

Antitumor Effects of Cannabidiol, a Nonpsychoactive Cannabinoid, on Human Glioma Cell Lines

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ABSTRACT

Recently, cannabinoids (CBs) have been shown to possess antitumor properties. Because the psychoactivity of cannabinoid compounds limits their medicinal usage, we undertook the present study to evaluate the *in vitro* antiproliferative ability of cannabidiol (CBD), a nonpsychoactive cannabinoid compound, on U87 and U373 human glioma cell lines. The addition of CBD to the culture medium led to a dramatic drop of mitochondrial oxidative metabolism [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide test] and viability in glioma cells, in a concentration-dependent manner that was already evident 24 h after CBD exposure, with an apparent IC_{50} of 25 μ M. The antiproliferative effect of CBD was partially prevented by the CB2 receptor antagonist *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528; SR2) and α -tocopherol. By contrast, the CB1 cannabinoid receptor antagonist *N*-(piperidin-1-yl)-5-(4-

chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride (SR141716; SR1), capsazepine (vanilloid receptor antagonist), the inhibitors of ceramide generation, or pertussis toxin did not counteract CBD effects. We also show, for the first time, that the antiproliferative effect of CBD was correlated to induction of apoptosis, as determined by cytofluorimetric analysis and single-strand DNA staining, which was not reverted by cannabinoid antagonists. Finally, CBD, administered *s.c.* to nude mice at the dose of 0.5 mg/mouse, significantly inhibited the growth of subcutaneously implanted U87 human glioma cells. In conclusion, the nonpsychoactive CBD was able to produce a significant antitumor activity both *in vitro* and *in vivo*, thus suggesting a possible application of CBD as an antineoplastic agent.

Marijuana and its derivatives have been used in medicine for many centuries, and currently there is a renewed interest in the study of the therapeutic effects of cannabinoids. Cannabinoids produce their effects by binding to specific plasma membrane G protein-coupled receptors. To date, two cannabinoid receptors have been characterized: the CB1 receptor, expressed primarily in the brain and in some peripheral tissues, and CB2 receptors, expressed by cells of the immune

system (Howlett et al., 2002; Pertwee and Ross, 2002). Ongoing research is determining whether cannabinoid ligands may be effective agents in the treatment of pain, glaucoma, the wasting and emesis associated with cancer chemotherapy and AIDS, and neurodegenerative disorders such as multiple sclerosis (Goutopoulos and Makriyannis, 2002). Among the potential therapeutic activities, one of the most exciting and promising areas of current cannabinoid research is the demonstrated ability of these compounds to affect a number of pathways involved in the cell survival/death decision (Bifulco and Di Marzo, 2002; Guzman et al., 2002).

Both natural and synthetic as well as endogenous cannabinoids have been found to affect the rate of cell proliferation in cell lines derived from the central nervous system. Very

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ABBREVIATIONS: CB, cannabinoid; CBD, cannabidiol; THC, Δ^9 -tetrahydrocannabinol; VR, vanilloid receptor; AEA, *N*-arachidonylethanolamide (anandamide); MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; PMA, phorbol 12-myristate 13-acetate; WIN 55,212-2, *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate; SR141716A (SR2), *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; SR144528 (SR1), *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; PBS, phosphate-buffered saline; PI, propidium iodide; ssDNA, single-strand DNA; ELISA, enzyme-linked immunosorbent assay; CPZ, capsazepine; PTX, pertussis toxin.

intriguing was the demonstration that THC and WIN-55,212-2 have been demonstrated to suppress the growth of rat glioma C6 cells inoculated intracerebrally in the rat or subcutaneously in immune-deficient mice, through a cannabinoid receptor-dependent mechanism (Sanchez et al., 1998; Galve-Roperh et al., 2000; Guzman et al., 2002). These observations were of the utmost interest for their possible impact on the clinical management of malignant gliomas, which represent the most common form of brain tumor associated with an unfavorable prognosis and refractoriness to surgical, radiological, and pharmacological treatment. However, the well known psychotropic effects of THC and related compounds raise a number of clinical and ethical considerations, thus limiting their medicinal usage. A subsequent work also highlighted that CB2 selective agonist can induce either *in vitro* or *in vivo* a significant tumor regression (Sanchez et al., 2001). However, the application of CB2 compounds is limited by their intrinsic immunosuppressive effects that would be expected to inhibit host antitumor immunity. As a matter of fact, Zhu et al. (2000) have recently reported that THC injection led to an accelerated growth of lung tumor implants in immune-competent mice through an involvement of CB2 receptors.

Therefore, one alternative therapeutic approach is represented by the use of nonpsychoactive cannabinoids. Among the bioactive constituents of marijuana, cannabidiol (CBD) does not have significant intrinsic activity over cannabinoid receptors (Howlett et al., 2002) and, thus, does not produce psychotropic and adverse side effects, which makes it one of the bioactive constituents with the highest potential for therapeutic use. Moreover, recent reports indicate that CBD can act as a neuroprotective agent in both *in vivo* (Braida et al., 2003) and *in vitro* studies (Hampson et al., 2000). Regarding the potential effects of CBD on the immune system, they appear somehow different and/or weaker, as compared with the classical cannabinoid effects. Although the work of Malfait et al. (2000) and Faubert Kaplan et al. (2003) suggested anti-inflammatory and/or immunosuppressive properties of CBD, other studies were not unequivocal. Smith et al. (1997) reported no effect of CBD in affecting the mortality of mice sublethally infected with *Legionella*, and a recent paper by Killestein et al. (2003) reported in multiple sclerosis patients, treated orally with a combination of THC/CBD, an increase in plasma interleukin-12 level, thus suggesting a pro- rather than anti-inflammatory effect of the therapy. Moreover, in accordance with these data and with our unpublished observations, Srivastava et al. (1998) found stimulation and inhibition in some cytokine levels induced by CBD. Thus, the nonpsychotropic cannabinoid seems to possess a minor impact on immune function. Therefore, the present study was undertaken to investigate, *in vitro* and *in vivo*, the possible antiproliferative effect of CBD on two glioma cell lines of human origin and characterize its mechanism of action.

Materials and Methods

Materials. CBD was a generous gift from GW Pharmaceuticals (Salisbury, UK). It was initially dissolved in ethanol to a concentration of 250 mM and stored at -20°C . CBD was further diluted with tissue culture medium for *in vitro* studies or PBS in *in vivo* studies to the desired concentration, keeping the ethanol concentration below 0.001%. SR141716 and SR144528 were kindly given by Dr. F. Barth (Sanofi Synthelabo Recherche, Montpellier, France).

L-Cycloserine, phorbol 12-myristate 13-acetate (PMA), fumonisin

B₁, desipramine, α -tocopherol, capsazepine, and pertussis toxin were purchased from Sigma-Aldrich (St. Louis, MO). Tissue culture media and all supplements were obtained from Sigma-Aldrich.

Cell Culture. U87 and U373 human glioma cells were used. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. Cells were cultured in 75-cm² culturing flasks in DMEM supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ 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.

Analysis of Cell Viability. To determine the effects of CBD upon cell viability, we carried out the MTT colorimetric assay ([3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide]; Sigma-Aldrich). Briefly, glioma cells were seeded in a 96-well flat bottom multiwell at a density of 6000 cells/well for U373 and 8000 cells/well for U87 cells. After 24 h, cells were treated with CBD and/or antagonists/inhibitors at the indicated concentrations and times. At the end of the incubation with the drugs, MTT (0.5 mg/ml final concentration) was added to each well and the incubation was then continued for 4 h. The insoluble formazan crystals were solubilized by the addition of 100 μl of 100% dimethyl sulfoxide. The plates were read at 570 nm using an automatic microtiter plate reader.

The viability of the cells was also estimated by the trypan blue dye-exclusion method. Cells were seeded in Petri dishes (40,000 cells/dish). After 24 h, cells were treated with CBD for the desired time. At the end of the exposure, the cells were harvested and incubated with 0.1% trypan blue for 2 to 5 min. The percentage of cells that excluded the vital dye trypan blue was determined microscopically.

Flow Cytometric Analysis. Tumor cells were cultured in 12-well plates in the presence or absence of CBD and/or cannabinoid antagonists for 24 h, as described above. The percentage of apoptotic cells on the total cell population (adhering + detached cells) was evaluated as previously described (Ceruti et al., 2000). Briefly, cells were collected, washed, and centrifuged at 200g. The cell pellet was gently resuspended in 1 ml of hypotonic fluorochrome solution [propidium iodide (PI), 50 $\mu\text{g/ml}$ in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma-Aldrich].

Cells were analyzed after a minimum of 30 min of incubation in the dark at room temperature, and apoptosis was detected in individual cells using a flow cytometer (equipped with a single 488-nm argon laser; BD Biosciences, San Jose, CA) by reduced fluorescence of PI in apoptotic nuclei.

Detection of Single-Strand DNA (ssDNA). To further confirm the presence of apoptosis and discriminate between necrotic and apoptotic cell death, we detected ssDNA fragments in the nuclei using an ELISA detection kit with a mouse monoclonal antibody to ssDNA (Chemicon International, Temecula, CA). This antibody does not recognize DNA in double-stranded conformation and provides specific detection of apoptosis (Frankfurt and Krishan, 2001).

Briefly, following the manufacturer's instructions, cells were seeded at 8000 cells/well in a 96-multiwell plate. After the incubation with the drug, the plates were centrifuged at 200g for 5 min and the cells were fixed with 80% methanol in PBS for 30 min at room temperature. The plates were dried and cells were incubated with formamide for 10 min at room temperature for an additional 10 min at 75°C , and then 5 min at 4°C . Cells were incubated for 1 h with 3% nonfat dry milk and then incubated with the antibody mixture (containing a primary monoclonal antibody to ssDNA and horseradish peroxidase-labeled secondary antibody) for 30 min. The addition of 2-2'-azino-bis[3-ethylbenziazoline-6-sulfonic acid solution for 40 min permitted the reading of the plates at 405 nm in a standard microtiter reader. As positive control, ssDNA was used, and as negative

control, necrotic cells were obtained by hyperthermia by heating the cells at 56°C for 1 h and then incubating them for 1 h at 37°C.

Nude Mouse Xenograft Model of Human Glioma. Athymic female CD-1 nude (nu/nu) mice (Charles River Laboratories, Calco, Italy), 8 weeks old, were used. The animals were injected subcutaneously on the left flank with 3×10^6 U87 human glioma cells in 0.1 ml of PBS. Tumors were measured using an external caliper, twice a week, and volume was calculated by the formula: $4\pi/3 \times (\text{length}/2) \times (\text{width}/2)^2$.

Seven days after the inoculation, when the tumor volume had reached an average volume of about 70 mm³, mice were randomly divided into two groups. The mice were treated peritumorally with CBD (dissolved in 0.1 ml of sterile PBS supplemented with 5 mg/ml defatted and dialyzed bovine serum albumin) or its vehicle, at a dose of 0.5 mg/mouse (day 0 of treatment). The injection was repeated once a day, 5 days per week, and the tumor volumes were checked twice a week until the animals were sacrificed.

Mice were monitored daily for health status, and 23 days after the beginning of the treatment, when the control group tumor burden was exceeding 10% of the host weight, the experiments were stopped and the animals sacrificed. This protocol was conducted in accordance with the Italian regulation for the welfare of animals in experimental neoplasia (Permission no. 94/2000A) and met the European Community directives regulating animal research.

Statistics. Statistical analysis for cell proliferation data was performed using one-way analysis of variance followed by the post hoc analysis Dunnett's *t* test.

In apoptotic studies, the differences between the groups were analyzed by Bonferroni's *t* test. All biochemical data are presented as the mean \pm S.E.M. of at least three separate experiments. In vivo data were analyzed by Mann-Whitney test to compare medians for nonparametric data. All statistical analyses were undertaken using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA).

Results

Inhibition of Human Glioma Cell Proliferation by CBD. The aim of initial experiments was to investigate whether CBD could affect the viability of the U87 and U373 human glioma cell lines. The addition of CBD to the culture medium of both human glioma cell lines for 24 h resulted in a concentration-dependent inhibition of the mitochondrial oxidative metabolism, as determined by MTT test. The range of concentrations tested was from 5 μ M to 40 μ M. For both cell lines, the concentration that started to be significant was 15 μ M, with a reduction in O.D. values of $20 \pm 0.6\%$ ($n = 12$) and $15 \pm 0.8\%$ ($n = 12$) for U87 and U373, respectively, as compared with the control. Further inhibition in the MTT test was observed, with 20 μ M ($28 \pm 2\%$ for U87 and $40 \pm 3.2\%$ for U373, $n = 12$), 30 μ M ($70 \pm 3.5\%$ for U87 and $70 \pm 3.43\%$ for U373, $n = 12$), and 40 μ M ($96 \pm 10\%$ for U87 and $94 \pm 10.34\%$ for U373, $n = 12$), with IC₅₀ values of 26.2 ± 2.8 μ M in U87 cells and 24.1 ± 2.16 μ M for U373.

In a subsequent series of experiments, we tested the effect of a single administration of CBD (using the mean IC₅₀ concentration of 25 μ M) to the cells following their growth during a 4-day period. When the MTT test was performed daily for 4 days, we found that the growth inhibition for both cell lines (Fig. 1, A–C) was still present during this period, with a maximum effect seen at 4 days. Interestingly, these results were positively correlated with the drop in cell number in a time-dependent manner (Fig. 1, B–D), as assessed by counting the cells by trypan blue exclusion. Taken together, these findings indicated that CBD induced its effects on gliomas in a concentration- and time-dependent manner,

suggesting a specific mechanism by which CBD could affect the viability of glioma cell lines.

Effects of Cannabinoid, Vanilloid Receptor Antagonists, and Pertussis Toxin upon Antiproliferative Effects of CBD. Most of the effects of cannabinoids on the central nervous system described so far are believed to be mediated by cannabinoid receptors. CBD has been reported to bind cannabinoid receptors with weak affinity, and recently, it has been demonstrated that it can also bind vanilloid receptors (Bisogno et al., 2001). Hence, we next studied whether the effects of CBD described in the present report were dependent on the stimulation of these receptors. Thus, we performed studies with the specific antagonist SR1, selective for CB1 receptor, SR2, selective for CB2 receptor, and capsazepine (CPZ), selective for the vanilloid receptor VR1, using in vitro concentrations that did not, per se, affect cell viability (data not shown).

As shown in Fig. 2 for U87 cells, the CBD-induced growth inhibition over a 24-h period was never prevented by SR1 and CPZ; by contrast, the SR2 antagonist appeared to significantly antagonize this effect, although in a noncomplete manner. Curiously, under the same experimental condition, the combination of SR2 with the other selective antagonists did not block the effect induced by CBD. However, the ability of SR2 in blocking the CBD growth inhibition was lost after 4 days of exposure to CBD (Fig. 2). Similar results were also found in U373 (Fig. 2), where, however, we observed only a nonsignificant trend for SR2 in limiting the CBD-inhibitory effect during 24 h of exposure.

Cannabinoid receptors are coupled to heterotrimeric G_i/G_o proteins that can be inactivated by pretreatment with pertussis toxin (PTX). We therefore compared the antiproliferative effect of CBD in the presence or absence of PTX. Pretreatment of U87 and U373 cells with 100 ng/ml PTX for 18 h was unable to limit the antimetabolic effect of CBD (Fig. 3).

Apoptosis Induced by CBD. To verify whether the CBD-induced reduction in glioma cell growth was indeed due to apoptotic cell death, both flow-cytometric analysis and ssDNA detection assay have been utilized. Flow-cytometric analysis was carried out on the total cell population (i.e., adhering + detached cells) and apoptosis was assessed as appearance of a hypodiploid DNA peak after PI staining of nuclei. In U87 control culture, after 24 h of incubation, only $2.66 \pm 1.38\%$ of the cells underwent a spontaneous apoptosis (Fig. 4). The exposure to the ineffective concentration of CBD in the MTT test at 10 μ M caused an induction of apoptosis overlapping that obtained in control cells ($7.25 \pm 4.03\%$). By contrast, culturing the cells with CBD for 24 h with the IC₅₀ concentration caused an induction of apoptosis in $51.58 \pm 4.82\%$ of the total cell population. Similar results were observed in the U373 cells, where in the control group the percentage of apoptosis was $2.09 \pm 0.16\%$, and the exposure to CBD was found to increase the apoptotic rate from $8.66\% \pm 7.77$ with 10 μ M to 41.36 ± 11.8 with 25 μ M CBD (data not shown). The addition of CB1 and CB2 cannabinoid antagonists never reversed the CBD-induced cell death (data not shown).

The presence of apoptosis was further confirmed by an ELISA apoptosis detection with a specific monoclonal antibody to ssDNA, which represents a specific and sensitive marker of apoptosis. As reported in Fig. 5, CBD at the concentration of 25 μ M induced in both cell lines a significant increase in the O.D. values of apoptosis, as compared with

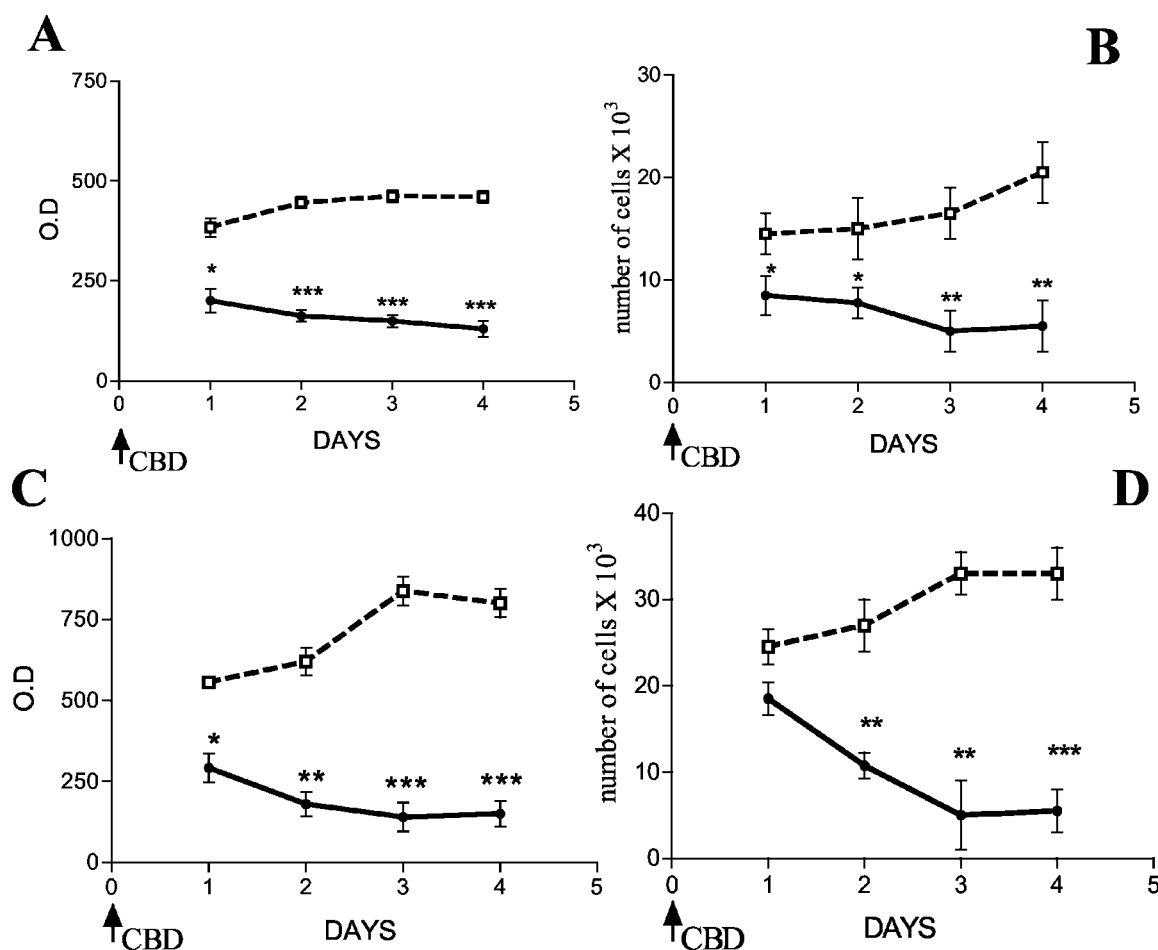


Fig. 1. Time-dependent inhibition of U87 and U373 cell proliferation induced by CBD. Cells were cultured in serum-free medium in the absence (□) or presence (●) of 25 μ M CBD, added at a day "0," for the time indicated. A and B, MTT and trypan blue tests on U87 glioma cell line; C and D, MTT and trypan blue tests on U373 glioma cell line. Results correspond to three different experiments and values are expressed as mean (O.D. or number of cells) \pm S.E.M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus untreated cells (□), Dunnett's t test.

the untreated cells. As expected, cells treated with hyperthermia and used as negative necrotic control did not show any degree of apoptosis.

Mechanism of Action of CBD. Since ceramide accumulation has been reported to mediate the apoptosis induced in C6 glioma cells by THC (Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002, Jacobsson et al., 2001), we started investigating whether CBD also acted through a similar pathway.

We performed experiments with selective inhibitors of ceramide synthesis, using concentrations previously reported to be effective in antagonizing cannabinoid effects (Gomez del Pulgar et al., 2002,) and not affecting, under our conditions, the cell viability. In our hands, L-cycloserine and fumonisin B₁ (inhibitors of serine-palmitoyl-transferase and ceramide synthase, respectively) had no effect on the cellular response evoked by CBD (Table 1). In addition, neither desipramine (an inhibitor of acid sphingomyelinase) nor PMA (an inhibitor of neutral sphingomyelinase via protein kinase C activation), prevented CBD-induced inhibition of cell viability in U87 cells (Table 1).

To evaluate whether events of oxidative stress were implicated in the mechanism of CBD action, we also tested the effect of a coinubation with the antioxidant agent α -tocopherol. We found that this compound at the concentration of 10 μ M significantly prevented, although in a partial manner, the antiproliferative effect of CBD (Fig. 6).

In Vivo Studies. Since tumor regression in animal experimental tumor models represents an important endpoint of clinical relevance, in a final set of experiments we evaluated the ability of in vivo CBD to reduce tumor growth. To address this point, tumors were induced in athymic nude mice by a subcutaneous inoculation of U87 cells into the flank region of the animals. We found that between 12 and 23 days of treatment, CBD-treated mice had significantly smaller tumors than did control mice (Fig. 7). The regression was of about 70% at day 18 (572 ± 147 mm³, $n = 7$, in CBD-treated mice, versus 1765 ± 259 mm³, $n = 7$, in control mice), although at 23 days of treatment this effect appeared weaker, with an inhibition of the tumor growth of about 50%, as compared with control animals (1210 ± 210 mm³, $n = 7$, in CBD-treated mice, versus 2212 ± 256 mm³, $n = 7$, in control mice).

Discussion

In the current study we demonstrated that the nonpsychoactive cannabinoid compound CBD can induce in vitro and in vivo inhibition of tumoral cell growth, and we also showed, for the first time, that CBD can trigger the apoptosis of human gliomas, a very aggressive tumor characterized by poor clinical prognosis and unsatisfactory response to the currently available pharmacological agents. Some very old

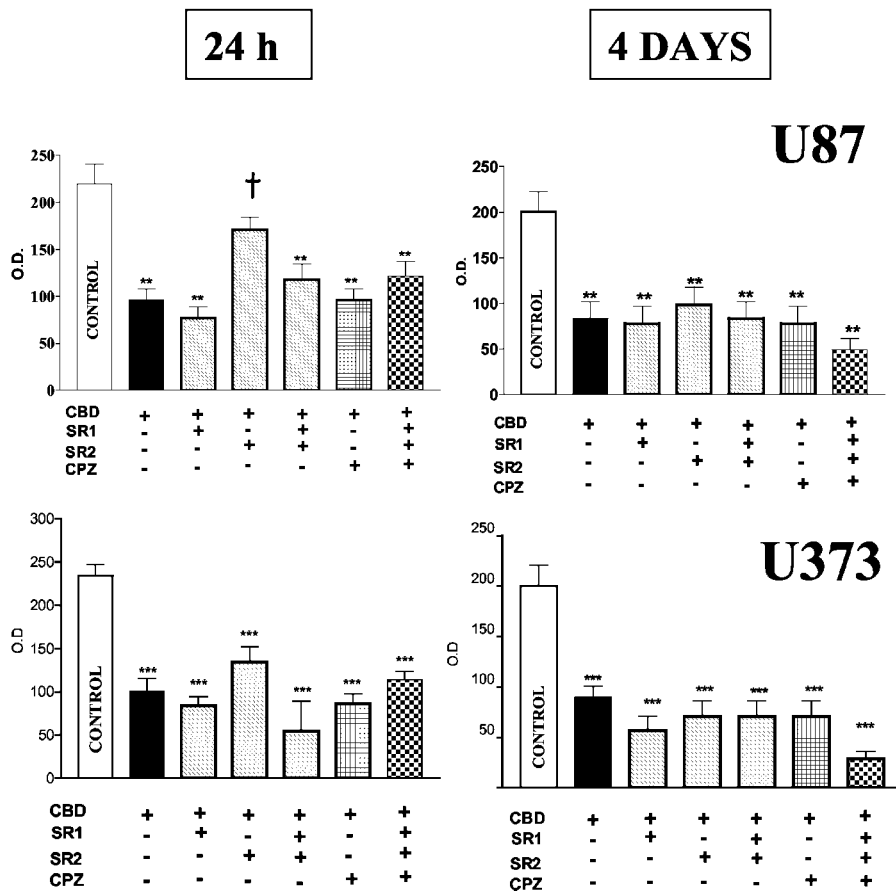


Fig. 2. Effect of concomitant treatment of U87 and U373 glioma cells cultured in serum-free medium with a combination of the selective antagonists 0.5 μ M SR1, 0.5 μ M SR2, and/or 0.625 μ M CPZ upon the sensitivity to the antiproliferative effects of CBD (MTT test). Cells were treated for 24 h or 4 days with 25 μ M CBD and the indicated antagonist compounds as reported in the figure. Results correspond to at least three different experiments and values are expressed as mean O.D. \pm S.E.M. ***, $p < 0.001$; **, $p < 0.01$, versus untreated cells (control); †, $p < 0.05$ versus 25 μ M CBD, Dunnett's t test.

data about the antiproliferative properties of CBD on transformed cells were first reported by Munson et al. (1975), who showed that the drug was ineffective, in vivo, in reducing the growth of Lewis lung adenocarcinoma or was a little effective in inhibiting DNA synthesis on Lewis lung cells and L1210 leukemia cells (Carchman et al., 1976). After that, to our knowledge, the only report that has studied CBD was the work of Jacobsson et al. (2000), demonstrating that in C6 rat glioma cells, CBD had a modest effect only evident after 6 days of incubation with the drug and only in a serum-free condition. At that time, no further investigation or discussion was put forward about the ability of this compound to alter the proliferation rate of glioma cells.

In our study, we demonstrated that CBD caused a concentration-related inhibition of the glioma cell viability under serum-free conditions to exclude any interaction with the reported direct interaction between serum proteins, such as albumin, and cannabinoids (Zheng et al., 1993). A question could be raised about the relatively high values of IC_{50} of CBD found in this work, as compared with the reported more potent THC (Sanchez et al., 1998). It is well known that nonpsychotropic cannabinoids are usually used at higher concentrations either in vitro or in vivo to obtain pharmacological effects (Malfait et al., 2000; Recht et al., 2001), probably due to the relatively low affinity of these compounds for CB1 and CB2 cannabinoid receptors (Showalter et al., 1996; Bisogno et al., 2001). Importantly, however, our results revealed that compared with THC, which caused an inhibition of cell viability during a 2- to 3-day or 4- to 5-day period (Sanchez et al., 1998; Ruiz et al., 1999), the inhibitory effects

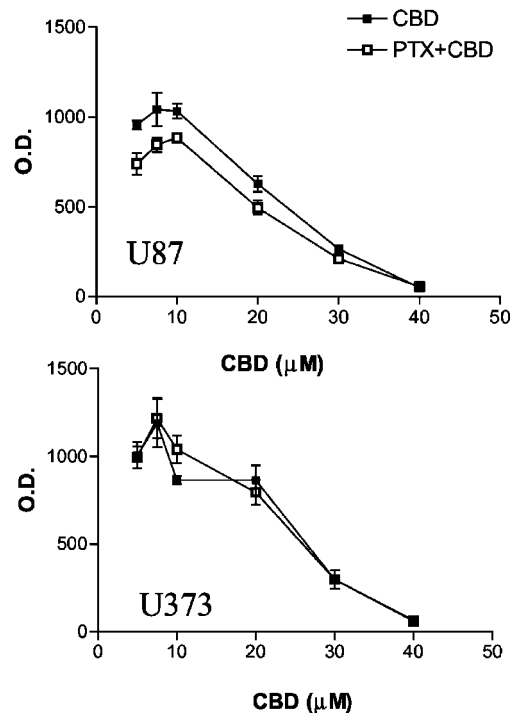


Fig. 3. Absence of an effect of PTX on CBD-induced antiproliferation. U87 and U373 cells were preincubated for 18 h with 100 ng/ml PTX and treated with different concentrations of CBD for 24 h. Viability was determined by MTT test. Results correspond to three different experiments and values (O.D.) are expressed as mean \pm S.E.M.

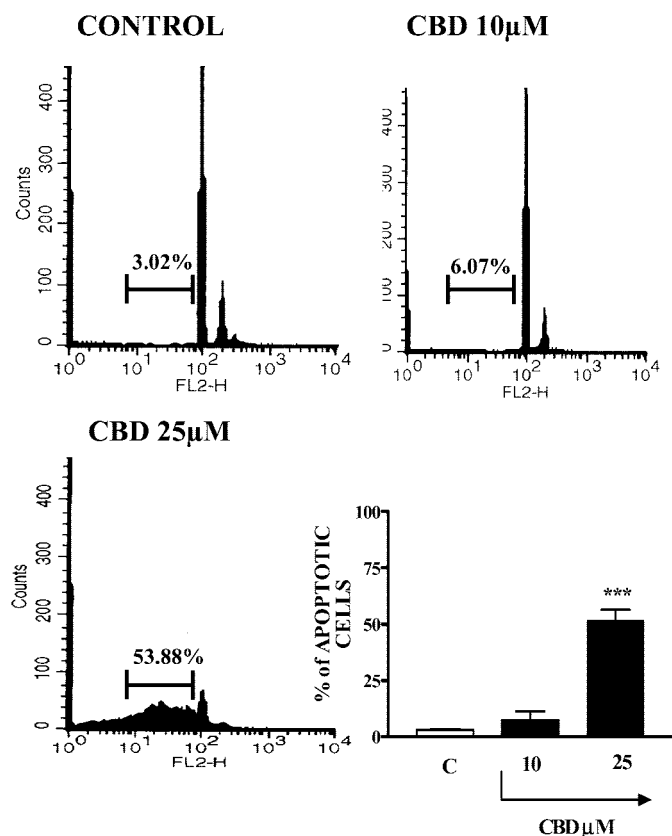


Fig. 4. CBD-induced apoptosis in U87 glioma cells. Cultures were grown in either serum-free medium alone (control) or in a medium containing 10 μM or 25 μM CBD. After 24 h of exposure, cells were detached, centrifuged, resuspended, and incubated with PI solution, and apoptosis was quantified as reduced fluorescence by flow cytometry on the total cell population (adhering + detached cells). A representative experiment is shown in the figure. Y values represent the relative cell number and X values represent the DNA content (PI fluorescence). Numbers on the graphs represent the percentage of apoptotic cells. Histograms represent the mean values \pm S.E.M. of the percentage of apoptotic cells obtained in four independent experiments. ***, $p < 0.001$ versus control (C), Student's t test. Similar results were obtained with U373 glioma cells (data not shown).

of CBD already became apparent after 24 h of exposure to the drug. The different potency of CBD versus THC was also confirmed in our experimental protocol, where THC affected

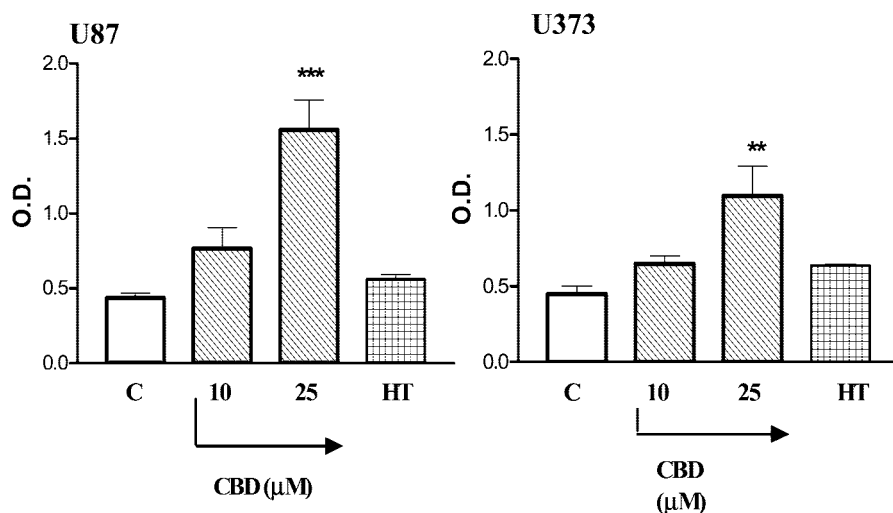


Fig. 5. ELISA-ssDNA monoclonal antibody detection of apoptosis induced by CBD on glioma cells. Cells were untreated (C) or exposed to CBD (10 μM and 25 μM) for 24 h, as previously described (see *Materials and Methods*). Cells treated with hyperthermia (HT; 56°C for 1 h, followed by an incubation at 37°C for 1 h) were used as necrotic cells-negative control of apoptosis. Data are expressed as O.D. (405 nm) and represent the mean \pm S.E.M. of at least three experiments. **, $p < 0.01$; ***, $p < 0.001$ versus control (C), Student's t test.

TABLE 1

Effect of inhibitors of ceramide synthesis upon the antiproliferative effects of CBD (25 μM) on U87 cell viability (MTT test)

U87 cells were incubated in the absence or presence of CBD and different concentrations of inhibitors. The values are expressed as mean O.D. \pm S.E.M. of at least three independent experiments.

| Test Compound | Control | CBD (25 μM) |
|--|--------------|----------------------------|
| L-Cycloserin (mM) | | |
| 0 | 651 \pm 29 | 337 \pm 50 ^a |
| 0.5 | 630 \pm 25 | 401 \pm 14 ^a |
| 1 | 645 \pm 28 | 402 \pm 8.5 ^a |
| 2 | 655 \pm 21 | 435 \pm 13 ^a |
| Desipramine (μM) | | |
| 0 | 650 \pm 30 | 316 \pm 45 ^a |
| 5 | 653 \pm 27 | 269 \pm 24 ^a |
| 10 | 645 \pm 31 | 337 \pm 43 ^a |
| Fumonisin B ₁ (μM) | | |
| 0 | 570 \pm 17 | 320 \pm 45 ^a |
| 1 | 585 \pm 20 | 300 \pm 30 ^a |
| 10 | 568 \pm 22 | 340 \pm 25 ^a |
| PMA (μM) | | |
| 0 | 590 \pm 13 | 293 \pm 43 ^a |
| 0.05 | 582 \pm 16 | 238 \pm 22 ^a |
| 0.1 | 588 \pm 18 | 245 \pm 13 ^a |
| 0.2 | 570 \pm 25 | 272 \pm 10 ^a |

^a $p < 0.001$ vs. the corresponding control values in the absence of L-cycloserine, desipramine, fumonisin B₁, and PMA.

the viability of our cell lines after 4 days of exposure and with an IC₅₀ of about 3 to 4 μM (data not shown).

In the present study we showed for the first time the ability of CBD to induce programmed cell death in human glioma cells through two independent methods based on the appearance of a hypodiploid DNA peak after PI staining of nuclei and detection of apoptotic DNA with monoclonal antibody to ssDNA. These results demonstrated that CBD was capable of inducing cell death and that this mechanism correlated with the reported inhibition of cell growth found in both in vitro and in vivo studies. These results are in accordance with previous reports demonstrating cannabinoids as agents able to cause apoptosis in both in vitro and in vivo experiments (Guzman et al., 2002). In fact, THC has been reported to trigger cell death in C6 glioma cells (Sanchez et al., 1998; Galve-Roperh et al., 2000), cortical neurons (Campbell, 2001), and human prostate PC-3 cells (Ruiz et al., 1999), mainly through a receptor-mediated mechanism. Thus it appears that nonpsychoactive compounds such as CBD also can share these apoptotic properties with THC.

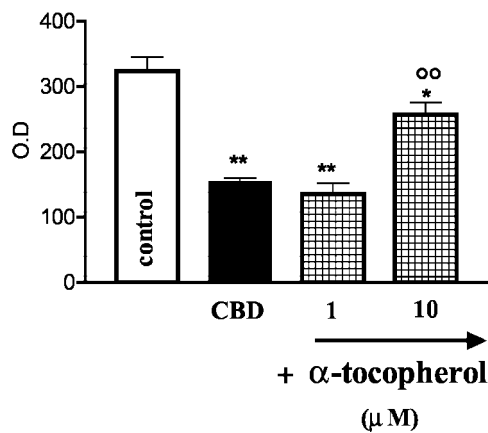


Fig. 6. Effect of increasing concentrations of α -tocopherol upon the anti-proliferative effect of 25 μ M CBD in U87 glioma cells. Cell viability was determined by MTT assay after 24 h of treatment. Results correspond to three different experiments, and values are expressed as mean O.D. \pm S.E.M. *, $p < 0.05$; **, $p < 0.01$, versus untreated cells (control); ∞ , $p < 0.01$ versus CBD alone, Dunnett's t test.

The exact mechanism by which CBD induces apoptosis and antiproliferative effects remains partially unclear. CBD has been reported to bind with relatively low-affinity CB1 and CB2 receptors, and recently, it has been demonstrated that it can also bind vanilloid receptors (Bisogno et al., 2001). However, because of its lipophilic properties, it cannot be ruled out that CBD can also act through an aspecific intercalation into the cell membrane. In our hands, neither the CB1 antagonist nor capsazepine was able to block the effect of CBD. By contrast, the CB2 antagonist appeared to affect the inhi-

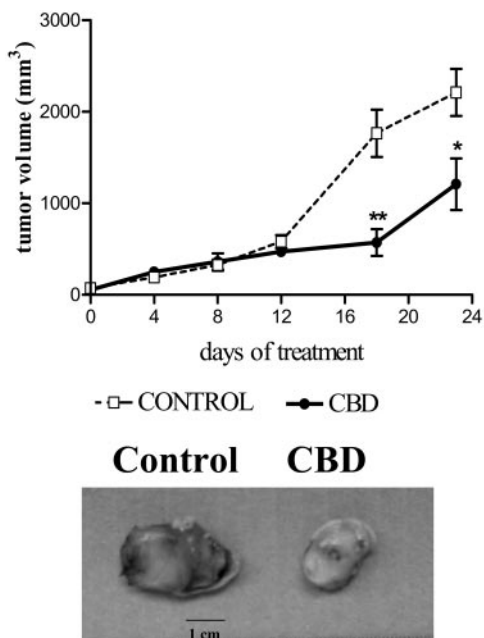


Fig. 7. CBD effect on subcutaneous U87 glioma cell growth. CBD (0.5 mg/mouse) administration began 7 days after U87 cell inoculation into the left flank of the athymic nude mice (day 0 of the treatment). CBD was injected in the peritumoral area once a day, 5 days per week. Tumor diameters were measured twice a week. Results represent the mean of seven mice in each group and are expressed as mean volume \pm S.E.M. *, $p < 0.05$; **, $p < 0.01$, versus control mice; Mann-Whitney nonparametric test. On the right of the graph is reported an example of s.c. gliomas after dissection. Tumors were grown in the presence of vehicle (control) or CBD after 23 days of treatment.

bition of mitochondrial oxidative metabolism induced by CBD during 24 h of exposure, but only to a partial extent. In any case, this antagonism was not observed in apoptotic studies. Thus, despite the presence of cannabinoid receptors in glioma cell lines, CBD appeared to exert its action through a small involvement of these receptors and no stimulation of vanilloid receptors. In accordance with the CBD insensitivity to antagonists, the inefficacy of PTX pretreatment in reversing CBD effects reinforces the notion of a cannabinoid G_i/G_o -coupled receptor-independent mechanism.

On the other hand, the role of cannabinoid receptors in THC-induced cell death also is controversial: whereas early reports seemed to point to an unidentified CB receptor-independent mechanism (Sanchez et al., 1998; Ruiz et al., 1999), further investigations have shown that both CB1 and CB2 receptors can contribute to this cytotoxic effect (Galve-Roperh et al., 2000), although in an unclear manner. Similar conflicting results were also found for AEA, which resulted in triggering cellular events through no involvement (Sancho et al., 2003; Sarker and Maruyama, 2003) or a partial involvement (Maccarrone et al., 2000; Jacobsson et al., 2001) of cannabinoid/vanilloid receptors. In addition, another very intriguing hypothesis, that cannot be ruled out, is that CBD could act through the stimulation of a novel non-CB1, non-CB2 receptor that could be present in these cells, although this hypothesis appears unlikely since CBD was insensitive to SR1 compound (Jarai et al., 1999). Also, the hypothesis that CBD could act indirectly, enhancing the AEA level through an inhibition of anandamide amidase activity (Watanabe et al., 1996), appears improbable since the SR1 compound and/or capsazepine, alone or in combination, did not reverse the effects of CBD.

Since apoptosis by THC and AEA has been demonstrated to be primarily related to ceramide generation (Galve-Roperh et al., 2000; Jacobsson et al., 2001; Gomez del Pulgar et al., 2002), in a first attempt to investigate through which mechanism CBD could induce its effects, we investigated whether CBD could also exert its effects through ceramide accumulation. We did not find any involvement of this intracellular messenger, thus suggesting that the CBD mechanism is clearly different from that described for THC and AEA. The protective effect of α -tocopherol we found suggests an implication of an oxidative stress mechanism in the antiproliferative effects of CBD and argues against a simple cell-toxic mechanism. On the other hand, similar results were also reported by Jacobsson et al. (2001), who described that the inhibition of cell growth induced by AEA on C6 cells was prevented by α -tocopherol. In accordance with this evidence, also, Sarker et al. (2000) found that AEA-induced apoptosis in PC-12 cells caused a rise in intracellular superoxide levels, and this effect was prevented by the antioxidant agent *N*-acetyl cysteine. These results led us to hypothesize that the effect of CBD could be attributed, in some way, to reactive oxygen species production that, in turn, can mediate the cell death in human glioma cells. To further clarify this point, experiments are now in progress to evaluate the mechanism underlying CBD-induced oxidative stress. Another possibility, as already reported for THC (Chan et al., 1998), is that CBD could induce cell death by a signal transduction cascade related to an increase in intracellular arachidonic acid induced by an activation of phospholipase A_2 . A further possi-

bility that we have to take into account is that CBD could induce an uncoupling of mitochondrial potential, as already reported for THC and AEA (Maccarrone et al., 2000; Sarafian et al., 2003).

Finally, a very important point of this work is the demonstration of the *in vivo* efficacy of CBD in reducing tumor growth. This evaluation of *in vivo* effect of CBD is essential, with the aim to develop a cannabinoid-based therapeutic strategy for gliomas devoid of CB-mediated psychotropic side effects. In our study, we observed a significant inhibition of the *in vivo* tumor growth over a 23-day period with a dose of CBD of 0.5 mg/mouse. In this study, we did not attempt to find the ideal dose for the treatment. Nevertheless, a significant delay in tumor growth was observed. Currently, it is not clear whether the apparent slighter effect induced by CBD seen at the end of the protocol is related to development of tolerance of tumor cells to the growth inhibition induced by CBD. This effect could in part reflect increased drug metabolism, drug regimen, dose used, or drug resistance. Nevertheless, the fact that this agent is not psychoactive to any degree and that it can be administered at high doses without apparent toxicity encourages further studies.

In conclusion, a cannabinoid-based therapeutic strategy for neural diseases devoid of undesired psychotropic side effects could find in CBD a valuable compound in cancer therapies along with the perspective of evaluating a synergistic effect with other cannabinoid molecules and/or with other chemotherapeutic agents as well as with radiotherapy. Whatever the precise mechanism underlying the CBD effects, the present results suggest a possible application of CBD as a promising, nonpsychoactive, antineoplastic agent.

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References

Bifulco M and Di Marzo V (2002) Targeting the endocannabinoid system in cancer therapy: a call for further research. *Nat Med* **8**:547–550.

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**:845–852.

Braida D, Pegorini S, Arcidiacono MV, Consalez GG, Croci L, and Sala M (2003) Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion and neuronal injury in gerbils. *Neurosci Lett* **346**:61–64.

Campbell VA (2001) Tetrahydrocannabinol-induced apoptosis of cultured cortical neurons is associated with cytochrome c release and caspase-3 activation. *Neuropharmacology* **40**:702–709.

Carchman RA, Harris LS, and Munson AE (1976) The inhibition of DNA synthesis by cannabinoids. *Cancer Res* **36**:95–100.

Ceruti S, Franceschi C, Barbieri D, Malorni W, Camurri A, Giammarioli AM, Ambrosini A, Racagni G, Cattabeni F, and Abbraccio MP (2000) Apoptosis induced by 2-chloro-adenosine and 2-chloro-2'-deoxy-adenosine in a human astrocytoma cell line: differential mechanisms and possible clinical relevance. *J Neurosci Res* **60**:388–400.

Chan GC, Hinds TR, Impey S, and Storm DR (1998) Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol. *J Neurosci* **18**:5322–5332.

Faubert Kaplan BL, Rockwell CE, and Kaminski NE (2003) Evidence for cannabinoid receptor-dependent and -independent mechanism of action in leukocytes. *J Pharmacol Exp Ther* **306**:1077–1085.

Frankfurt SO and Krishan A (2001) Enzyme-linked immunosorbent assay (ELISA) for the specific detection of apoptotic cells and its application to rapid drug screening. *J Immunol Methods* **253**:133–144.

Galve-Roperh I, Sanchez C, Cortes ML, Gomez del Pulgar T, 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.

Gomez del Pulgar T, Velasco G, Sanchez C, Haro A, and Guzman M (2002) De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis. *Biochem J* **363**:183–188.

Goutopoulos A and Makriyannis A (2002) From cannabis to cannabinergics: new therapeutic opportunities. *Pharmacol Ther* **95**:103–117.

Guzman M, Sanchez C, and Galve Roperh I (2002) Cannabinoids and cell fate. *Pharmacol Ther* **95**:175–184.

Hampson AJ, Grimaldi M, Lolic M, Wink D, Rosenthal R, and Axelrod J (2000) Neuroprotective antioxidants from marijuana. *Ann N Y Acad Sci* **899**:274–282.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, et al. (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54**:161–202.

Jacobsson SO, Rognard E, Stridh M, Tiger G, and Fowler CJ (2000) Serum-dependent effects of tamoxifen and cannabinoids upon C6 glioma cell viability. *Biochem Pharmacol* **60**:1807–1813.

Jacobsson SO, Wallin T, and Fowler CJ (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.

Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, et al. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci USA* **96**:14136–14141.

Killestein J, Hoogervorst ELJ, Reif M, Blauw B, Smits M, Uitdehaag BMJ, Nagelkerken L, and Polman CH (2003) Immunomodulatory effects of orally administered cannabinoids in multiple sclerosis. *J Neuroimmunol* **137**:140–143.

Maccarrone M, Lorenzon T, Bari M, Melino G, and Finazzi-Agro 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.

Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreaskos E, Mechoulam R, and Feldmann M (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritis therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci USA* **97**:9561–9566.

Munson AE, Harris LS, Friedman MA, Dewey WL, and Carchman RA (1975) Antineoplastic activity of cannabinoids. *J Natl Cancer Inst* **55**:597–602.

Pertwee RG and Ross RA (2002) Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids* **66**:101–121.

Recht LD, Salmonsens R, Rosetti R, Jang T, Pipia G, Kubiawski T, Karim P, Ross AH, Zurier R, Litofsky NS, and Burstein S (2001) Antitumor effects of ajulemic acid (CT3), a synthetic non-psychoactive cannabinoid. *Biochem Pharmacol* **62**:755–763.

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**:400–404.

Sanchez C, de Ceballos ML, Gomez del Pulgar T, Rueda D, Corbacho C, Velasco G, Galve-Roperh I, Huffman JW, Ramon y Cajal S, and Guzman M (2001) Inhibition of glioma growth *in vivo* by selective activation of the CB(2) cannabinoid receptor. *Cancer Res* **61**: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**:6–10.

Sancho R, Calzado MA, Di Marzo V, Appendino G, and Munoz E (2003) Anandamide inhibits nuclear factor- κ B activation through a cannabinoid receptor-independent pathway. *Mol Pharmacol* **63**:429–438.

Sarafian TA, Kouyoumjian S, Khoshaghid F, Tashkin DP, and Roth MD (2003) Delta 9-tetrahydrocannabinol disrupts mitochondrial function and cell energetics. *Am J Physiol* **284**:L298–L306.

Sarker KP 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.

Sarker KP, 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.

Showalter VM, Compton DR, Martin BR, and Abood ME (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. *J Pharmacol Exp Ther* **278**:989–999.

Smith MS, Yamamoto Y, Newton C, Friedman H, and Klein T (1997) Psychoactive cannabinoids increase mortality and acute phase cytokine responses in mice sublethally infected with *Legionella pneumophila*. *Proc Soc Exp Biol Med* **214**:69–75.

Srivastava MD, Srivastava BIS, and Brouhard B (1998) Δ^9 Tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. *Immunopharmacology* **40**:179–185.

Watanabe K, Kayano Y, Matsunaga T, Yamamoto I, and Yoshimura H (1996) Inhibition of anandamide amidase activity in mouse brain microsomes by cannabinoids. *Biol Pharm Bull* **19**:1109–1111.

Zheng ZM, Spector S, and Friedman H (1993) Serum proteins affect the inhibition by delta-tetrahydrocannabinol of tumor necrosis factor alpha production by mouse macrophages. *Adv Zhu Exp Med Biol* **335**:89–93.

Zhu LX, Sharma S, Stolina M, Gardner B, Roth MD, Tashkin DP, and Dubinett SM (2000) Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. *J Immunol* **165**:373–380.

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