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Cannabidiol inhibits RAD51 and sensitizes glioblastoma to temozolomide in multiple orthotopic tumor models

Liliana Soroceanu, Eric Singer, Pratiksha Dighe, Max Sidorov, Chandani Limbad, Aida Rodriquez-Brotons, Peter Rix, Rinette W. L. Woo, Lawrence Dickinson, Pierre-Yves Desprez, and Sean D. McAllister

California Pacific Medical Center Research Institute, San Francisco, CA 94107, USA (L.S., E.S., P.D., M.S., C.L., A.R.-B., R.W.L.W., P.Y.D., S.D.M.); Launch Bioscience, San Diego, CA 92130, USA (P.R.); Eden Medical Center Sutter Research, Castro Valley, CA 94546, USA (L.D.)

Corresponding Authors: Sean D. McAllister, California Pacific Medical Center Research Institute, 475 Brannan Street, Suite 130, San Francisco, CA 94107, USA (mcallis@cpmcri.org); Liliana Soroceanu, California Pacific Medical Center Research Institute, 475 Brannan Street, Suite 130, San Francisco, CA 94107, USA (sorocel@cpmcri.org).

Abstract

Background. Cannabidiol (CBD), a nonpsychoactive cannabinoid with a low toxicity profile, has been shown to produce antitumor activity across cancers in part through selective production of reactive oxygen species (ROS) in tumor cells. The alkylating agent, temozolomide (TMZ), is standard of care for treatment of glioblastoma (GBM). It can trigger increased ROS to induce DNA damage. It has also been reported that downregulating the expression of RAD51, an important DNA damage repair protein, leads to sensitization of GBM to TMZ.

Methods. We determined the extent to which CBD enhanced the antitumor activity of TMZ in multiple orthotopic models of GBM. In addition, we investigated the potential for CBD to enhance the antitumor activity of TMZ through production of ROS and modulation of DNA repair pathways.

Results. CBD enhanced the activity of TMZ in U87 MG and U251 GBM cell lines and in patient-derived primary GBM163 cells leading to stimulation of ROS, activation of the ROS sensor AMP-activated protein kinase (AMPK), and upregulation of the autophagy marker LC3A. CBD produced a sensitization of U87 and GBM163-derived intracranial (i.c.) tumors to TMZ and significantly increased survival of tumor-bearing mice. However, these effects were not observed in orthotopic models derived from GBM with intact methylguanine methyltransferase (MGMT) expression. We further demonstrate that CBD inhibited RAD51 expression in MGMT-methylated models of GBM, providing a potential mechanism for tumor sensitization to TMZ by CBD.

Conclusion. These data support the potential therapeutic benefits of using CBD to enhance the antitumor activity of TMZ in GBM patients.

Key Points

- CBD produces enhancement of TMZ antitumor activity selectively in MGMT-methylated GBM leading to prolong survival.
- CBD inhibited RAD51 expression in MGMT-methylated GBM, providing a potential mechanism for tumor sensitization to TMZ by CBD.

In the management of glioblastoma (GBM), surgery with postoperative radio- and chemotherapy, primarily with the alkylating agent temozolomide (TMZ), are the treatment of choice.[1] Additional tumor treatment modalities have also been FDA approved for the treatment of GBM.[2] Despite these aggressive treatments, 90% of the patients die within 2 years.[1] Specifically, patients with an unmethylated O6-methylguanine–DNA methyltransferase (MGMT) promoter respond poorly

Importance of Study

Development of effective treatment strategies for GBM is a high unmet need. This study demonstrates that CBD produces enhancement of TMZ antitumor activity selectively in MGMT-methylated GBM leading to prolong survival. This effect may in part be explained by CBD targeting of RAD51. Additional studies in GBM with the antitumor agent CBD, a brain penetrant cannabinoid with a low toxicity profile, are warranted.

to alkylating agents such as TMZ.[3] Thus, there is an urgent need for novel therapeutics strategies targeting GBM.

The cannabinoid (CB) Δ^9-tetrahydrocannabinol (THC) activates 2 known CB receptors (CB_1 and CB_2), which leads to the inhibition of cell proliferation and induction of apoptosis resulting in the reduction of tumor burden in vivo in multiple cancers, including GBM.[4–6] These antitumor effects of CB_1 and CB_2 receptor agonists are thought to occur primarily through the endoplasmic reticulum (ER) stress-dependent upregulation of autophagy-mediated cell death pathways (a caspase-independent form of programmed cell death).[6–8] However, the clinical utility of THC is limited by its psychoactive effects. There are more than 60 CBs in Cannabis sativa and a majority are not psychoactive.[9] Nonpsychoactive CBs found in reasonable abundance include cannabidiol (CBD), cannabigerol (CBG), and cannabichromene (CBC).[9] CBD has negligible affinity for the cloned CB_1 and CB_2 receptors and does not directly target the classical endoCB system.[10–12] We determined that in a screen of plant-based cannabiniods, that CBD was the most active at inhibiting cell viability/proliferation (viability) in cancer lines from different origins.[13] This effect has been shown to exhibit significantly lower potency in noncancer cells.[14] The initial site CBD interacts with to produce antitumor activity is unknown. Multiple target sites have been implicated,[15] but the most unifying downstream mechanism in culture is the initial CBD-dependent selective production of reactive oxygen species (ROS) in tumor cells.[16]

This led us to hypothesize that nonpsychoactive CBs could be as effective as psychoactive CBs at inhibiting cancer progression and could reduce or replace the amount of psychoactive CBs used in the treatment of cancer. In support of this hypothesis, we previously reported that CBD can reduce the concentration of THC needed to inhibit GBM cell growth and induce apoptosis in culture.[17] This result was confirmed by additional investigations in vivo[18] in subcutaneous xenograft tumor models, while our group has also demonstrated that CBD alone can inhibit human GBM progression in an intracranial xenograft mouse model.[19]

As a result of the growing preclinical body of evidence of direct antitumor activity produced by THC and CBD, multiple clinical trials have evaluated the activity of CBs in GBM. In a pilot study, intracranially administered THC was shown to be safe, in addition to inhibiting markers of tumor cell proliferation.[20] Sativex, a plant-based extract containing a 1:1 ratio of THC:CBD, was also shown to prolong survival for GBM patients in a Phase IIb clinical trial, but the detailed results of the trial have not been publish.[21] The direct antitumor activity produced by CBD has also recently been evaluated in clinical case studies across cancers, including GBM. Clinical responses were observed in a significant subset of patients supporting the need for future controlled clinical trials.[22,23] Taken together, these findings underscore the importance of understanding mechanims of CB antitumor activity, which may further improve the activity of the CBs and potentially lead to the development of more active second-generation compounds.

In this study, we investigated the antitumor activity of CBs alone and in combination with TMZ for targeting GBM progression in intracranial models using GBM cell lines and primary patient-derived GBM. In comparison to standard GBM cell lines, primary patient-derived GBM grown under glioma stem cell conditions more readily recapitulate the genotype, gene expression patterns (transcriptome), and in vivo growth patterns of human GBM.[24] Mechanistically, we focused on the antitumor properties of the nonpsychoactive CB, CBD. We found CBD alone, in comparison to THC and the combination of CBD + THC, was as effective at sensitizing GBM tumors to TMZ in multiple intracranial xenograft models. However, CBD did not sensitize TMZ-resistant GBM to TMZ in vivo. CBD-dependent stimulation of ROS led to inhibition of GBM cell viability in cell lines and primary patient-derived cultures. CBD enhanced the ability of TMZ to inhibit cell viability in part through production of ROS. CBD alone in combination with TMZ upregulated the expression of the ROS sensor AMP-activated protein kinase (AMPK), and the autophagy marker LC3A. CBD alone also inhibited RAD51 in TMZ-sensitive cell lines, but not in TMZ-insensitive cell lines. Importantly, CBD has a low toxicity profile in humans and is already being tested in clinical trials for GBM. Taken together, these data support CBD as a potential adjuvant therapy for targeting GBM.

Methods

Drugs

CBD was obtained from INSYS Therapeutics, and THC was obtained from National Institutes of Health (NIH; Bethesda, MD) through the National Institute of Drug Abuse. All other chemicals and drugs were obtained through Selleckchem (Houston, TX).

Primary Cell Culture and In Vivo Passaging

Cultures were generated in-house from tissue samples obtained during surgical resection of patients diagnosed

with GBM. As previously described,[25] tumors were then subjected to enzymatic digest, mechanically dissociated and cultured as neurospheres as previously described.[26] Tumor lines were maintained as subcutaneous flank xenografts in athymic nu/nu mice and processed as stated above.

In Vivo Studies

Six- to 8-week-old female athymic nu/nu mice with a weight range of 20–25 g were obtained from Envigo (Indianapolis, IN). Human U87, U251, and T98 GBM cells were grown in RPMI media with 10% FBS and were harvested from dishes while in their exponential growth phase in culture with 0.1% trypsin/EDTA, and washed twice with serum-free RPMI media. Primary lines were grown as described above. For the intracranial model, tumors were generated in female athymic nu nu mice by the intracranial injection of cells as detailed in the figure legends. Survival studies were carried out in accordance with the National Institutes of Health's guidelines involving experimental neoplasia and our approved IACUC protocol. Animals in all groups were removed from the study when they demonstrated any single sign indicative of significant tumor burden development, including hunched back, sustained decreased general activity, or a significant decrease in weight. For drug treatment studies, CBs were dissolved in a mixture of 2.5% ethanol, 2.5% Tween 80, and 95% saline, and TMZ was dissolved in 30% DMSO and 70% saline. Treatments were initiated based on the known progression of the tumors determined through pilot studies. For luciferase-labeled GBM tumors, mice were randomized based on imaging as previously described.[27] When tumor cells where not luciferase-labeled, they were randomized based on their body weight. Animal health observations and removal from the study up first incidence of tumor burden development is described in Supplementary Material.

Western Blotting

Western analysis was performed as previously described.[13] Western blots were probed with the antibodies described in Supplementary Material. Anti-actin and anti-GAPDH were used as loading controls. The relative amounts of proteins were quantified using densitometry and the software program ImageJ (NIH).

Pharmacokinetic Studies

To assess the pharmacokinetic (PK) of CBD, female BALBc mice were injected i.p with 15 or 7 mg/kg of CBD. Blood samples were collected at 5, 15, 30 min and 1, 2, 5, 12, 24, and 48 h after i.p. administration and analyzed as described in Supplementary Material.

Immunohistochemistry and Immunofluorescence

Animal brains were harvested, fixed in 10% formalin and processed for immunohistochemistry and immunofluorescene as previously published by our group[28] and as outlined in Supplementary Material.

Ethics Approval and Consent to Participate

Patient tumor tissue was collected under an IRB-approved research protocol in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient in the study. All patient data were de-identified for the study. In vivo studies were carried out in accordance with the National Institutes of Health guidelines, Health Research Extension Act of 1985 and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Policy), Office of Laboratory Animal Welfare assurance, and an approved Institutional Animal Care and Use Committee (IACUC) protocol.

Data and Statistical Analyses

The IC_{50} values with corresponding 95% confidence limits were compared by the analysis of logged data using GraphPad Prism (La Jolla, CA). Significant differences were also determined using a one-way ANOVA or the unpaired Student's *t*-test, where suitable. Survival data were evaluated using Kaplan–Meir curves and a log-rank Mantel–Cox test. *P*-values < .05 defined statistical significance. To test for synergism, the combination index (CI) was also calculated using Compusyn (Paramus, NJ) where CI < 1, = 1, and >1 indicates synergism, additive effect, and antagonism, respectively, as previously described[29] and as previously published by our group.[17]

Results

CBD Enhances the Activity of TMZ in U87and U251 GBM Cells

We determined whether CBD could enhance the activity of the first-line agent TMZ by evaluating the effects of the drugs alone or in combination (Figure 1A and B). U87 and U251 cells were treated for 3 days with a range of concentrations of either CBD, TMZ, or CBD + TMZ and the ability of the drugs to inhibit cell viability was assessed using the MTT assay. Using the calculated IC_{50} values, various dose ratios of CBD and TMZ were combined in both U87 and U251 cells, and viability was evaluated and a CI throughout the dose ratios was calculated (Figure 1C). A CI value of <1, 1, and >1 indicates synergism, additivity, and antagonism, respectively.[30] The combination of CBD and TMZ led primarily to additive inhibition of cell viability across multiple dose ratios in U87 and U251 cells.

CBD-Dependent Stimulation of ROS Leads to Inhibition of GBM Cell Viability

CBD produces a sustained upregulation of ROS in a concentration-dependent manner in U251 GBM cells leading to inhibition of cell viability (Figure 1D). This effect

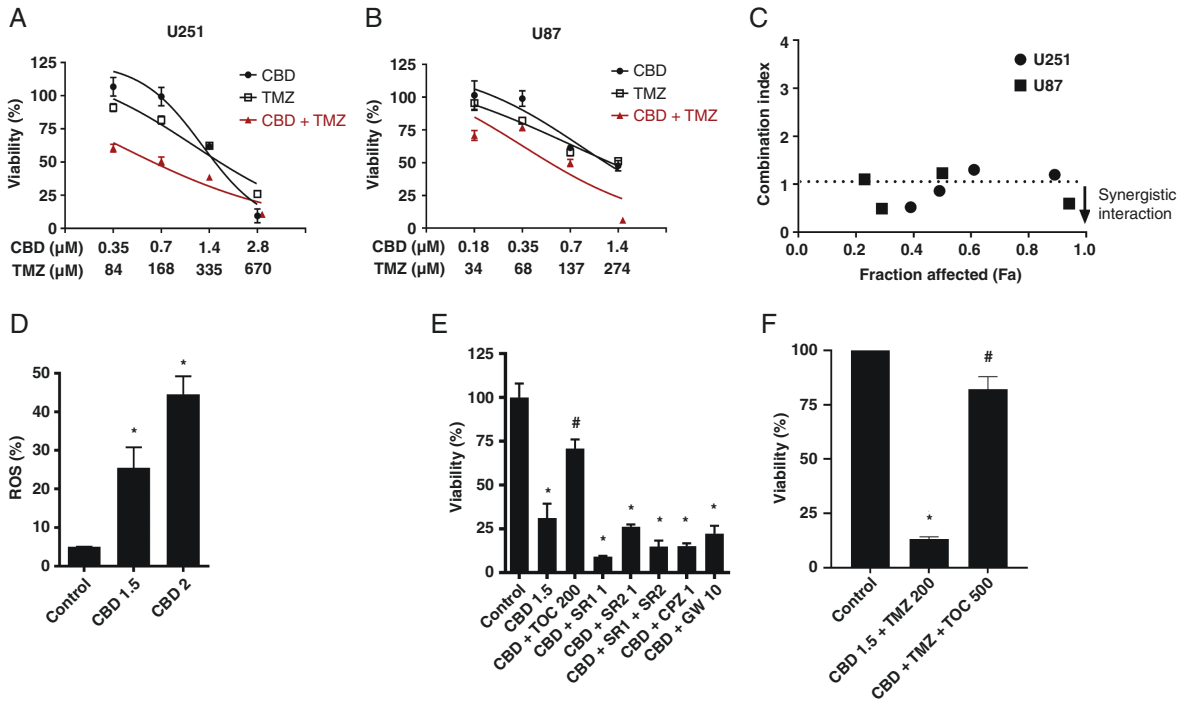

Figure 1. CBD enhances the inhibitory effects of TMZ on GBM cell growth through upregulation of ROS. (A) U251 and (B) U87 cells were treated for 3 days with vehicle, CBD, TMZ, or CBD + TMZ at specific combined dose ratios and cell viability (%) was calculated as absorbance in the treated cells/control cells × 100. (C) These data were used to calculate combination index (CI) values as described previously by our group[17,31] using Compusyn software. A CI value of <1, 1, and >1 indicates synergism (downward arrow), additivity (hashed line), and antagonism, respectively.[30] (D) U251 cells were treated for 2 days with vehicle or CBD (μM) and production of ROS was measured using 2′-7′dichloro-dihydrofluorescein. (E) U251 cells were treated for 2 days with 1.5 μM CBD (CBD) in the presence or absence of 200 μM μ-tocopherol (TOC), 1 μM CB1 receptor antagonist (SR141716A—SR1), 1 μM CB2 receptor antagonist (SR144528—SR2), 1 μM vanilloid receptor antagonist (capsazepine—CPZ), or 10 μM PPRg antagonist (GW 9662—GW). (E) U251 cells were treated for 3 days with 1.5 μM CBD (CBD) + 200 μM TMZ in the presence or absence of 500 μM TOC. Data are the mean of 3 independent experiments; bars ± SE. *,#Statistically significant difference between control and CBD, respectively ($P < .05$).

is not the result of interaction with CB_1 and CB_2 receptors, VR_1 receptors, or PPRγ, but was reversed by the ROS scavenger α-tocopherol (TOC) (Figure 1E). Inhibition of cell viability produced by the combination of CBD + TMZ was also blocked by TOC (Figure 1F). The ability of CBs to sensitize GBM to TMZ in orthotopic models has not been evaluate; therefore, we next studied the effects of CBs to sensitize human GBM to TMZ in vivo.

CBs Sensitize Human U87-Derived Intracranial Tumors to TMZ

We evaluated the ability of CBD alone or CBD + THC in a 1:1 ratio (CBD/THC) in combination with TMZ to inhibit tumor growth and extend survival in an intracranial (i.c.) model of GBM utilizing human U87 cells. In vivo imaging of luciferase-labeled U87 cells evaluated reduction in radiance (indirect measure of tumor size) in each treatment group (Figure 2A and B). CBD or CBD/THC did not inhibit GBM progression; however, these CBs produced a sensitization of U87-derived tumors to TMZ, leading to more effective inhibition of tumor progression and prolonged survival (Figure 2C). The mean survival for vehicle, CBD, CBD/THC, TMZ, CBD + TMZ, and CBD/THC + TMZ was 39, 43, 40, 46, 53, and 53 days, respectively (Figure 2C). Treatment with CBD + TMZ ($P < .043$) or CBD/THC + TMZ ($P < .030$) produced a significant improvement in survival in comparison to treatment with TMZ alone. Treatment with CBD + TMZ also produced full regression in 2 of the 11 tumors.

To determine whether the effect of CBD *in vivo* was dose dependent, we treated mice bearing U87-derived i.c. tumors with 3.75 and 7.5 mg/kg of CBD alone or in combination with TMZ (Figure 2D and E). We observed full regression of a tumor in 1 of 11 mice in the CBD treatment group but overall there was no significant increase in survival in this group compared to control. We also observed full regression of tumor growth in 2 of 11 mice in the TMZ-treated group. In contrast to 15 mg/kg of CBD (Figure 2C), lower doses of CBs did not enhance the antitumor activity of TMZ in an orthotopic mouse model of human GBM. These data demonstrate that the ability of CBD to enhance the antitumor activity of TMZ is dose dependent. PK parameters for CBD in plasma (Supplementary Table 1 and Supplementary Figure 1A and B) were compared between the 15 mg/kg (produced sensitization to TMZ) and 7.5 mg/kg (did not produce sensitization to TMZ) dosage levels to determine PK parameters that correlate with CBD-dependent

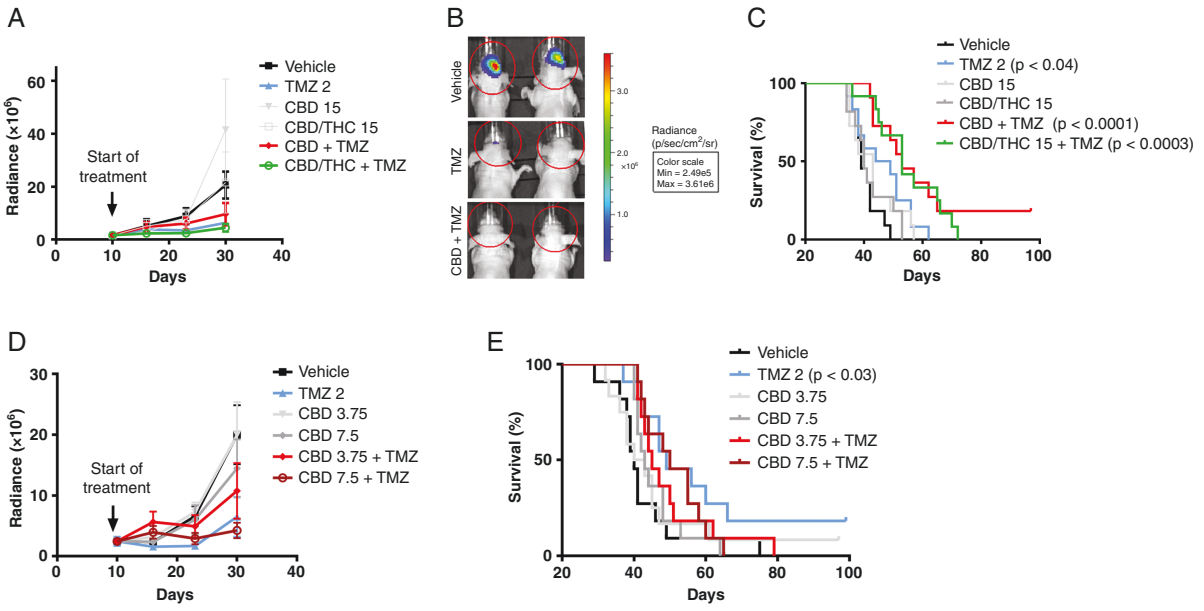

Figure 2. Cannabinoids (CBs) sensitize human GBM to TMZ in orthotopic mouse tumor models. Tumors were generated in female athymic nu/nu mice (n = 11–12) by the intracranial (i.c.) injection of 0.3 × 10^6 U87 luciferase-labeled cells or U251 cells in 4 µl of RPMI. Starting on day 9, CBs were administered i.p. 5 days a week until completion of the experiment. Starting on day 9, TMZ was administered i.p. 5 days a week for one week. Mice were treated with vehicle, 15 mg/kg CBD, 15 mg/kg CBD/THC (1:1), 2 mg/kg TMZ, 15 mg/kg CBD + 15 mg/kg TMZ, and 15 mg/kg CBD/THC + 2 mg/kg TMZ and (A) tumor progression, (B) i.c. luciferase-based imaging of vehicle (top), TMZ at 2 mg/kg (middle panel) 15 mg/kg CBD + 2 mg/kg TMZ (bottom) at day 30, and (C) survival were assessed. Mice bearing U87-derived i.c. tumors (n = 11) were treated with 3.75 and 7.5 mg/kg of CBD alone or in combination with TMZ and (D) tumor progression and (E) survival were assessed. Mice bearing U251-derived i.c. tumors (n = 10–11) were treated with vehicle, 15 mg/kg CBD, 1.5 mg/kg TMZ, 15 mg/kg CBD + 1.5 mg/kg TMZ, and (F) survival was assessed between vehicle and treated animals. Survival data were evaluated using Kaplan–Meier curves and the long-rank Mantel–Cox test. P < .05 defined statistically significantly differences.

production of TMZ sensitization. Plasma levels of CBD using LC/MS/MS analysis were determined and converted into time–concentration plots, and PK parameters were calculated. To model the in vivo experiments reported in this investigation, i.p administration was used to evaluate drug levels. Therefore, it was not possible to determine absolute bioavailability or the true elimination half-life because intravenous administration was not used. The dose of 15 mg/kg of CBD produced a C_{max} of 3.3 µg/ml or 10.6 µM in plasma, with a calculated $AUC_{0\text{-}t}$ of 1.63 µg·h/ml. The C_{max} and $AUC_{0\text{-}t}$ were 2.5 and 2.7 time higher, respectively, in the 15 mg/kg compared with the 7.5 mg/kg CBD dosing group. The T_{max} was similar between both dosing groups. Based on the antitumor studies, we chose to use a CB dose of 15 mg/kg for the remainder of the in vivo studies.

CBD Sensitizes Primary Patient-Derived GBM to TMZ

CBs produce a sensitization of human U87-derived intracranial tumors to TMZ. GBM initiation, resistance to therapy, and recurrence has been shown to be driven by a subpopulation of tumor cells with stem-like characteristics, the glioma stem-like cells (GSC).[32–34] In comparison to standard GBM cell lines, primary GBM grown under GSC conditions more readily recapitulated the genotype, gene expression patterns (transcriptome), and in vivo growth patterns of human GBM.[24] We therefore evaluated whether CBD could enhance the antitumor activity of TMZ in a primary patient-derived xenograft mouse model of GBM.

GBM163X cells were treated for 3 days with a range of concentrations of either CBD or TMZ, and the ability of the drugs to inhibit cell viability was assessed. Using the calculated IC_{50} values, various dose ratios of CBD and TMZ were combined and viability was evaluated and a CI throughout the dose ratios was calculated (Figure 3A).

In GBM163X cells, the combination of CBD and TMZ led to slightly antagonist to additive inhibition of cell viability, as the fraction affected (reduction in cell viability) was increased. Inhibition of GBM163X cell viability produce by CBD or CBD +TMZ was blocked in the presence of TOC (Figure 3B). We next evaluated the ability of CBD alone or in combination with TMZ to inhibit tumor growth and prolong survival in an i.c. model of GBM utilizing GBM163X. As shown in Figure 3C, the mean survival for vehicle, CBD, THC, CBD/THC, TMZ, CBD + TMZ, THC + TMZ, and CBD/THC + TMZ was 44, 43, 42, 42, 66, 78, 73, and 82 days, respectively. Treatment with TMZ alone prolonged survival (P < .0001); however, treatment with CBD, THC, or CBD/THC alone did not. Treatment with CBD + TMZ (P < .003), THC + TMZ (P < .005), or CBD/THC + TMZ (P < .0003) produced a significant improvement

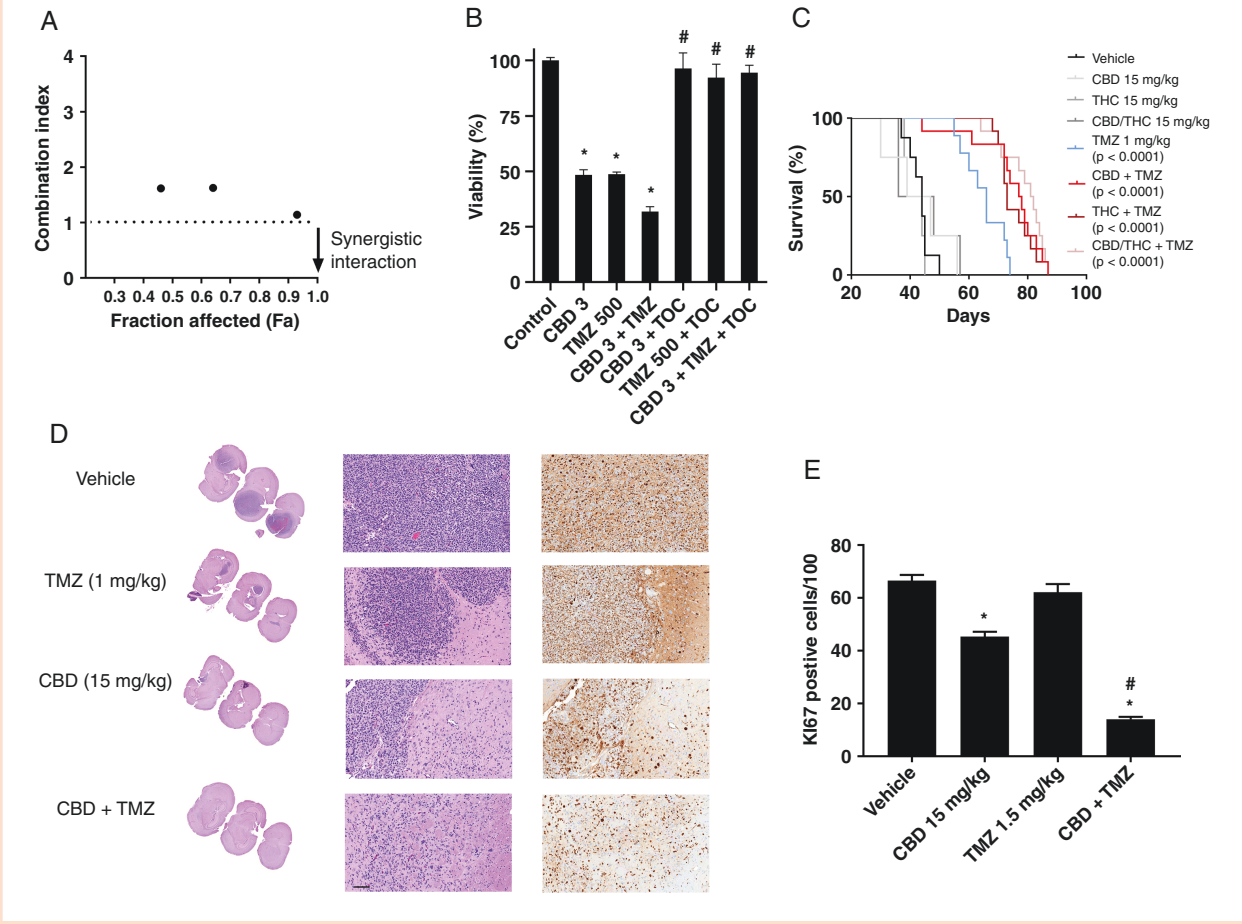

Figure 3. Cannabinoids enhance the activity of TMZ in patient-derived orthotropic mouse tumor models. (A) GBM163X cells were treated for 3 days with vehicle, CBD or TMZ alone at specific combined dose ratios (3 μM CBD combined with 250, 500, or 750 μM of TMZ) and cell viability (%) was calculated as absorbance in the treated cells/control cells × 100. These data were used to calculate combination index (CI) values as described previously by our group[17,31] using CompuSyn software. A CI value of <1, 1, and >1 indicates synergism (downward arrow), additivity (hashed line), and antagonism, respectively.[30] (B) GBM163X cells were treated for 3 days with vehicle (control), 2 μM CBD, 500 μM TMZ, or 2 μM CBD + 500 μM TMZ in the presence and absence of TOC and cell viability was evaluated. (C) Tumors were generated in female athymic nu/nu mice by the intracranial (i.c.) injection of 0.3×10^6 GBM163X cells in 4 μl of RPMI. Starting day 21, CBs were administered i.p. 5 days a week until completion of the experiment. Starting on day 21, 1 mg/kg TMZ was administered i.p. 5 days a week for 1 week. Mice (n = 8–12 for vehicle, TMZ, and drug combinations) were treated with vehicle, 15 mg/kg CBD, 15 mg/kg THC, 15 mg/kg CBD/THC (1:1), 1 mg/kg TMZ, 15 mg/kg CBD + 1 mg/kg TMZ (CBD + TMZ), 15 mg/kg THC + 1 mg/kg TMZ (THC + TMZ), and 15 mg/kg CBD/THC + 1 mg/kg TMZ (CBD/THC + TMZ) and (C) survival was assessed. (D) Representative microphotographs of H&E staining from GBM163-derived i.c. tumors treated as indicated (left and center panels). Right panels show Ki67 staining of the same tissue samples from mice treated with vehicle, CBD, TMZ, and the combination of CBD + TMZ. Samples were collected at the time the animals succumbed to disease. Bar = 200 μm. (E) Bar graph shows quantification of Ki67 positive cells counting 6 fields from 2 different animals in each group (20×); the plotted numbers represent averages of Ki67 positive cells for 100 total human tumor cells/field. $^{**}P < .02$ when compared with vehicle. Survival data were evaluated using Kaplan–Meir curves and the long-rank Mantel–Cox test. Data are the mean of 3 independent experiments; bars, ± SE. $^{*,\#}$Statistically significant from control and CBD, respectively ($P < .05$).

in survival in comparison to treatment with TMZ alone. While the combination of CBD/THC + TMZ improved survival to the greatest extent, there was no significant difference between CBs (CBD, THC, or CBD/THC) in the ability to sensitize GBM to TMZ. This is in agreement with what was observed in the U87-derived i.c. model. Both CBD alone and CBD + TMZ produced a sustained inhibition of tumor cell proliferation in vivo as evidenced by reduced Ki67 staining (Figure 3D and E). We next determined the antitumor activity of CBs alone or in combination with TMZ in GBM resistant to the antitumor activity of TMZ.

CBD Does Not Sensitize TMZ-Resistant, MGMT-Unmethylated GBM to TMZ

We tested the activity of the combination of CBD + TMZ in the TMZ-resistant GBM T98 cell line and in the TMZ-resistant primary GBM 3832 cells (Figure 4). Unlike U87 and U251 cells, where MGMT levels are undetectable, 3832 and T98 cells express MGMT to a variable extent (Supplementary Figure 2). TMZ is an alkylating agent prodrug, delivering a methyl group to purine bases of DNA (O6-guanine; N7-guanine, and N3-adenine). The primary cytotoxic lesion, O6-methylguanine (O6-MeG),

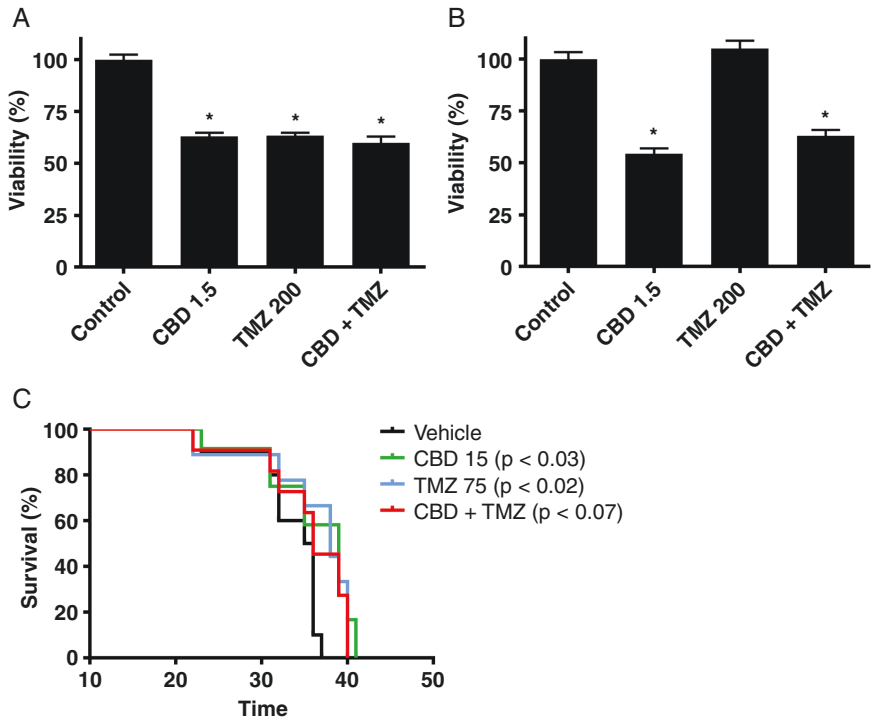

Figure 4. CBD does not enhance the antitumor effects of TMZ in TMZ-resistant GBM. (A) T98 cells and (B) 3832 primary cells where treated with vehicle (control), 1.5 μM CBD, 200 μM TMZ, or 1.5 μM CBD + 200 μM TMZ for 3 days, and cell viability was evaluated. Data are the mean of 3 independent experiments; bars, ± SE. *Statistically significant interaction ($P < .05$). (C) Athymic nu/nu mice ($n = 10$) were injected i.c. with 1×10^4 patient-derived GSC 3832 cells labeled with luciferase. Treatments started at day 9 following BLI confirmation of tumor presence (using bioluminescence measurements) and randomization of mice. Mice were treated with vehicle, CBD (15 mg/kg), TMZ (75 mg/kg) or CBD (15 mg/kg) + TMZ (75 mg/kg), and survival was assessed. Survival data were evaluated using Kaplan–Meir curves and the long-rank Mantel–Cox test.

can be removed by MGMT through direct DNA repair mechanism. Thus, in tumors expressing this protein, the antitumor activity of TMZ is reduced.[35] In contrast to the effect of CBD + TMZ in U87 and U251 cells, the drug combination did not demonstrate additive or synergistic effects in T98 and 3832 cells (Figure 4A and B). We next evaluated the ability of CBD alone or in combination with TMZ to inhibit tumor growth and prolong survival in an i.c. model of primary GBM utilizing 3,832 cells. In vehicle-treated mice, this highly aggressive line produces tumors with a median survival of 35 days even at inoculations of 5,000 cells, with 50% of the populations of vehicle-treated mice demonstrating symptoms of tumor burden between day 35 and 36. As shown in Figure 4C, the mean survival for vehicle, CBD, TMZ, and CBD + TMZ was 35.5, 39, 38, and 36 days, respectively. In comparison to GBM tumors with undetectable levels of MGMT, tumors derived from 3,832 cells, which express MGMT, demonstrated resistance to the antitumor activity of TMZ. Therefore, mice were administered a maximum tolerated dose (MTD) of 75 mg/kg TMZ in vivo (Figure 4C), and treatment with TMZ alone prolonged survival ($P < .02$). CBD treatment increased animal survival ($P < .03$), consistent with our previously published work[26]; however, CBD in combination with TMZ was not significantly more effective than either drug alone (Figure 4C). Overall, the data demonstrate that CBD produces more robust enhancement of TMZ activity in GBM cells with lower levels of MGMT expression, which are more responsive to TMZ.

The Combination of CBD + TMZ Activates AMPK and Upregulates the Autophagy Marker LC3-II

To further understand the mechanisms underlying the CBD-induced sensitization to TMZ, we next investigated the effects of CBD and TMZ on the AMPK and authophagy pathways. TMZ-sensitive U87 and GBM163X (Figure 5A and B) and TMZ-insensitive T98 and GBM3832 (Figure 5C and D) cells were treated with vehicle (control), CBD, TMZ or CBD + TMZ. The combination of CBD + TMZ was most efficient at activating the redox (ROS) sensor AMPK (via phosphorylation) in all the tumor cells tested, with varying degrees of AMPK activation produced by CBD and TMZ alone. Stimulation of APMK leads to autophagy-mediated cell death.[36] The conversion of the soluble form of LC3 (LC3-I) to the lipidated and autophagosome-associated form (LC3-II; lower band in the Western blot

image) is considered one of the hallmarks of autophagy. A majority of the LC3-I to LC3-II conversion resulted from CBD treatment alone, with the exception of T98 cells where conversion was not observed. We next investigated additional pathways that may contribute to CBD-dependent sensitization of GBM to TMZ in TMZ-sensitive cells.

CBD Inhibits the FOXM1-RAD51 Pathway in TMZ-Sensitive Cell Lines

FOXM1 inhibition sensitizes resistant brain cancer cells to the first-line DNA damage agent TMZ by downregulating the expression of RAD51 gene, which encodes an important DNA damage repair protein.[37] Our recently published work demonstrated that across multiple cancers CBD regulated a consistent set of transcription factors controlling tumor progression, including FOXM1.[38] We therefore investigated whether CBD regulates the FOXM1-RAD51 pathway in this study. In TMZ-sensitive lines U87 and GBM163X (Figure 5A and B), CBD effectively inhibited RAD51 expression when cells were treated with CBD alone or in combination with TMZ. In contrast, CBD was ineffective at downregulating RAD51 upon cotreatment with TMZ in TMZ-resistant cells (Figure 5C and D). The inhibitory effects of CBD on FOXM1 expression were only observed in the CBD alone treatment group in GBM163X, suggesting this target was not consistently modulated in either TMZ-sensitive or resistant GBM.

In the presence of the ROS scavenger TOC, CBD was not effective at downregulating RAD51 in GBM163X demonstrating dependence on production of ROS (Supplementary Figure 3A and B). RAD51 levels were also modulated by the combination of CBD and TMZ in vivo, following treatment of mice bearing GBM163X intracranial tumors with 15 mg/kg CBD and 2 mg/kg TMZ (Supplementary Figure 4).

Discussion

GBMs are a heterougenous group of high-grade brain neoplasms that are notoriously resistant to conventional therapies.[39] Therefore, the identification of nontoxic agents which can improve the efficacy of first-line therapies such

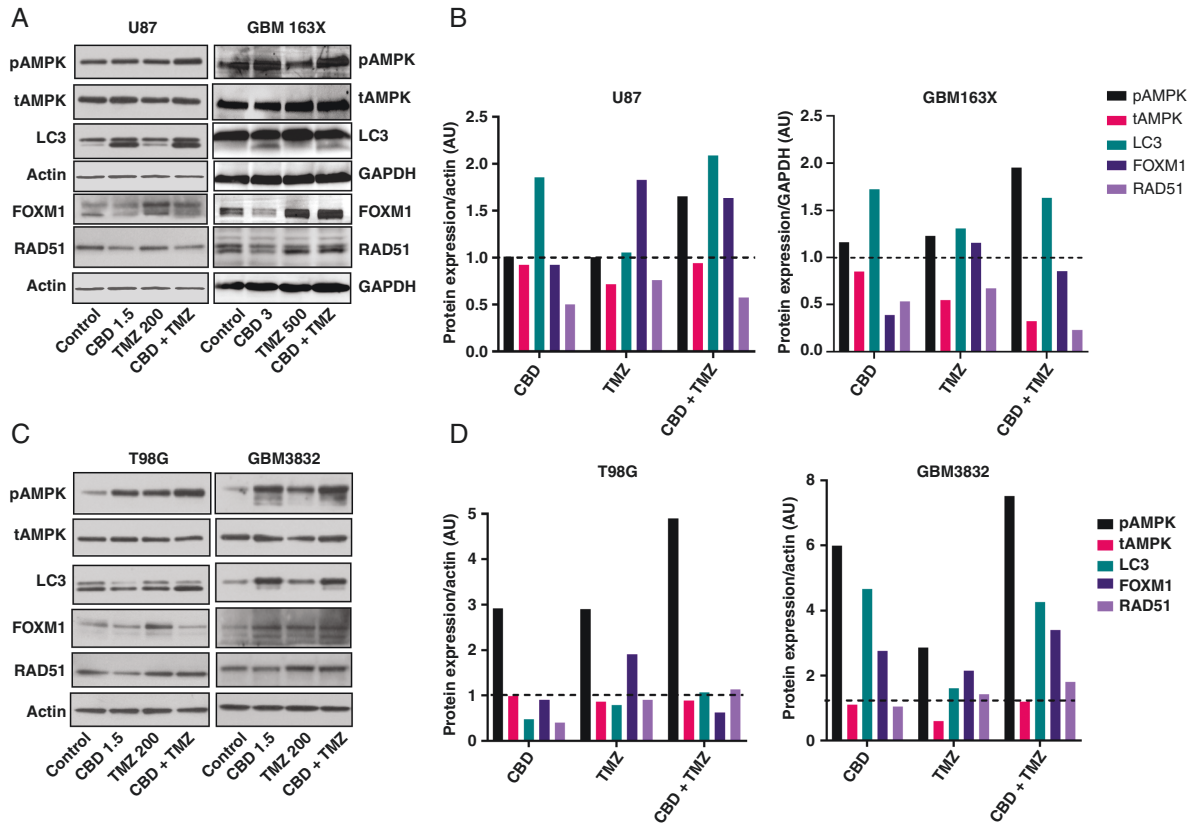

Figure 5. CBD stimulates AMPK, upregulates LC3-II conversion, and inhibits FOXM1 and RAD51 expression in TMZ-sensitive GBM cell lines. (A) U87 were treated for 3 days with vehicle (control), 1.5 μM CBD, 200 μM TMZ, or 1.5 μM CBD + 200 μM TMZ and (B) GBM163X were treated for 3 days with vehicle (control), 3 μM CBD, 500 μM TMZ, or 1.5 μM CBD + 500 μM TMZ. C, D) T98G and(3832 cells were treated for 3 days with vehicle (control), 1.5 μM CBD, 200 μM TMZ, or 1.5 μM CBD + 200 μM TMZ. The expression of pAMPK, tAMPK, LC3A, FOXM1, and RAD51 were evaluated using Western analysis and Image J, respectively. Actin or GAPDH served as a loading control.

as TMZ, possibly allowing for dose lowering, is urgently needed. Several other agents have been tested in combination with TMZ, none of them had both the low toxicity[40] profile and high brain–blood barrier penetrance of CBD.[41]

In this study, we determined whether CBD could enhance the activity of the first-line agent TMZ using multiple culture models of human GBM, including patient-derived tumors. The combination of CBD and TMZ led to primarily additive inhibition of cell viability in U87 and U251 cell culture models. There was some slight antagonism at lower dose ratios in primary GBM163X cells, which may be a result of the increased resistance of primary GBM cells versus serum-derived cell lines.[24] CBD produced a sustained upregulation of ROS in a concentration-dependent manner in GBM cells leading to inhibition of cell viability. The inhibition of cell viability produced by CBD or the combination of CBD + TMZ was blocked by the ROS scavenger TOC.

We next evaluated the ability of CBs alone or in combination with TMZ to inhibit tumor growth and extend survival i.c. models of MGMT-methylated GBM utilizing cell lines and patient-derived tumors. This study is the first to perform a direct comparison of the antitumor activity of CBD, THC, and CBD + THC alone and in combination with TMZ in orthotopic models. CBD, THC, nor CBD/THC alone inhibited GBM progression; however, these CBs produced a similar sensitization of tumors to TMZ, leading to more effective inhibition of tumor progression and prolonged survival. This result is in agreement with previous studies targeting GBM with different ratios of CBD:THC + TMZ.[42,43] In these investigations, treatments incorporating different ratios of CBD:THC alone targeting subcutaneous implanted GBM significantly inhibited tumor progression; all ratios tested, including treatments incorporating ratios with higher levels of CBD, for example 5:1 CBD:THC, were equally effective. Importantly, the antitumor activity of CBs alone was not observed in intracranial (orthotopic) models; however, the ability of CBs to sensitize GBM to TMZ was still observed. This difference is most likely the result of the difference in the tumor microenvironment. Indeed, using live-cell imaging, we have previously shown that after initial inhibition of tumor growth by CBD, intracranial GBM tumors appear to resume a more rapid growth rate in spite of continuous CBD administration,[26] whereas treatment of GBM tumors implanted subcutaneously leads to a more stable inhibition.[26,42] Taken together, these data suggest that CBD alone, or in combination with lower concentrations of THC that do not produce unwanted pyshcoativity, may be a preferred treatment regimen for development of clinical trials targeting GBM.

The ability of CBD to sensitize GBM to TMZ was dose dependent in the U87 MG i.c. model. The PK parameters corresponding to the active dose of 15 mg/kg and the inactive dose of 7.5 mg/kg were compared allowing for an understanding of drug exposure needed to produce CBD-dependent sensitization of tumors to TMZ. CBD was rapidly absorbed following ip injection in mice, with maximal concentrations observed at the first sampling time point of 5 min postdose. Systemic CBD exposure (plasma C_{max} and AUC) was dose proportional between the 7.5 and 15 mg/kg dosage levels. Concentrations in the high dose decreased with a terminal half-life of 7.5 h to a final concentration of 0.019 μg/ml by 12 h postdose.

In contrast to the effects of CBD + TMZ in MGMT-methylated GBM lines, the drug combination did not demonstrate additive or synergistic inhibitory effects in MGMT-unmethylated culture and in vivo models. These data demonstrate CBD produces enhancement of TMZ only in MGMT-methylated GBM, suggesting that MGMT-unmethylated GBM cells activate additional pathways leading to therapeutic resistance to the effect of CBD.

While subthreshold doses of TMZ are commonly used to study drug interactions in vivo,[42,43] it should be noted that using a higher dose of TMZ, approaching the MTD, in combination with CBD would have been a preferred treatment regimen to more closely model clinical care. There is the potential that CBs would not further improve survival if the combination treatment included a significantly higher dose of TMZ. However, in TMZ-sensitive tumors, treatment with a higher dose of TMZ, approaching MTD, would result in a significant extension of survival for many months requiring long-term chronic treatment with CBs, which is not feasible due to animal welfare concerns when considering i.p. injections of CBs 5 days a week over many months.

The initial site CBD interacts with to produce antitumor activity is unknown, and the most unifying downstream mechanism in culture is the initial CBD-dependent production of ROS.[16] Downstream of production of ROS, CBD has been shown to target multiple genes implicated in controlling tumor progression including TIMP1, PAI, ERK, AKT/mTOR, PUMA, and CHOP[19,44–48] as well as other pathways.[15] We investigated the potential pathways modulated downstream of ROS that may explain why CBD is more effective at enhancing the activity of TMZ in MGMT-methylated versus MGMT-unmethylated GBM cells. The ROS sensor AMP-activated protein kinase (AMPK) is activated as a result of cellular stress, and through inhibition of the Akt-mTORC1 complex, lead ultimately to autophagy-mediated cell death.[36] Both THC and CBD have been shown to upregulate autophagy-mediated cell death markers in human GBM,[18,26] and CB_1 and CB_2 receptor-induced apoptosis across cancers has been show to rely on stimulation of autophagy.[49] CB_1 and CB_2 receptor agonists have been demonstrated to sensitize tumors to DNA damaging agents through mechanisms including AMPK autophagy-mediated cell death.[50,51] In MGMT-methylated GBM cells the combination of CBD + TMZ was most efficient at activating AMPK, with varying degrees of AMPK activation produced by CBD and TMZ alone. In comparison to CBD, the combination of CBD + TMZ did not further improve the conversion of LC3-I to LC3-II, suggesting that a majority of the LC3-I to LC3-II conversion is the result of the treatment with CBD.

The Forkhead box protein M1 (FOXM1) transcription factor has been shown to promote tumorigenesis.[52] FOXM1 inhibition sensitized resistant brain cancer cells to the first-line DNA damage agent TMZ by downregulating the expression of RAD51 gene, which encodes an important DNA damage repair protein.[37] Since across cancers, CBD has been shown to inhibit the expression of specific

transcription factors controlling tumor progression,[13,26] including FOXM1,[38] we investigated whether CBD modulates expression of FOXM1 and RAD51 in GBM. In MGMT-methylated lines, CBD effectively downregulated RAD51, but not FOXM1, alone and in combination with TMZ. In contrast, CBD was ineffective at targeting RAD51 expression in MGMT-unmethylated GBM lines.

In this study, we demonstrate in multiple orthotopic models of MGMT-methylated GBM that CBs can sensitize tumors to the chemotherapeutic agent TMZ. We also show for the first time that in MGMT-unmethylated GBM cells where MGMT expression is intact, CBD did not enhance the therapeutic effect of TMZ in culture or in orthotopic models. As clinical trial data for targeting GBM with CBs becomes available, it would of interest to determine whether MGMT expression correlates with response to CBs. CBD, a CB that does not produce psychoactive side effects, was equally as effective as THC and the combination of CBD + THC at producing sensitization to TMZ in orthotopic mouse models of GBM. CBD and the combination of CBD +THC are already being tested in GBM in clinical trials or clinical case studies. The current investigation suggests that CBD alone, or combinations limiting the concentration of THC, may be a preferred treatment regimen that would limit psychotropic side effects. We also discovered that CBD effectively downregulates RAD51 in MGMT-methylated, but not in MGMT-unmethylated GBM cells. The targeting of RAD51 in MGMT-methylated GBM may in part explain why CBs can sensitize tumors to TMZ in culture and in vivo in preclinical models of GBM.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Advances* online.

Keywords

cannabidiol | glioblastoma | patient-derived-xenograft | sensitization to temozolomide

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