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Research Article

Molecular Cancer Therapeutics

Cannabidiol Enhances the Inhibitory Effects of Δ^9-Tetrahydrocannabinol on Human Glioblastoma Cell Proliferation and Survival

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Abstract

The cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptor agonist Δ^9-tetrahydrocannabinol (THC) has been shown to be a broad-range inhibitor of cancer in culture and *in vivo,* and is currently being used in a clinical trial for the treatment of glioblastoma. It has been suggested that other plant-derived cannabinoids, which do not interact efficiently with CB_1 and CB_2 receptors, can modulate the actions of Δ^9-THC. There are conflicting reports, however, as to what extent other cannabinoids can modulate Δ^9-THC activity, and most importantly, it is not clear whether other cannabinoid compounds can either potentiate or inhibit the actions of Δ^9-THC. We therefore tested cannabidiol, the second most abundant plant-derived cannabinoid, in combination with Δ^9-THC. In the U251 and SF126 glioblastoma cell lines, Δ^9-THC and cannabidiol acted synergistically to inhibit cell proliferation. The treatment of glioblastoma cells with both compounds led to significant modulations of the cell cycle and induction of reactive oxygen species and apoptosis as well as specific modulations of extracellular signal-regulated kinase and caspase activities. These specific changes were not observed with either compound individually, indicating that the signal transduction pathways affected by the combination treatment were unique. Our results suggest that the addition of cannabidiol to Δ^9-THC may improve the overall effectiveness of Δ^9-THC in the treatment of glioblastoma in cancer patients.

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Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC) and other cannabinoids can act as direct anticancer agents in multiple types of cancer in culture and *in vivo* (1). Specifically, activation of the two cloned cannabinoid receptors, CB_1 and CB_2, by Δ^9-THC can lead to the inhibition of cell proliferation, invasion, and induction of apoptosis in cancer cell lines, resulting in the reduction of tumor burden *in vivo* (2–4). The promising preclinical therapeutic potential of Δ^9-THC, as an inhibitor of glioblastoma, has prompted a human clinical trial (5).

The CB_1 and CB_2 receptors are members of the G-protein coupled receptor (GPCR) superfamily, and can interact with five structurally distinct classes of compounds. These include the plant-derived classical cannabinoids, such as Δ^9-THC; the nonclassical bicyclic cannabinoids, such as CP55,940; the endogenous cannabinoids, such as anandamide; the aminoalkylindoles, such as WIN55,212-2; and the antagonist/inverse agonists, such as SR141716A (6). Interaction sites, independent of CB_1 and CB_2 receptors, also seem to be responsible for the anticancer activity of cannabinoids (7–10). There are >60 cannabinoids in *Cannabis sativa*. In addition to Δ^9-THC, cannabidiol, cannabinol, and cannabigerol are also present in the plant (11). Cannabinol has low affinity for CB_1 and CB_2 receptors, whereas the nonpsychotropic cannabinoids, cannabidiol and cannabigerol, have negligible affinity for the cloned receptors (12–14). Whereas cannabinol and cannabigerol have not been tested for their ability to inhibit human brain cancer, cannabidiol has been reported to inhibit the growth of a human glioblastoma in a xenograft model (7, 15, 16).

There are conflicting reports as to what extent other cannabinoids can modulate the activity of Δ^9-THC, and it has been suggested that nonpsychoactive cannabinoids can either potentiate or inhibit the actions of Δ^9-THC (17–20). Cooperative effects have also been observed with endogenous cannabinoids (21). The potential benefits of using a cannabinoid-based medicine comprising multiple cannabinoids has been a driving force in recent

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human clinical trials (20, 22, 23). Investigations have shown that nonpyschoactive cannabinoids can alter the physiologic response to Δ^9-THC, potentially by altering its metabolism (17–19, 24, 25). However, no investigation to date has provided the molecular mechanisms to explain how cannabinoids, acting through distinct pathways, could converge onto a shared pathway resulting in a modulation of activity unique to the combination.

In this study, we sought to determine whether cannabidiol, the plant-derived cannabinoid, would modulate the ability of Δ^9-THC to inhibit glioblastoma cell proliferation and survival. We found that cannabidiol enhanced the ability of Δ^9-THC to inhibit glioblastoma cell growth and induce apoptosis. The molecular mechanisms associated with these specific effects are presented.

Material and Methods

Cell Culture and Treatments

The human glioblastoma cell lines used were SF126, U251, and U87. The cell lines were maintained at 37°C and 5% CO_2. In all experiments, the different cell populations were first cultured in RPMI media containing 10% fetal bovine serum. Glioblastoma cells were then seeded into 96-well plates in 10% fetal bovine serum and on the first day of treatment the media were replaced with vehicle control or drug in RPMI and 0.1% fetal bovine serum as previously reported (8). The media with the appropriate compounds were replaced every 24 h. Δ^9-THC and cannabidiol were obtained from NIH through the National Institute of Drug Abuse.

MTT Assay

Assays were done as previously described (26). Percent control was calculated as the MTT absorbance of the treated cells/control cells ×100.

Apoptosis Analysis

Cells were grown in 6-well culture dishes and treated with the appropriate compounds every 24 h for 3 d. Cells attached to the plate and the cells in media were collected, pelleted, washed once with PBS, and processed for labeling with FITC-tagged annexin and propidium iodide (PI) by use of an Apo-Direct apoptosis kit obtained from Phoenix Flow Systems. Briefly, the cell pellet was resuspended in 300 μL of the supplied reaction buffer along with 3 μL of both PI and FITC-tagged annexin. After a 15-min incubation period at room temperature, the labeled cells were analyzed by flow cytometry using a FITC detector (FL1) and a PI emission signal detector (FL2). Cell flow cytometry in combination with PI and annexin staining was used to quantify the percentage of cells undergoing apoptosis in control and treatment groups. Percent control was calculated as annexin-positive staining in treated cells/control cells ×100. PI staining was used to distinguish necrotic cells from those undergoing apoptosis.

Cell Cycle Analysis

U251 cells were grown in Petri dishes (100 mm × 15 mm) and received drug treatments for 2 d. On the third day, the cells were harvested and centrifuged at 1,200 rpm for 5 min. The pellet was washed once with PBS plus 1% bovine serum albumin, and centrifuged again. The pellet was resuspended in 0.5 mL of 2% paraformaldehyde and fixed overnight at room temperature. The next day the cells were pelleted and resuspended in 0.5 mL 0.3% Triton in PBS and incubated for 5 min at room temperature. The cells were then washed twice with PBS plus 1% bovine serum albumin. The cells were finally suspended in PBS (0.1% bovine serum albumin) with 10 μg/mL PI and 100 μg/mL RNAse. The cells were incubated for 30 min at room temperature before being stored at 4°C. Cell cycle was measured using a Fluorescence Activated Cell Sorting (FACS) Calibur, Cell Quest Pro and Modfit software.

Boyden Chamber Invasion Assay

Assays were done in modified Boyden Chambers (BD Biosciences) as previously described (26). Data were presented as relative invasiveness of the cells through the Matrigel, where the respective controls were set as 100%.

Reactive Oxygen Species Measurements

The production of cellular reactive oxygen species (ROS)/H_2O_2 was measured using 2′-7′Dichlorodihydrofluorescein (DCFH-DA; Sigma Aldrich). DCFH-DA is deacylated intracellularly into a nonfluorescent product, which reacts with intracellular ROS to produce 2′-7′Dichlorofluorescein, which remains trapped inside the cell, and can be measured quantitatively. Cells were plated onto 6-well dishes and received drug treatments for 3 d. On the third day, 10 mmol/L DCFH-DA was added to the media (RPMI with 0.1% fetal bovine serum) and the cells were incubated with DCFH-DA overnight. The next day, the cells were trypsinized, washed with PBS, and the fluorescent intensity was measured using FACS and Cell Quest Pro software.

Western Analysis

Western analysis was done as previously described (26). Anti–phospho-JNK, anti–phospho-p38, anti–phospho-ERK1/2, and anti-ERK1/2 were obtained from Millipore. Anti–cleaved caspase 3, 7, 9 and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling. Antibodies were added according to the manufacturer's protocol.

PCR

Total cellular RNA was isolated from glioblastoma cancer cells treated with vehicle control or with cannabidiol. Transcripts for *p8* and for β-*actin* were reverse-transcribed using SuperscriptII Reverse TranscriptaseII (Gibco-BRL), and PCR was done. The 5′ and 3′ PCR primers were GAAGAGAGGCAGGGAAGACA and CTGCCGTGCGTGTCTATTTA for *p8*; and GCGGGAAATCGTGCGTGACATT and GATGGAGTTGAAGGTAGTTTCGTG

for β-*actin*. PCR was done in buffer containing 1 μmol/L of each of the 5′ and 3′ PCR primers and 0.5 U of Taq polymerase using 18 cycles for amplification of *p8* and β-*actin* cDNAs. The cycle conditions were 45 s denaturation at 94°C, 45 s annealing at 55°C, and 1 min extension at 72°C.

Pharmacologic and Statistical Analyses

In the proliferation assays, IC_{50} values with corresponding 95% confidence limits were calculated using nonlinear analysis of logged data (GraphPad Prism). When just the confidence limits of the IC_{50} values overlapped significant differences were determined using unpaired Student's *t*-test. Significant differences were also determined using one-way ANOVA where suitable. Bonferroni's multiple comparison posthoc analyses were conducted when appropriate. *P* values <0.05 defined statistical significance. Positive and negative aspects of constituent interaction were determined in a 2 × 2 design using two-way ANOVA as previously described (27). IC_{20} and IC_{80} values were calculated using the equation $IC_F = (F/100\text{-}F)^{1/H} \times IC_{50}$, where F is the fractional response expressed as a percentage, IC_F is the quantity of drug needed to inhibit an F percentage response, and H is the hillslope. Treatment groups were divided into (*a*) no treatment (control), (*b*) Δ^9-THC alone, (*c*) cannabidiol alone, and (*d*) Δ^9-THC and cannabidiol combined. Data were analyzed using a two-way ANOVA with an interaction term that was used to test for whether the combination of Δ^9-THC and cannabidiol differed from the additive effects of each alone (GraphPad Prism).

To further test for synergism, the combination index (CI) was calculated where CI <1, = 1, and >1 indicate synergism, additive effect, and antagonism, respectively (28, 29). Based on the classic isobologram for mutually exclusive effects relative to the end point of measurement, the CI value for x% inhibition is calculated as: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$; where $(D)_1$ and $(D)_2$ represent Δ^9-THC and CBD, respectively, and $(Dx)_1$ and $(Dx)_2$ are the doses for x% growth that can be obtained using the IC_F equation described above, and $(D)_1$ and $(D)_2$ are the concentrations in the combination that also inhibit cell growth by x% (29).

A cell cycle analysis program was used to estimate the proportions of cells in each of three compartments: G_0-G_1, S, and G_2-G_M. The experiments were conducted on four different dates and there were two replicates for each date. It was noticed that in the control (vehicle) experiments the percentage of cells in each compartment varied significantly from day to day, therefore each treatment compartment percentage estimate was standardized by dividing it by the average percentage for the vehicle on that date. This procedure was carried out for data from each experiment on each day. Multivariate analysis of variance (MANOVA) on the vector of cell cycle compartment standardized ratios (G_0-G_1, S, and G_2-G_M) was done to control the multiple comparison type 1 (false positive) error rate. Because this produced a significant result (at $P < 0.05$), it was concluded that there were differences due to treatments. Standardized ratios for each compartment were then tested separately using univariate one-way ANOVA with treatment as the explanatory factor. These tests were also significant at $P < 0.05$ for each compartment except G_2-G_M. It was concluded that there was evidence for a treatment effect in each of the three cell cycle compartments G_0-G_1, S, and G_2-G_M. We then tested the ratios for each treatment within a compartment to determine if they significantly differed from 1.0, indicating a treatment effect for that particular treatment. Finally, we tested for a significant interaction of cannabidiol and Δ^9-THC, each at its lowest dose by ANOVA with an interaction term. This was carried out after transforming the standardized ratios to logarithms so a test for additive interactions could be done.

Results

Δ^9-THC and Cannabidiol Inhibit the Growth of Multiple Glioblastoma Cell Lines

The CB_1 and CB_2 receptor agonist Δ^9-THC can inhibit glioblastoma cell proliferation in culture and *in vivo*, and is currently being used in a clinical trial (4). Cannabidiol, a cannabinoid constituent with negligible affinity for CB_1 and CB_2 receptors, can also inhibit the proliferation of glioblastoma in culture and *in vivo* (7, 16). SF126, U251, and U87 cells were treated for three days with a range of concentrations of either Δ^9-THC or cannabidiol. The antiproliferative activity of the compounds was assessed using the MTT assay and the corresponding IC_{50} values were calculated as previously described (26). The IC_{50} values for Δ^9-THC in SF126, U251, and U87 cells were 2.5 μmol (1.8–3.4), 3.3 μmol/L (2.4–4.6), and 3.3 μmol/L (2.3–4.8), respectively. The IC_{50} values for cannabidiol in SF126, U251, and U87 cells were 1.2 μmol/L (1.1–1.3), 0.6 μmol/L (0.5–1.0), and 0.6 μmol/L (0.5–0.7), respectively. Cannabidiol was therefore a more potent inhibitor of cell growth than Δ^9-THC in the three cell lines studied.

Cannabidiol Enhances the Inhibitory Effects of Δ^9-THC on Glioblastoma Cell Growth

It has been suggested that nonpsychoactive cannabinoid constituents can either potentiate or inhibit the actions of Δ^9-THC (11, 17–19). Therefore, the glioblastoma cell lines that were originally used to test the antiproliferative activity of individual cannabinoids were used to determine the effects of combination treatments. The positive and negative aspects of constituent interaction were tested by analyzing the activity of different combinations of Δ^9-THC and cannabidiol in a 2 × 2 design (Fig. 1). The concentrations used for the treatments were IC_{80} or IC_{20} values calculated from the IC_{50} values as described in Materials and Methods. When applied in combination at the predicted IC_{80} concentration, Δ^9-THC and cannabidiol produced a greater than additive inhibition of cell growth in SF126 and U251 cells. This was not observed in U87 cells

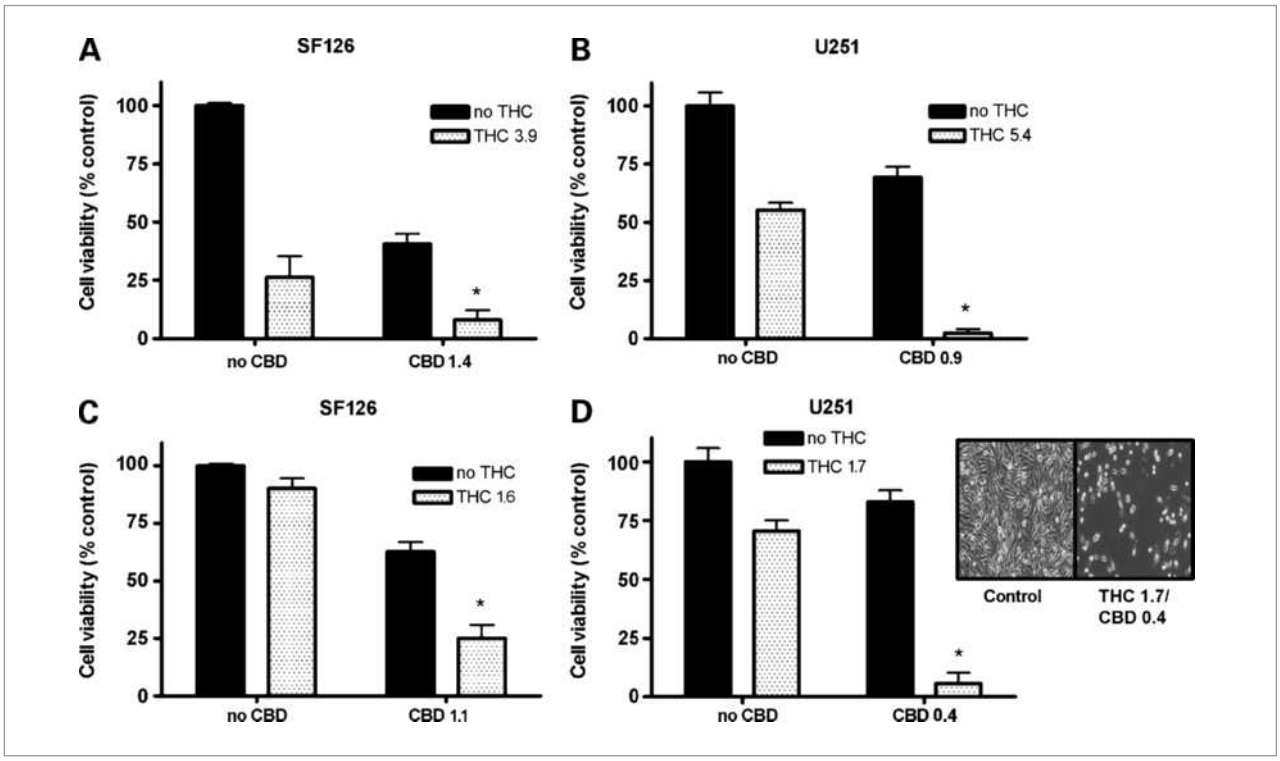

Figure 1. Cannabidiol (CBD) enhances the inhibitory effects of Δ^9-THC on glioblastoma cell growth. To test for positive and negative interactions, a 2 × 2 factorial design using specific μmol/L concentrations of drug was used as described in Materials and Methods. Cell proliferation was measured using the MTT assay. SF126 (**A**) and U251 (**B**) cells were treated for 3 d with vehicle/no drug, Δ^9-THC, cannabidiol, or a combination of Δ^9-THC and cannabidiol. Concentrations of Δ^9-THC and cannabidiol that produce only minimal effects on cell proliferation were also tested in 2 × 2 factorial design in SF126 (**C**) and U251 (**D**) cells. Percent control was calculated as the MTT product absorbance in the treated cells/control cells ×100. Data are the mean of at least three independent experiments; bars, ± SE. Data were analyzed using two-way ANOVA (GraphPad Prism). *, statistically significant interaction ($P < 0.01$). **D,** *inset*, representative light microscope image of the effects of the combination treatment on U251 cells (×40).

(data not shown). In SF126 cells, cell viability was 26 ± 9% in the presence of Δ^9-THC (3.9 μmol/L), 40 ± 4% in cannabidiol (1.4 μmol/L), and 8 ± 4% in Δ^9-THC (3.9 μmol/L) plus cannabidiol (1.4 μmol/L; Fig. 1A). In U251 cells, cell viability was 55 ± 3% in the presence of Δ^9-THC (5.4 μmol/L), 69 ± 4% in cannabidiol (0.9 μmol/L), and 2 ± 2% in Δ^9-THC (5.4 μmol/L) plus cannabidiol (0.9 μmol/L; Fig. 1B). Predicted IC_{20} concentrations of Δ^9-THC and cannabidiol that alone produce only minimal effects on cell growth were combined and further tested in a 2 × 2 factorial design in the positive responding cell lines (SF126 and U251). Again, greater than additive effects were observed in SF126 and U251 cells. In SF126 cells, cell viability was 90 ± 4% in the presence of Δ^9-THC (1.6 μmol/L), 63 ± 4% in cannabidiol (1.1 μmol/L), and 25 ± 6% in Δ^9-THC (1.6 μmol/L) plus cannabidiol (1.1 μmol/L; Fig. 1C). In U251 cells, cell viability was 71 ± 4% in the presence of Δ^9-THC (1.7 μmol/L), 83 ± 5% in cannabidiol (0.4 μmol/L), and 7 ± 4% in Δ^9-THC (1.7 μmol/L) plus cannabidiol (0.4 μmol/L; Fig. 1D). In the cell lines showing significant interactions (SF126 and U251 cells), we further tested for synergism using the CI described in Materials and Methods. As shown in Supplementary Table S1, a synergistic increase in the antiproliferative activity of the cannabinoids was observed in both U251 and SF126 cells. CI values of <1, 1, and >1 indicate synergism, additivity, and antagonism, respectively (29). Synergistic activity was observed at all the concentration ranges tested in U251 cells; therefore, this cell line was used primarily in the remaining experiments.

Cannabidiol Does Not Enhance the Inhibitory Effects of Δ^9-THC on Glioblastoma Cell Invasiveness

In addition to uncontrolled cell growth, a hallmark of the aggressive phenotype of glioblastoma cells is their ability to migrate away from the primary tumor of origin and invade into neighboring central nervous system tissue (30). Thus, we sought to determine whether the addition of cannabidiol to Δ^9-THC would improve the activity of the compound to inhibit migration and invasion through a reconstituted basement membrane in a Boyden chamber assay (Fig. 2). Both Δ^9-THC and cannabidiol could significantly inhibit the invasiveness of U251 cells. The predicted IC_{50} values for Δ^9-THC and cannabidiol to inhibit U251 cell invasiveness were 85 nmol/L (range, 49–150) and 126 nmol/L (range, 20–796), respectively. Concentrations of 100 nmol/L Δ^9-THC and cannabidiol

(their approximate IC_{50} values) were used to test for positive or negative interactions. These concentrations were chosen, as compared with the predicted IC_{80} values, to ensure significant increases in cell death would not be produced, because this could confound the results of the invasion assay, i.e., dead cells would not migrate and invade. Whereas both Δ^9-THC and cannabidiol were able to inhibit U251 cell invasiveness, cannabidiol did not enhance the activity of Δ^9-THC when the compounds were combined. In U251 cells, invasiveness was 48 ± 3% in the presence of Δ^9-THC (0.1 μmol/L), 72 ± 3% in cannabidiol (0.1 μmol/L), and 36 ± 3% in Δ^9-THC (0.1 μmol/L) plus cannabidiol (0.1 μmol/L). Because Δ^9-THC and cannabidiol acted synergistically to inhibit glioblastoma cell growth, but not to inhibit cell invasiveness, mechanistic experiments were focused on understanding the reduction in cell viability produced by the combination treatment. The 4:1 ratio of Δ^9-THC (1.7 μmol/L) and cannabidiol (0.4 μmol/L; as described above in Fig. 1D) was primarily used as the combination treatment for the remaining experiments.

The Combination Treatment of Δ^9-THC and Cannabidiol Leads to the Modulation of Specific Mitogen-Activated Protein Kinases

The regulation of extracellular signal-regulated kinase (ERK), c-*jun*-N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) activity plays a critical role in controlling cell growth and apoptosis (31). Modulation of these pathways has been indicated in cannabinoid control of cancer cell growth and survival (9, 32–34). We used U251 cells to determine whether modulation of ERK, JNK, and p38 MAPK activity occurred. Treatment with the combination of cannabinoids led to a substantial downregulation of phosphorylated ERK (pERK), but produced no significant change in total ERK (Fig. 3A). Additionally, no inhibition of p38 MAPK or JNK activity was observed. When U251 cells were treated with individual concentrations of Δ^9-THC and cannabidiol, instead of the combination, no changes in pERK were observed (Fig. 3B). These data show that the modulation of pERK was specific for the combination treatment. Downregulation of pERK in the presence of the combination treatment was first observed after two days treatment in U251 cells (Fig. 3C). The downregulation of pERK was also observed in SF126 cells using a combination treatment of Δ^9-THC and cannabidiol (Fig. 3D). Therefore, the modulation of pERK by the combination treatment of THC and cannabidiol seems to represent a common mechanism shared by different glioblastoma cell lines.

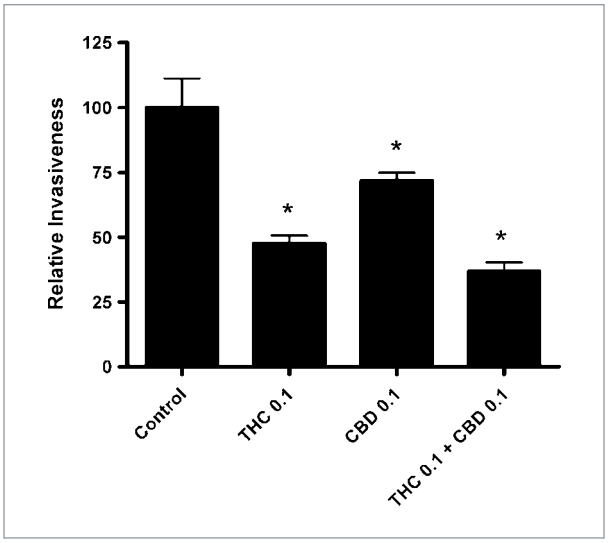

Figure 2. Δ^9-THC in combination with cannabidiol does not produce a greater overall inhibition of glioma invasiveness. To test for positive and negative interactions, a 2 × 2 factorial design was used as described in Materials and Methods. The Boyden chamber invasion assay was used to determine the effects of treatment on the invasiveness of U251 cells. U251 cells were treated for 3 d with Δ^9-THC (0.1 μmol/L), cannabidiol (0.1 μmol/L), or a combination of Δ^9-THC (0.1 μmol/L) and cannabidiol (0.1 μmol/L). Data are presented as relative invasiveness of the cells through the Matrigel, where the respective controls are set as 100%. Data are the mean of at least three independent experiments; bars, ± SE. *, statistically significant differences from control ($P < 0.05$).

The Combination Treatment of Δ^9-THC and Cannabidiol Inhibits Cell Cycle and Induces Apoptosis

Significant reductions in ERK activity have been shown to lead to growth arrest and induction of apoptosis (31). The large reduction in glioblastoma cell viability and ERK activity, observed in the presence of the combination treatment, led us to hypothesize there would be a corresponding modulation of the cell cycle and programmed cell death. Therefore, U251 cells were treated with Δ^9-THC and cannabidiol alone or with the combination of the two drugs, and cell cycle was analyzed using cell flow cytometry (Table 1). When administered separately, Δ^9-THC and cannabidiol both produced increases in the population of cells in G_0-G_1 phase, but not in the S and G_2-G_M phases. The combination of Δ^9-THC and cannabidiol produced a greater than additive increase in the population of cells in G_0-G_1 phase and G_2-G_M phase and a decrease in cells in S phase.

In addition to producing cell cycle arrest, the combination treatment may reduce cell viability through induction of apoptosis. We therefore measured apoptosis using annexin staining in combination with cell flow cytometery (Fig. 4A). There was a minor increase in apoptosis produced with 1.7 μmol/L Δ^9-THC, but it was not found to be significantly different from control ($n = 7$). No increase in apoptosis was observed in the presence of 0.4 μmol/L cannabidiol. However, when Δ^9-THC and cannabidiol were combined, a greater than additive increase in apoptosis was observed. In a time course analysis studying the induction of apoptosis produced by the combination treatment, we observed only a small increase with the combination treatment after two days (Supplementary Fig. S1A and B), whereas a strong induction of apoptosis was observed by day 3 (Fig. 4A).

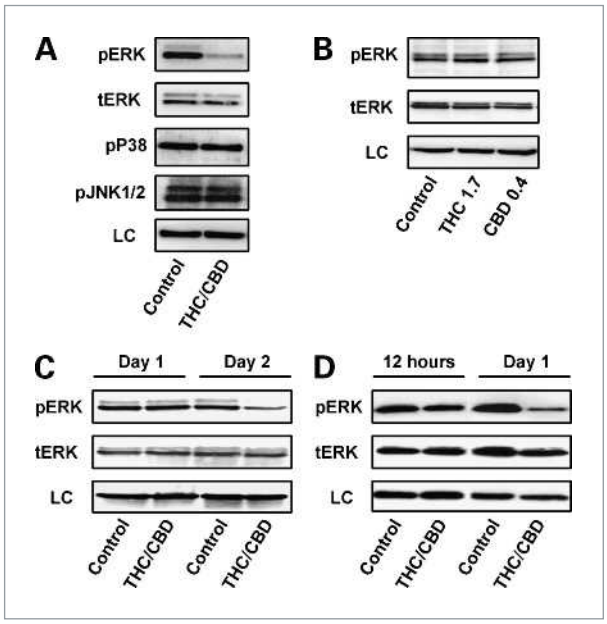

Figure 3. The combination treatment of Δ^9-THC and cannabidiol specifically inhibits ERK activity. The effects of cannabinoids on mitogen-activated protein kinases were analyzed using Western analysis. **A,** U251 cells were treated with vehicle or a combination of Δ^9-THC (1.7 μmol/L) and cannabidiol (0.4 μmol/L) for 3 d. Proteins were then extracted and analyzed for pERK, total ERK, pJNK1/2, and pP38 MAPK. **B,** U251 cells were treated with Δ^9-THC (1.7 μmol/L) or cannabidiol (0.4 μmol/L) alone for 3 d and analyzed for pERK and total ERK. **C,** U251 cells were treated with vehicle or a combination of Δ^9-THC (1.7 μmol/L) and cannabidiol (0.4 μmol/L) for 1 and 2 d. **D,** SF126 cells were treated with vehicle or a combination of Δ^9-THC (1.6 μmol/L) and cannabidiol (1.1 μmol/L) for 12 h or 1 d. Either α-tubulin or β-actin was used as a loading control (LC). Blots are representative of at least three independent experiments.

The Inhibitory Effects of the Combination Treatment Are the Result of CB_2 Receptor Activation and Production of ROS

Depending on the glioblastoma cell line used, studies have linked the inhibitory activity of plant-derived cannabinoids to activation of CB_1 and/or CB_2, modulation of MAPKs, and induction of cellular stress through increases in ROS and additional stress-related proteins, leading to activation of caspases (15, 32, 35). We used the measure of apoptosis to investigate mechanisms by which cannabidiol enhanced the activity of Δ^9-THC.

Apoptosis produced by the combination of Δ^9-THC and cannabidiol was partially blocked by the CB_2 receptor antagonist SR144528, but almost complete reversal was observed in the presence of the antioxidant α-tocopherol (TCP; Fig. 4B). The cannabinoid receptor antagonists and TCP had no effect on apoptosis on their own at 0.5 μmol/L and 20 μmol/L, respectively (data not shown). As predicted by TCP blockade, the combination of Δ^9-THC and cannabidiol produced a significant increase in the formation of ROS as assessed by DCDHF-DA oxidation using FACS analysis (Supplementary Fig. S1C and D). A small increase in ROS was observed after one day of treatment, with a major induction observed by day 2, preceding the majority of the observed apoptotic cell death.

In an attempt to match levels of apoptosis produced by the combination treatment, the concentrations of the individual cannabinoids (Δ^9-THC and cannabidiol) were next increased. The purpose of these experiments was to determine whether the compounds alone recruited similar pathways as compared with the combination of Δ^9-THC and cannabidiol. When U251 cells were treated with Δ^9-THC alone, the induction of apoptosis was almost completely blocked by TCP and partially blocked by the CB_2 antagonist SR144528 (Fig. 4C). However, Δ^9-THC alone could not produce the level of apoptosis observed with the combination treatment (Fig. 4B and C). This finding was not simply an issue of the treatment concentration used because application of Δ^9-THC up to 5 μmol/L did not produce a greater induction of apoptosis (data not shown). When U251 cells were treated with cannabidiol alone, the induction of apoptosis was blocked by TCP but no reversal was observed with SR144528 (Fig. 4D). This result was expected because cannabidiol does not interact efficiently with either CB_1 or CB_2 receptors.

The ability of the higher concentrations of Δ^9-THC (2.5 μmol/L) and cannabidiol (2.0 μmol/L) to inhibit pERK was also studied and compared with the combination treatment (Supplementary Fig. S2). Again, the combination treatment produced a substantial downregulation of pERK. However, the higher concentration of Δ^9-THC alone had no effect on pERK. The higher concentration of cannabidiol produced only a small inhibition of pERK. Taken together, these data suggest that a unique pathway was activated by the combined administration of Δ^9-THC and cannabidiol, which led to the downregulation of pERK.

Cannabinoids Mediate Apoptosis through *p8* and Caspases

The induction of the stress-associated gene, *p8*, has been shown to be a specific event in THC-induced apoptosis, but its involvement in cannabidiol-induced apoptosis has not been determined (35, 36). This pathway was evaluated to determine the role *p8* plays in the observed increase in apoptosis during cannabinoid treatments (Fig. 5A and B). Treatment of U251 cells with cannabidiol led to a small reduction in *p8* expression compared with control, but this change was not statistically significant ($n = 4$). Treatment with Δ^9-THC alone or the combination of Δ^9-THC and cannabidiol led to an upregulation of *p8* expression. The magnitude of the effect was similar between treatment groups. These data show that the modulation of *p8* was not specific for the combination treatment.

Multiple caspase pathways were next evaluated to determine additional mechanisms by which the combination

Table 1. Cannabinoid modulation of cell cycle

Treatment, μmol/L	Mean (G_0-G_1)	Mean (S)	Mean (G_2-G_M)
CBD 0.4	1.08* ± 0.03	0.90 ± 0.05	0.97 ± 0.28
Δ^9-THC 1.7	1.12* ± 0.04	0.78 ± 0.11	1.28 ± 0.37
Δ^9-THC 1.7/CBD 0.4	1.23*,† ± 0.02	0.49*,† ± 0.08	2.69*,† ± 0.56

NOTE: Cell cycle was measured using PI staining and FACS analysis, and Modfit was used to determine the percentage of cells in G_0-G_1, S, and G_2-G_M phase. U251 cells were treated for 3 d with CBD (0.4 μmol/L), Δ^9-THC (1.7 μmol/L), or a combination of CBD (0.4 μmol/L) and Δ^9-THC (1.7 μmol/L). The percentage of cells in each compartment was standardized by dividing it by the average percentage for the vehicle. This procedure was carried out for data from each experiment on each day. Statistical analysis was done as described in the Material and Methods.
Abbreviation: CBD, cannabidiol.
*$P < 0.05$.
†Significant interaction.

treatment increased apoptosis (Fig. 5C). In the presence of cannabidiol alone, no significant changes in caspase activity were observed. Small increases in the activity of caspase 7, caspase 9, and PARP, but not caspase 3, were observed when U251 cells were treated with Δ^9-THC alone. Treatment with the combination of Δ^9-THC and cannabidiol led to a substantial upregulation of caspase 3, 7, and 9 activities as well as an increase in PARP expression. These data show that a unique modulation of caspase activity is produced when glioblastoma cells are treated with the combination of Δ^9-THC and cannabidiol as opposed to the individual cannabinoids.

Discussion

We observed that plant-derived cannabinoids inhibit the proliferation of human glioblastoma cell lines. Compared with Δ^9-THC, cannabidiol was significantly more potent at inhibiting cancer cell growth. This finding is in agreement with studies using models of aggressive breast cancers (26, 37). Δ^9-THC is currently being used in a clinical trial for treatment of recurrent glioblastoma (5). Past studies have suggested that nonpsychoactive cannabinoids can modulate the actions of Δ^9-THC (17–20). We hypothesized that the cannabinoid therapy utilizing

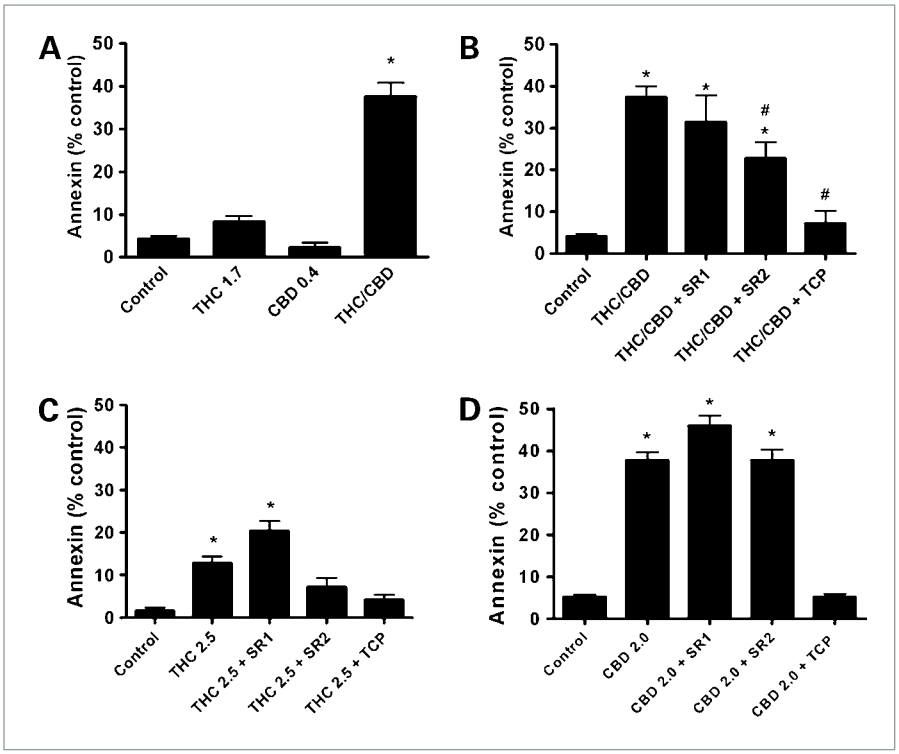

Figure 4. The effects of the combination treatment are the result of CB_2 receptor activation. The number of U251 cells positive for annexin (apoptosis) staining after 3 d treatment was measured using FACS analysis. Cells were treated with **A,** Δ^9-THC (1.7 μmol/L), cannabidiol (0.4 μmol/L), or a combination of Δ^9-THC (1.7 μmol/L) and cannabidiol (0.4 μmol/L) denoted as THC/CBD; **B,** a combination of Δ^9-THC (1.7 μmol/L) and cannabidiol (0.4 μmol/L) denoted as THC/CBD; **C,** 2.5 μmol/L Δ^9-THC; and **D,** 2.0 μmol/L cannabidiol. In **B, C,** and **D,** cells were also treated in the presence of 0.5 μmol/L of the CB_1 antagonist SR141716A (SR1), 0.5 μmol/L of the CB_2 antagonist SR144528 (SR2), or 20 μmol/L TCP. Percent control was calculated as positive annexin staining of the treated cells minus control cells. Data are the mean of at least three independent experiments; bars, ± SE. Data were compared using one-way ANOVA with Bonferroni's multiple comparison posthoc analyses. *, statistically significant differences from control ($P < 0.05$); #, statistically significant differences from the combination treatment of THC/CBD ($P < 0.05$).

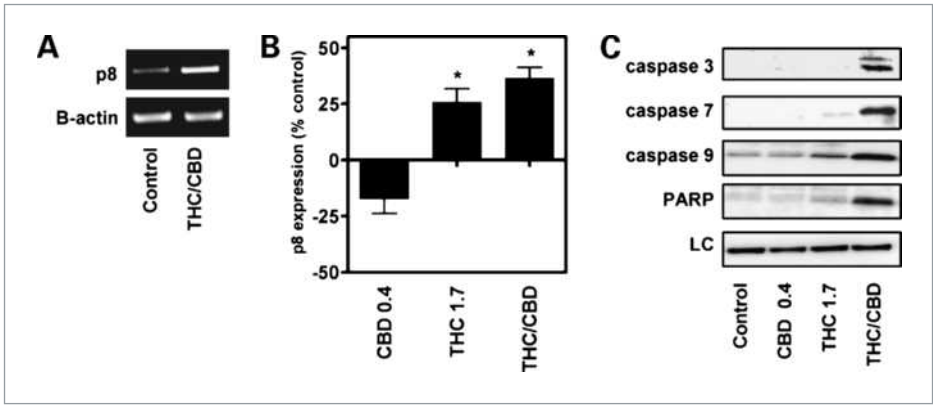

Figure 5. When combined, Δ^9-THC and cannabidiol produce an increase in activation of *p8* and multiple caspases. The effects of cannabinoids on *p8* and caspase expression were analyzed using semiquantitative reverse transcriptase-PCR and Western analysis, respectively. RNA and protein were collected from U251 cells treated for 3 d with cannabidiol (0.4 μmol/L), Δ^9-THC (1.7 μmol/L), or a combination of Δ^9-THC (1.7 μmol/L) and cannabidiol (0.4 μmol/L). **A,** reverse transcriptase-PCR was run on RNA extracted from control-treated and Δ^9-THC/cannabidiol-treated samples. Expression of the β-actin gene product was used as a control for equal loading. **B,** data are represented as percentage *p8* expression of the treated cells/control cells ×100, and all values were normalized against β-*actin*. Blots and PCR reactions are representative of at least three independent experiments. Data were compared using one-way ANOVA with Dunnett's multiple comparison posthoc analyses. *, statistically significant differences from control ($P < 0.05$). **C,** proteins were extracted from treated cells and analyzed for cleaved caspase 3, 7, 9, and PARP expression.

Δ^9-THC alone could be improved using a strategy of combination treatments.

We discovered that cannabidiol enhanced the ability of Δ^9-THC to inhibit cell proliferation and induce cell cycle arrest and apoptosis. This activity occurred in two of three glioblastoma cell lines tested. Treatment of U251 cells with the combination led to a substantial downregulation of ERK activity, but not p38 MAPK and JNK1/2. The reduction in the phosphorylation of ERK was specific for the combination treatment and occurred in more than one glioblastoma cell line. Importantly, continuing to increase the concentration of Δ^9-THC alone did not result in the inhibition of phosphorylated ERK. These data indicate that the enhanced effects observed were not solely due to an increase in the potency of Δ^9-THC in U251 cells on coapplication with cannabidiol. Further support for this conclusion was observed when studying the activity of Δ^9-THC and cannabidiol on U251 cell invasiveness. Both compounds were effective at inhibiting the invasiveness of U251 cells, but there was no evidence that cannabidiol improved the activity of Δ^9-THC on coapplication.

In human glioblastoma cells, the ability of Δ^9-THC to inhibit growth and induce apoptosis has been linked to the initial activation of CB_1 and CB_2 receptors (35). Similar effects produced by cannabidiol have been linked in part to CB_2 receptor activation, but the initial interaction site for the additional activity of cannabidiol remains to be clarified (7). We observed that increases in apoptosis produced by Δ^9-THC alone, or the combination of Δ^9-THC and cannabidiol, were partially dependent on CB_2 receptor activation. Apoptosis produced by cannabidiol alone was not dependent on CB_2 receptor activation. Importantly, the induction of apoptosis in the presence of the combination treatment was significantly greater than that observed with Δ^9-THC alone. Apoptosis produced by the combination of Δ^9-THC and cannabidiol was dependent on the production of oxidative stress and resulted in a unique activation of both intrinsic and extrinsic caspases.

Studies have shown that the inhibitory activity of cannabinoids in glioblastoma is dependent on activation of CB_1 and CB_2 receptors, modulation of MAPKs, and induction of multiple types of cellular stresses leading to apoptosis (32, 35, 38, 39). In the case of Δ^9-THC, upregulation of *p8* seems to be a specific event that leads to apoptosis in multiple types of cancers (35, 36). Treatment of U251 cells with the combination of Δ^9-THC and cannabidiol led to an upregulation of *p8* expression, but similar activity was seen with Δ^9-THC alone. This is in contrast to what we observed when studying modulation of caspase activity, and suggests that the enhanced apoptotic activity produced by the combination treatment was not the result of an interaction with the p8 pathway.

The ability of cannabidiol to inhibit growth and induce apoptosis in glioblastoma and additional cancers has been primarily associated with the upregulation of ROS and multiple caspases, and has been linked to alterations in NADPH oxidases (9, 15). A link between ROS production and modulation of the LOX pathway has been hypothesized as a potential mechanism for the antitumor activity of cannabidiol in glioblastoma (16). In this study, an initial increase in ROS was clearly linked to a latter induction of apoptosis. Individually, both Δ^9-THC and cannabidiol could increase apoptosis through the production of ROS, but Δ^9-THC was significantly less efficient at inducing this process as a single agent as compared with when it was used in combination with cannabidiol. Although the concentration of cannabidiol used in the combination treatment did not significantly

stimulate ROS, it may have primed this pathway for Δ^9-THC through a convergence on shared signal transduction pathways. A similar hypothesis could explain the unique downregulation of phosphorylated ERK that was produced by the combination treatment. Alternatively, cannabidiol may have potentiated the activity of Δ^9-THC by inhibiting pathways that impart drug resistance in glioblastoma. For instance, a recent study showed that amphiregulin expression was associated with increased ERK activation, which mediated resistance to THC-induced apoptosis in gliomas (40). Therefore, cannabidiol may have potentiated the activity of THC-induced apoptosis by inhibiting amphiregulin-regulated increases in ERK activation. Future studies will be needed to elucidate the detailed mechanism associated with the unique effects of the Δ^9-THC and cannabidiol combination treatment.

Individually, Δ^9-THC and cannabidiol can activate distinct pathways in glioblastoma cells that ultimately culminate in inhibition of cancer cell growth and invasion as well as induction of cell death (2, 4, 41). We hypothesized that, if the individual agents were combined, a convergence on shared pathways may ensue, leading to an enhanced ability of the combination treatment to inhibit certain cancer cell phenotypes. We found this to be true in this investigation. Cannabidiol significantly improved the inhibitory effects of Δ^9-THC on glioblastoma cell proliferation and survival, but not on cell invasiveness. The data suggest that the improved activity observed with the combination treatment is the result of a specific modulation of ERK and ROS activity leading to inhibition of cell cycle and induction of apoptosis.

Combinations, compared with individual drug treatments with specific cannabinoid-based compounds, may represent an improvement for the treatment of patients with glioblastoma and perhaps additional cancers. It is also possible that other constituents of *C. sativa* that are not structurally related to cannabinoids could improve antitumor activity when combined. An important next step will be to carry out studies testing for synergistic antitumor activity of cannabinoids in additional preclinical models of glioblastoma. Even if synergism is not evident, combination treatments may allow for increased dosing due to nonoverlapping toxicities and decrease the development of resistance to the activity of Δ^9-THC or cannabidiol when administered alone. With the growing evidence showing cannabinoids are effective inhibitors of multiple types of cancer, it is likely that additional clinical trials will be carried out. Combination treatments with cannabinoids may improve overall efficacy in these future clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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