy. Exp. Cell Res. 256, 42–49.
2 Guzman, M. (2003) Cannabinoids: potential anticancer agents. Nat. Rev. Cancer 10, 745–755.
3 Parolaro, D., Massi, P., Rubino, T. and Monti, E. (2002) Endocannabinoids in the immune system and cancer. Prostaglan. Leuk Essen. Fatty Acids 66, 319–332.
4 Galve-Roperh, I., Sanchez, C., Cortes, M. L., Del Pulgar, T. G., 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, 313–319.
5 Sarafian, T. A., Tashkin, D. P. and Roth, M. D. (2001) Marijuana smoke and Delta(9)-tetrahydrocannabinol promote necrotic cell death but inhibit Fas-mediated apoptosis. Toxicol. Appl. Pharmacol., 174, 264–272.
6 Sarafian, T. A., Kouyoumjian, S., Tashkin, D. and Roth, D. M. (2002) Synergistic cytotoxicity of Δ^9-tetrahydrocannabinol and butylated hydroxyanisole. Toxicol. Lett. 133, 171–179.
7 Maccarrone, M., Lorenzon, T., Bari, M., Melino, G. and Finazzi-Agrò, A. (2000) Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. J. Biol. Chem. 275, 31938–31945.
8 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. USA 95, 8375–8380.
9 Massi, P., Vaccani, A., Ceruti, S., Colombo, A., Abbracchio, M. P. and Parolaro, D. (2004) Antitumor effects of cannabidiol, a nonpsychoactive cannabinoid, on human glioma cell lines. J. Pharmacol. Exp. Ther. 308, 838–845.
10 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. **7**, 248–254.
11 Goetze, B., Grunewald, B., Kiebler, M. A. and Macchi, P. (2003) Coupling the iron-responsive element to GFP-an inducible system to study translation in a single living cell. Sci. STKE 14, PL12.
12 Hissin, P. J. and Hilf, R. (1976) A fluorimetric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–226.
13 Colman, R. F. (1981) Glutathione reductase. Methods Enzymol. 17, 500–508.
14 Wendel, A. (1981) Glutathione peroxidase. Methods Enzymol. 77, 325–333.
15 Pertwee, R. G. (2004) The pharmacology and therapeutic potential of cannabidiol. In: Cannabinoids, pp. 32–83, Di Marzo, V. (ed.), Kluver Academic/Plenum Publishers, New York.
16 Chen, M. and Wang, J. (2002) Initiator caspases in apoptosis signaling pathways. Apoptosis 7, 313–319.
17 Sancho, R., De La Vega, L., Appendino, G., Di Marzo, V., Macho, A. and Munoz, E. (2003) The CB1/VR1 agonist arvanil induces apoptosis through an FADD/caspase-8-dependent pathway. Br. J. Pharmacol. 140, 1035–1044.
18 Huang, H. L., Fang, L. W., Lu, S. P., Chou, C. K., Luh, T. Y. and Lai, M. Z. (2003) DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation. Oncogene 22, 8168–8177.
19 Lombard, C., Nagarkatti, M. and Nagarkatti, P. S. (2005) Targeting cannabinoid receptors to treat leukemia: role of cross-talk between extrinsic and intrinsic pathways in Δ^9-tetrahydrocannabinol (THC)-induced apoptosis of Jurkatt cells. Leuk. Res. 29, 915–922.
20 Pertwee, R. G., Thomas, A., Stevenson, L. A., Maor, Y. and Mechoulam, R. (2005) Evidence that (–)-7-hydroxyl-4′-dimethylheptyl-cannabidiol activates a non-CB(1), non-CB(2), non-TRPV1 target in the mouse vas deferens. Neuropharmacology 48, 1139–1146.
21 Drysdale, A. J., Ryan, D., Pertwee, R. G and Platt, B. (2006) Cannabidiol-induced intracellular Ca^{2+} elevation in hippocampal cells. Neuropharmacology 50, 621–631.
22 Kogan, N. M. (2005) Cannabinoids and cancer. Mini Rev. Med. Chem. 5, 941–952.
23 Sarker, K. P. and Maruyama, I. (2003) Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. Cell. Mol. Life Sci. 60, 1200–1208.
24 Ruiz, L., Miguel, A. and Diaz-Laviada, I. (1999) Delta-9-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism. FEBS Lett. 458, 400–404.
25 Bifulco, M., Laezza, C., Pisanti, S. and Gazzero, P. (2006) Cannabinoids and cancer: pros and cons of an antitumor strategy. Br. J. Pharmacol. 148, 123–135.
26 Marsicano, G., Moosmann, B., Hermann, H., Lutz, B. and Behl, C. (2002) Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. J. Neurochem. 80, 448–456.
27 Hampson, A. J., Grimaldi, M., Axelrod, J and Wink, D. (1998) Cannabidiol and (–)delta9-tetrahydrocannabinol are neuroprotective antioxidants. Proc. Natl. Acad. Sci. USA 95, 8268–8273.
28 Braida, D., Pegorini, S., Arcidiacono, V., Consalez, G. G. Croci, L. and Sala, M. (2003) Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion and neuronal injury in gerbils. Neurosci. Lett. 31, 61–64.
29 Costa, B., Colleoni, M., Conti, S., Parolaro, D., Franke, C., Trovato, A. E. and Giagnoni, G. (2004) Oral anti-inflammatory activity of cannabidiol, a non psychoactive constituent of cannabis, in acute carrageenan–induced inflammation in the rat paw. Naunyn Schmiedebergs Arch. Pharmacol. 369, 294–299.
30 Malfait, A. M., Gallily, R., Sumariwalla, P. F., Malik, A. S., Andreakos, E., Mechoulam, R. and Feldmann, M. (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. Proc. Natl. Acad. Sci. USA 95, 9561–9566.
31 Velasco, G., Galve-Roperh, I., Sanchez, C., Blasquez, C. and Guzman, M. (2004) Hypothesis: cannabinoid therapy for the treatment of gliomas? Neuropharmacology 47, 315–323.
32 Sarker, K. P., Obara, S., Nakata, M., Kitajima, I. and Maruyama, I. (2000) Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. FEBS Lett. 472, 39–44.
33 Biswas, K. K., Sarker, K. P., Abeyama, K., Kawahara, K., Iino, S., Otsubo, Y., Saigo, K., Izumi, H., Hashiguchi, T., Yamakuchi, M., Yamaji, K., Endo, R., Suzuki, K., Imaizumi, H. and Maruyama, I. (2003) Membrane cholesterol but not putative receptors mediates anandamide-induced hepatocyte apoptosis. Hepatology 38, 1167–1177.
34 Jacobsson, S. O. P., Wallin, T. and Fowler, C. J. (2001) Inhibition of rat C6 glioma cell proliferation by endogenous and synthetic cannabinoids. Relative involvement of cannabinoid and vanilloid receptors. J. Pharmacol. Exp. Ther. 299, 951–959.
35 Maccarrone, M., Pauselli, R., Di Rienzo, M. and Finazzi-Agrò, A. (2002) Binding, degradation and apoptotic activity of stearoylethanolamide in rat C6 glioma cells. Biochem. J. 366, 137–144.
36 Chan, G. C-K., Hinds, T. R., Impey, S. and Storm, D. R. (1998) Hippocampal neurotoxicity of Δ^9-tetrahydrocannabinol. J. Neurosci. 18, 5322–5332.
37 Sarafian, T. A., Magallanes, J. A. M., Shau, H., Tashkin, D. and Roth, M. D. (1999) Oxidative stress produced by marijuana smoke. Am. J. Respir. Cell Mol. Biol. 20, 1286–1293.
38 Kogan, N. M., Rabinowitz, R., Levi, P., Gibson, D., Sandor, P., Schlesinger, M. and Mechoulam, R. (2004) Synthesis and

antitumor activity of quinonoid derivatives of cannabinoids. J. Med. Chem. 47, 3800–3806.
39 Sanchez, C., Galve-Roperh, I., Canova, C., Brachet, P. and Guzman, M. (1998) Delta-9 tetrahydrocannabinol induces apoptosis in C6 glioma cells. FEBS Lett. 436, 6–10.
40 Casanova, M. L., Blasquez, C., Martinez-Palacio, J., Villanueva, C., Fernandez-Acenero, M. J., Huffman, J. W., Jorcano, J. L. and Guzman, M. (2003) Inhibition of skin tumor growth and angiogenesis *in vivo* by activation of cannabinoid receptors. J. Clin. Invest. 111, 43–50.
41 Gallily, R., Even-Chena, T., Katzavian, G., Lehmann, D., Dagan, A and Mechoulam, R. (2003) γ-irradiation enhances apoptosis induced by cannabidiol, a non psychotropic cannabinoid, in cultured HL-60 myeloblastic leukemia cells. Leuk. Lymphoma 44, 1767–1773.
42 Duntsch, C., Divi, M. K., Jones, T., Zhou, Q., Krishnamurthy, M., Boehm, P., Wood, G., Sills, A. and Moore, B. M. (2005) Safety and efficacy of a novel cannabinoid chemotherapeutic, KM-233, for the treatment of high-grade glioma. J. Neurooncol. 29, 1–10.
43 Cabral, G. A. and Marciano-Cabral, F. (2005) Cannabinoid receptors in microglia of the central nervous system: immune functional relevance. J. Leukoc. Biol. 78, 1192–1197.
44 Maresz, K., Carrier, E. J., Ponomarev, E. D., Hillard, C. J. and Dittel, B. N. (2005) Modulation of the cannabinoid CB2 receptor in microglial cell in response to inflammatory stimuli. J. Neurochem. 95, 437–445.
45 Herdener, M., Heigold, M., Saran, M. and Bauer, G. (2000) Target cell-derived superoxide anions cause efficiency and selectivity of intracellular induction of apoptosis. Free Radical. Biol. Med. 29, 1260–1271.
46 Schimmel, M. and Bauer, G. (2002) Proapoptotic and redox state-related signaling of reactive oxygen species generated by transformed fibroblasts. Oncogene 20, 5886–5896.