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Small Molecule Therapeutics

Molecular Cancer Therapeutics

The Combination of Cannabidiol and Δ^9-Tetrahydrocannabinol Enhances the Anticancer Effects of Radiation in an Orthotopic Murine Glioma Model

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Abstract

High-grade glioma is one of the most aggressive cancers in adult humans and long-term survival rates are very low as standard treatments for glioma remain largely unsuccessful. Cannabinoids have been shown to specifically inhibit glioma growth as well as neutralize oncogenic processes such as angiogenesis. In an attempt to improve treatment outcome, we have investigated the effect of Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) both alone and in combination with radiotherapy in a number of glioma cell lines (T98G, U87MG, and GL261). Cannabinoids were used in two forms, pure (P) and as a botanical drug substance (BDS). Results demonstrated a duration- and dose-dependent reduction in cell viability with each cannabinoid and suggested that THC-BDS was more efficacious than THC-P, whereas, conversely, CBD-P was more efficacious than CBD-BDS. Median effect analysis revealed all combinations to be hyperadditive [T98G 48-hour combination index (CI) at FU_{50}, 0.77–1.09]. Similarly, pretreating cells with THC-P and CBD-P together for 4 hours before irradiation increased their radiosensitivity when compared with pretreating with either of the cannabinoids individually. The increase in radiosensitivity was associated with an increase in markers of autophagy and apoptosis. These *in vitro* results were recapitulated in an orthotopic murine model for glioma, which showed dramatic reductions in tumor volumes when both cannabinoids were used with irradiation (day 21: 5.5 ± 2.2 mm^3 vs. 48.7 ± 24.9 mm^3 in the control group; $P < 0.01$). Taken together, our data highlight the possibility that these cannabinoids can prime glioma cells to respond better to ionizing radiation, and suggest a potential clinical benefit for glioma patients by using these two treatment modalities. *Mol Cancer Ther; 13(12); 2955–67. ©2014 AACR.*

Introduction

Cannabinoids is a broad term used to describe a group of naturally occurring compounds extracted and isolated from the *Cannabis sativa* and *Cannabis indica* plants (1). Δ^9-Tetrahydrocannabinol (THC) is the major bioactive component in this group of diverse compounds, and is primarily responsible for the psychoactive effects of the plant (2). Since its isolation in the 1960s, THC has been shown to exert a variety of effects through its strong interactions with the cannabinoid receptors, which are expressed in a variety of tissues. In recent times, numerous reports highlighting potent activity *in vitro* and in *in vivo* models have established it as a potential anticancer therapeutic agent in a number of cancer types (3–6). Mechanistically, binding of THC and the other receptor-dependent cannabinoids to these receptors can elicit a number of pathways through which these compounds can work to ultimately reduce tumor growth. These vary with each cannabinoid but generally include the induction of apoptosis and/or autophagy via engagement of the mitogen-activated protein kinase (MAPK) and the endoplasmic reticulum stress-related pathways (5, 7). Cannabinoids have also been reported to be antiangiogenic (8) as well as anti-inflammatory (9). Furthermore, limited clinical trial data have reinforced the concept that THC possesses therapeutic potential. Understandably, however, the psychotrophic effects of the compound continue to attract controversy; even though a pilot trial of its therapeutic use in patients with glioblastoma multiforme (GBM), an advanced type of glioma, showed feasibility "...without overt psychoactive effects." (10).

Other prominent cannabinoids such as cannabidiol (CBD) and cannabigerol (CBG) also exhibit anticancer activity (11); however, importantly they are devoid of the psychoactivity that troubles THC. Their bioactivities seem to occur through similar mechanisms and signaling pathways evoked by THC, yet their actions may not be receptor dependent as they lack significant binding affinities for them. This suggests that receptor activation by some of the cannabinoids may not be a requisite for

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anticancer activity (3), which highlights the possibility of using cannabinoids in a way that maximizes the anticancer action while simultaneously minimizing psychoactivity (11). In particular, the evidence of the anticancer activity of CBD has grown steadily these past few years (11–15). These studies have involved its use as a single agent or in combination with other cannabinoids or other treatment modalities, and have included a number of cancer types (5). A lot of interest has been shown in the activity that cannabinoids have on GBM, which has resulted in phase Ib/IIa clinical trials in late 2013. These trials involve the already-licensed cannabinoid preparation Sativex that contains CBD and THC at a ratio of 1:1 (www.clinicaltrials.gov Identifier, NCT01812603 and NCT01812616).

High-grade glioma is one of the most aggressive cancers in adult humans, and long-term survival rates are very low. Statistics for U.K. patients indicate 36% of adults with glioma live for at least a year; with the 5-year survival rate of 10%. Specifically, in patients with the more aggressive glioma, GBM, life expectancy falls to below 1 year and the 5-year survival rate is just 6% (16). Treatment can consist of surgery, radiotherapy, and/or chemotherapy depending on the individual circumstance; however, due primarily to the intricate localization of the tumor in the brain and its invasive behavior, these treatments remain largely unsuccessful. Developing new drugs to be used in this disease is thus of importance, and the fact that cannabinoids have shown activity in this disease, supports the call for further studies to establish the clinical value of this potential therapeutic agent.

Therefore, the aim of the current study is first to assess the antiproliferative effect of CBD and THC in an *in vitro* glioma setting, used alone and in combination with radiotherapy. Second, the combination of CBD, THC, and irradiation will also be assessed in a murine orthotopic glioma model, and efficacy over the duration of treatment studied with the aid of magnetic resonance. Overall, these studies would help to evaluate CBD and THC as drugs to combat glioma.

Materials and Methods

Cell culture, mice, and cannabinoids

The human cancer cell lines T98G (GBM) and U87MG (glioblastoma astrocytoma) were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom), whereas the mouse glioma cell line GL261, which is syngeneic to the C57BL/6 mouse, was acquired from the National Cancer Institute (Bethesda, MD). Authentications of the human cell lines were performed by the service providers using the AmpFISTR Identifier Plus PCR amplification kit looking for the presence of <10 known loci for each cell line. The cell lines were grown in DMEM (Sigma-Aldrich) medium supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 2 mmol/L L-glutamine (Life Technologies) and were incubated in a humidified atmosphere with 5% CO_2 in air at 37°C, and discarded every 6 weeks. For experiments with cannabinoids, the FBS was reduced to 5%.

Female C57BL/6 mice (~9 weeks of age) were maintained by the Biological Research Facility at St George's University of London. Mice were acclimatized for at least 7 days before commencement of any procedure, which were conducted in strict accordance with and approved by the Home Office of the United Kingdom (PPL 70/7562).

The activities of CBD and THC were assessed in a pure (P) and a botanical drug substance (BDS) form (GW Pharmaceuticals Ltd.), and were dissolved in ethanol. Final ethanol concentrations in cell cultures were <0.1%. Both the P and BDS forms were prepared and used at the specified concentrations according to their total molecular weight rather than according to the weights of the active THC or CBD component within them. The pure forms of CBD and THC contained >96% of each cannabinoid. Conversely, a number of other cannabinoids were found in the BDS form of CBD, the breakdown of which were 63.5% CBD, 3.6% THC, 1.1% CBG, 5.2% CBC, 1.3% CBDV, 0.4% CBDA, and 0.1% CBD. THC-BDS contained 65.4% THC, 0.4% CBD, 1.3% CBG, 1.8% CBC, 0.9% THCV, 0.4% THCA, 2.0% CBN, and 0.2% CBO. For *in vivo* experiments, both cannabinoids were administered in combination in their pure form [at a final concentration of 200 μmol/L (~4 mg/kg), made up from 100 μmol/L CBD and 100 μmol/L THC], and final ethanol concentration was <2%.

Viability assays

To study the effect of each agent on cell growth, exponentially growing cells were added to 384-well black/clear bottom plates (BD Biosciences) at a density of 2,000 cells per well and left to adhere for 24 hours. CBD or THC (0.1–100 μmol/L) was then added to the wells, ensuring an equal total volume of 60 μL across the plate. Cell viability was assessed at 24, 48, and 72 hours by measuring the fluorescence generated following the cellular reduction of resazurin (10 μmol/L; Sigma) to resarufin. Fluorescence was measured after 4 hours of incubation at 37°C, using a GloMax Multi Plus microplate reader (ex/em: 540 nm/590 nm; Promega). Data were analyzed using the GraphPad Prism curve fitting software.

Flow cytometric analysis of the cell cycle

Exponentially growing cells were seeded into 6-well plates (BD Biosciences) at a density of 2×10^5 cells per well and left to adhere overnight. Cells were then treated for 24 hours with CBD or THC, before harvesting, washing in ice-cold PBS, and fixing in 70% (v/v) ethanol in PBS. Cells were then rewashed and resuspended in 1 mg/mL propidium iodide and 1 mg/mL RNAse-A (both Sigma) for flow cytometric analysis. Acquisition of data was performed within 1 hour using a Becton Dickinson FACSCalibur (BD Biosciences), and gating on fluorescence width and area was used to

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remove doublet artefacts and to discriminate cells from debris. The percentages of cells in each phase were determined using the nonproprietary cell-cycle analysis program WinMDI v2.9 (http://facs.scripps.edu/software.html).

Combination studies: fixing the ratio of the drug concentrations

Exponentially growing cells were added to 384-well black/clear bottom plates at a density of 2,000 cells per well and left to adhere overnight. CBD-P or CBD-BDS was combined with THC-P or THC-BDS at an equal ratio of their respective IC_{50}s (e.g., $^1/_2 \times IC_{50}$ of CBD-P was combined with $^1/_2 \times IC_{50}$ of THC-P) and added to the plates to achieve a final volume per well of 60 μL. Fixing the ratio in this manner ensured that the amount of one drug respective to the other was kept constant (11, 17). Cells were incubated for 24, 48, and 72 hours and viability was assessed using resazurin as described earlier. The efficacy of each of the drug combinations was established and the nature of drug–drug interactions was then assessed by calculating a combination index (CI) using the median-effect equation (18).

Immunoblotting analysis

Cells were treated for 4 hours with CBD-P, THC-P, or a combination of the two. They were then harvested by scraping into lysis buffer (New England Biolabs), and standard Western blot analysis protocols were followed as described previously (11). Primary antibody probing was performed with anti-total and phosphorylated AKT, anti-total and phosphorylated ERK, and anti-LC3B and anti-caspase-3 (all New England Biolabs). All antibodies were used at a dilution of 1:1,000, followed by appropriate HRP-conjugated secondary antibodies (New England Biolabs) at a dilution of 1:1,000 and bands were visualized by the SuperSignal Chemiluminescent Detection System (Thermo Scientific).

Clonogenic survival assays

To demonstrate the effects of irradiation, long-term colony formation assays were performed. Exponentially growing cells were seeded into small flasks at a density of 2×10^5 cells per flask and left to adhere overnight. Cells were then irradiated with 0 to 20 Gy using a ^{137}Cs cell irradiator (IBL 437C; CIS Bio International). Cells were then harvested using trypsin, and reseeded at low densities (50–800 cell per well) in duplicate into 6-well plates allowing for the appropriate correction for each irradiation insult. The plates were then incubated for approximately 14 days until sufficiently large colonies were formed (>50 cells/colony), at which point samples were fixed in ethanol, stained with 0.5% methylene blue (Sigma), and colonies counted manually. To assess whether combining CBD and/or THC modifies the efficacy of irradiation, cells were pretreated with the drugs in their pure form for 4 hours before irradiation and the same protocol was then followed. Colony formation efficiency for each treatment regimen was normalized to the relevant control, and radiation dose–response curves were generated on GraphPad Prism using the linear quadratic equation modeling. To quantify the effect of the cannabinoids on irradiation efficacy, the sensitizer enhancement ratios at 10% survival (SER_{10}) were calculated. SER_{10} is, mathematically, the quotient of the radiation doses required to kill 90% of the cells (LD_{90}) when comparing controls with individual treatments.

Immunofluorescence and γ-H2AX signal quantification

Exponentially growing cells were seeded onto coverslips (VWR) at a density of 2×10^5 cells per well and allowed to attach overnight. Cells were concomitantly irradiated at a dose of 10 Gy and then treated with CBD-P and/or THC-P and incubated for 1 or 5 hours, before fixation in formalin (Sigma). Cells were then simultaneously blocked and permeabilized with 5% (w/v) BSA and 0.3% (v/v) Triton-X (both Sigma-Aldrich) in PBS for 1 hour at room temperature. Coverslips were washed three times in PBS and then incubated with anti–γ-H2AX antibody (1:400 dilution; New England Biolabs) overnight at 4°C. The coverslips were then washed again and incubated with a FITC-conjugated secondary antibody (1:750; Life Technologies) for 1 hour at room temperature in the dark. Coverslips were mounted on the microscope slide with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Labs), then cells were visualized and images captured using a fluorescence microscope and nonproprietary imaging software. The fluorescence intensity, which was relative to the number of γ-H2AX foci present, was assessed from 50 nuclei viewed in multiple random fields of view.

In vivo syngeneic orthotopic glioma model

Exponentially growing GL261 murine glioma cells were resuspended in PBS at a concentration of 7.5×10^7 cells/mL. Only cells with a viability of >90%, as assessed by trypan blue exclusion analysis, were used. Female C57BL/6 mice were anaesthetized using an intraperitoneal injection of a 1:2:1 mixture of Hypnorm (0.315 μg/mL fentanyl citrate and 10 mg/mL fluanisone), sterile water, and Hypnovel (5 mg/mL Midazolam) at 10 mg/kg bodyweight, and then fixed in a stereotactic head frame appropriate for the animal. A midline scalp incision was made and bregma was identified. A small hole was then made in the skull using a 21-gauge needle at a position that was 1 mm lateral and 3 mm anterior to bregma. A 10 μL Hamilton syringe/needle that was fixed to the frame was positioned above this opening and advanced to a depth of 3 mm. Two microliters of the cell suspension (150,000 cells) was then delivered slowly over a course of 3 to 4 minutes. The incision was then closed using glue. Animals were routinely observed for signs of discomfort and the study ended no later than 21 days postimplantation. Brains were extracted and frozen on dry-ice before being stored at −196°C in preparation for future analysis.

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Animals were randomly assigned into a control (vehicle only) and three treatment groups: (i) Irradiation—4 Gy focused to to the head; (ii) Cannabinoids—2 mg/kg each of CBD-P and THC-P administered together in 100 μL and 4 Gy focused irradiation. (iii) Irradiation + cannabinoids—2 mg/kg each of CBD-P and THC-P administered together in 100 μL and 4 Gy focused irradiation. Cannabinoids were administered by intraperitoneal injection 9, 13, and 16 days after tumor implantation, whereas irradiation was administered under general anesthetic only on day 9. The irradiator used was the AGO HS 320/250 X-ray machine (PTW), and each mouse was subject to irradiation using 250 kVp X-rays delivered at 12 mA (dose rate of 0.44 Gy/min). Tumor volume was assessed 9, 13, 16, and 21 days after tumor implantation by using a 4.7 T Varian horizontal bore magnetic resonance imaging (MRI) system with a mouse-brain coil setup (RAPID Biomedical GmBH). Animals were anesthetized as previously described before placing them into the imaging coil apparatus. Body temperature was maintained using a warming bed, which was integrated into the system. Images of nine contiguous coronal slices of 1 mm thickness were acquired, with the most anterior slice being positioned just dorsal to the olfactory bulb. T2-weighted images were acquired and the tumor area on each of the slices measured by the ImageJ software. The sum of the tumor areas from sequential slices was used as an estimation of tumor volume at each time point, and growth curves were compiled for each treatment group.

Immunohistochemistry of the brains from C57BL/6 mice

As described previously, the brains from the mice were frozen slowly on dry-ice and stored in liquid nitrogen. Cryosections (10 μm) were fixed onto positively charged slides for routine hematoxylin and eosin staining (Department of Pathology, St George's Hospital, London, United Kingdom). Extra slides were prepared, fixed in ice-cold acetone, and then blocked and permeabilized as described earlier, before an overnight incubation with anti-Ki67 (1:50; Abcam PLC) or anti-CD31 (1:25; BD Biosciences). After three washing steps in PBS, slides were incubated for 3 hours with a species-appropriate secondary fluorochrome–conjugated antibody (1:500; New England Biolabs), washed three times, then coverslips attached using DAPI-based mountant. Apoptosis was detected using a proprietary *in situ* cell death detection kit following fixation of the slides in formalin, according to the manufacturer's instructions (Roche Diagnostics Ltd.). Sections were visualized and images captured using a fluorescence microscope and a nonproprietary imaging software. TUNEL was assessed qualitatively by two independent operators and were scored 0, +, ++, or +++ according to staining intensity.

Statistical analysis

All statistical analyses were performed using GraphPad Prism or Microsoft Excel. Datasets were tested for normality by the Shapiro–Wilk testing, and differences between variable and control groups determined by using appropriate analysis of variance. Paired tests were then used to further determine any difference. All sets consisted of data from at least three separate experiments.

Results

CBD and THC reduce the number of glioma cells when used alone

Two human cell lines representing glioma at various stages of this multistep malignancy were selected for investigation, as was the murine GL261 cell line syngeneic to the C57BL/6 mouse. The activities of CBD and THC were assessed in these cells both in their pure (P) form (>96% purity) and in a less refined formulation designated as the botanical drug substance (BDS) that typically contained between 60% and 72% (w/w) of the specific cannabinoid. The remaining mass of the BDS was made up mainly of other cannabinoids, such as CBG and cannabichromene (CBC), and other plant material.

There were dose-dependent reductions in cell numbers in all three cell lines cultured with the cannabinoids (Fig. 1A). Generally, of the two human cell lines, T98G (GBM) was more sensitive to the treatments, with concentrations required to reduce cell numbers by 50% (IC_{50}) being approximately 10 μmol/L. Flow cytometric analysis revealed that culturing the cells with CBD or THC caused no significant changes to the DNA profile (Fig. 2B), suggesting drug activity involved an element of cytostasis. Western blot analysis of caspase-3 showed that the induction of apoptosis was minimal following treatment with CBD and THC; however, a dose-dependent increase in the levels of LC3Bii indicated the induction of autophagy (Fig. 3D). Closer examination of the IC_{50}-values for the cannabinoids identified a divergence of the activities of the P and BDS forms. In all the three cell lines tested, CBD was more active in the P form; this was most apparent in T98G cells. This suggests that the minor cannabinoids in the BDS form of the compound, namely CBC (∼5%), THC (∼4%), and CBG (∼1%), may have interfered with its activity (Fig. 1B). Conversely, the activity of THC was greater in the BDS form, which contained, among its extra constituents, the cannabinoids, CBC (∼2%), and CBG (∼1%), indicating that these extra components may aid THC in its action.

CBD and THC act together to reduce cell numbers

We also assessed the effect of combining the two compounds in our glioma cell lines by means of the median effect model. This required combining CBD with THC at equivalent ratios of their IC_{50}s, and computing a CI that signified the nature of any interaction. These values were generally below 1 for the cell lines, indicating a hyperadditive effect when combining CBD with THC (Fig. 2A). Each permutation of combination between the P and BDS

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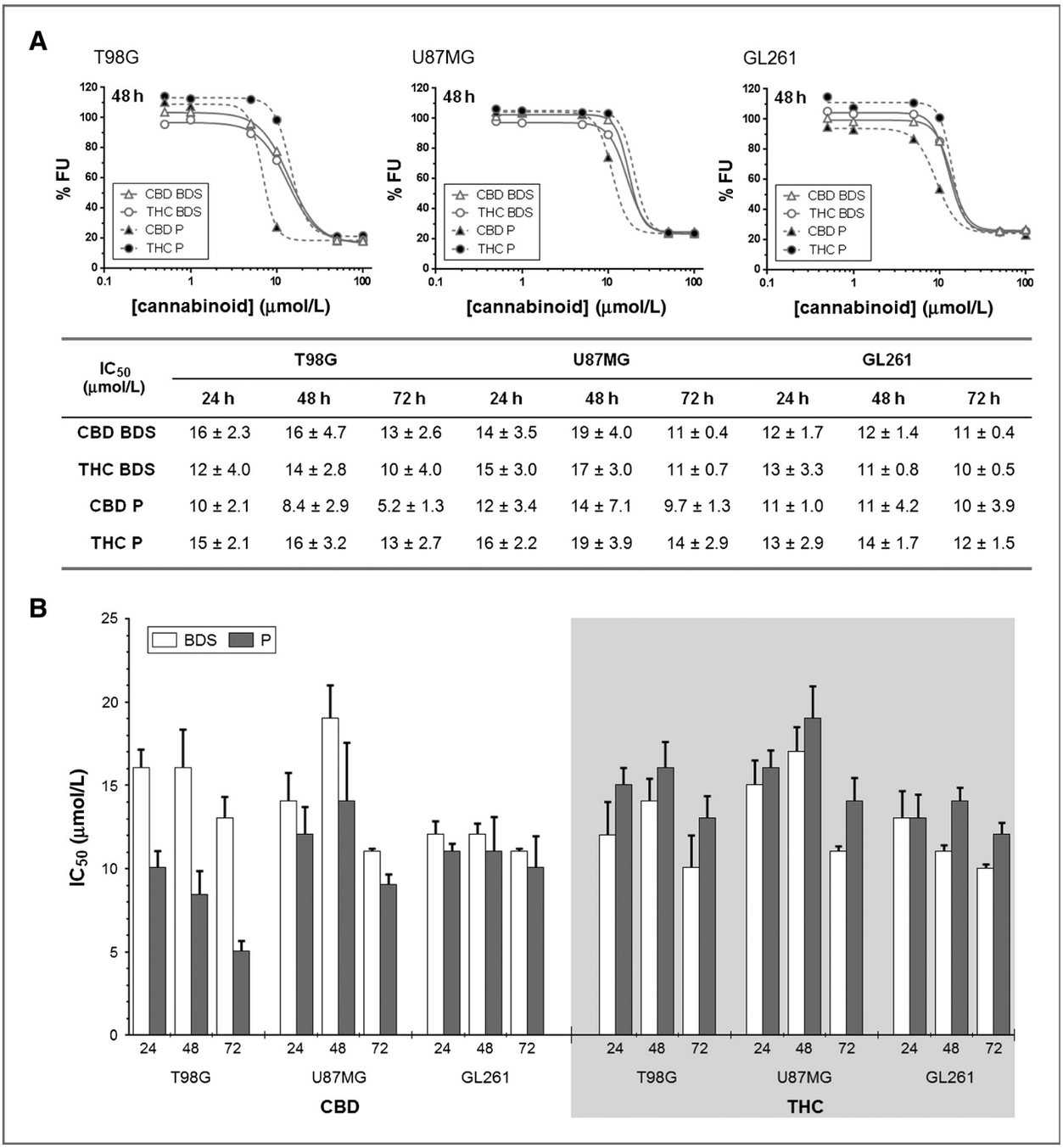

IC_{50} (μmol/L)	T98G			U87MG			GL261		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CBD BDS	16 ± 2.3	16 ± 4.7	13 ± 2.6	14 ± 3.5	19 ± 4.0	11 ± 0.4	12 ± 1.7	12 ± 1.4	11 ± 0.4
THC BDS	12 ± 4.0	14 ± 2.8	10 ± 4.0	15 ± 3.0	17 ± 3.0	11 ± 0.7	13 ± 3.3	11 ± 0.8	10 ± 0.5
CBD P	10 ± 2.1	8.4 ± 2.9	5.2 ± 1.3	12 ± 3.4	14 ± 7.1	9.7 ± 1.3	11 ± 1.0	11 ± 4.2	10 ± 3.9
THC P	15 ± 2.1	16 ± 3.2	13 ± 2.7	16 ± 2.2	19 ± 3.9	14 ± 2.9	13 ± 2.9	14 ± 1.7	12 ± 1.5

Figure 1. Effect of CBD and THC on cell proliferation and cell-cycle dynamics. T98G, U87MG, and GL261 were cultured with CBD or THC in both the P and BDS forms. Cell number was measured at 24, 48, and 72 hours, and the concentration required to reduce cell numbers by 50% (IC_{50}) were calculated using curve fitting software. The dose–response curves shown are from the 48-hour time point, where % FU represents the fraction of cells that were unaffected (A). Closer examination of the efficacy of the P and BDS forms of each cannabinoid revealed CBD was more active in the P form, whereas THC was more active in the BDS form (B). Each data point is the mean and SD of at least four separate experiments.

forms was tested and no significant differences were observed; however, combinations were typically more synergistic in U87MG cells than in T98G cells. In addition, combining CBD with THC did not alter the DNA profile of the cells (Fig. 2B).

CBD and THC alter intracellular signaling molecules

To investigate whether the effects that cannabinoids had on cell proliferation were associated with changes to intracellular signaling pathways, we analyzed lysates harvested from appropriately treated cells for the

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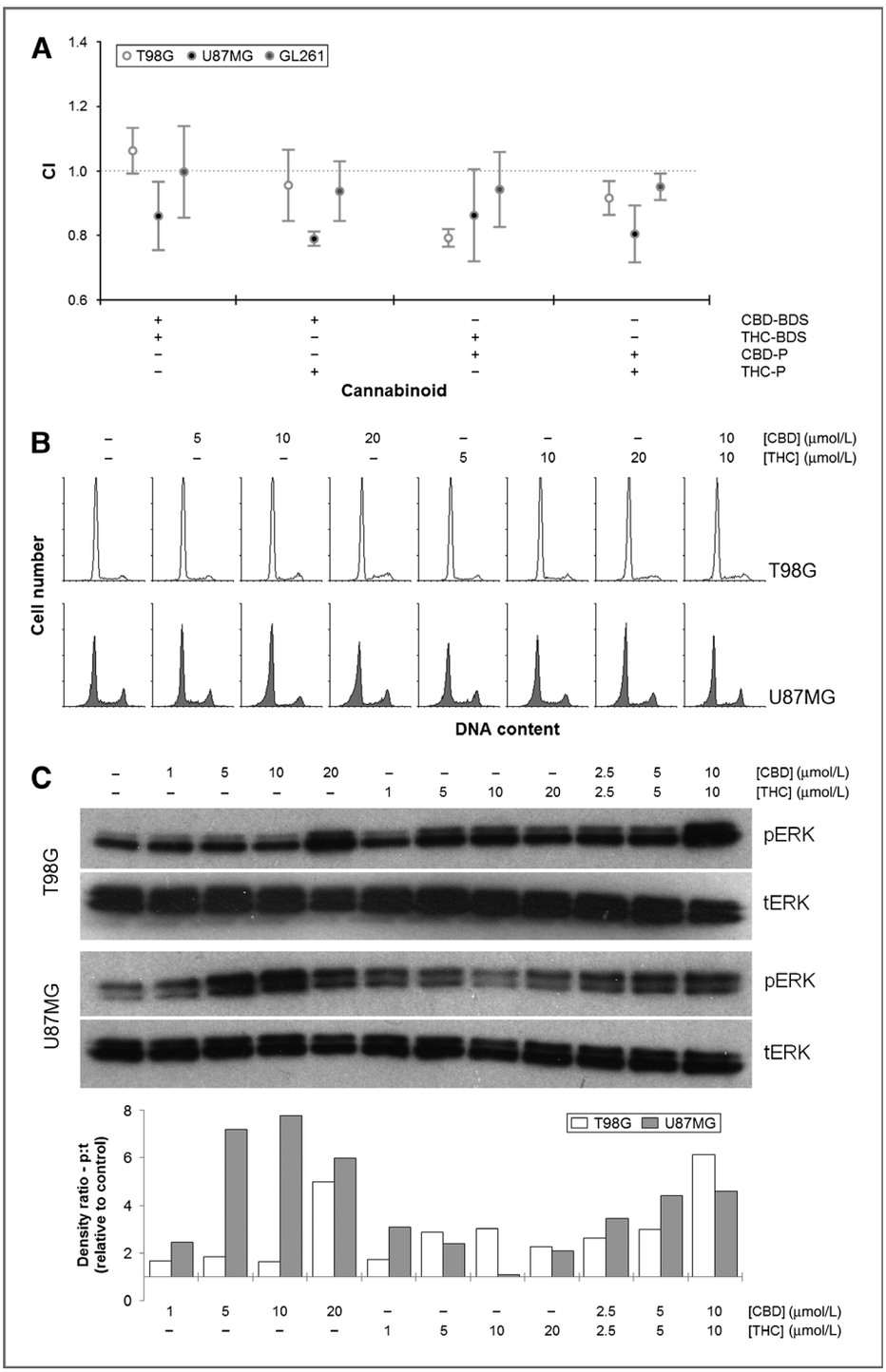

Figure 2. Effect of combining CBD with THC on cell number and cell signaling molecules. T98G, U87MG, and GL261 were cultured with both forms of each cannabinoid concomitantly, and the nonexclusive CI for the 50% unaffected fraction was assessed by median effect modeling. CI value of >1 indicates antagonism; CI of 1 indicates additivity, and CI < 1 synergy. Most combinations resulted in outcomes that were hyperadditive (A). Cell-cycle profiles determined by propidium iodide staining in T98G and U87MG are shown in B. Only the pure forms of THC and CBD were studied. The figure also details the effect of combining equimolar amounts of each cannabinoid. T98G and U87MG cells were also cultured with CBD and THC in their P form either individually or together at the concentrations indicated for 4 hours before Western blotting for pERK and tERK (C). The levels of pERK appeared to be the protein changed most often. Each data point/blot represents the mean and SDs of at least three separate experiments.

expression of key protagonists in signaling cascades that we have previously identified as being involved in the function of cannabinoids (3, 4, 11). Results showed that the greatest change was seen in $MAPK^{ERK1/2}$, where culturing with either CBD-P or THC-P for 4 hours altered its phosphorylation (Fig. 2C). Specifically, dose-dependent increases in the ratio of phosphorylated to total ERK was seen when T98G was cultured with either CBD or THC. A similar increase in levels was seen in U87MG cultured with CBD; however, they were reduced when treated with THC. The effect of AKT expression was less clear, nevertheless, there was a hint that cannabinoids reduced the expression/phosphorylation (data not shown).

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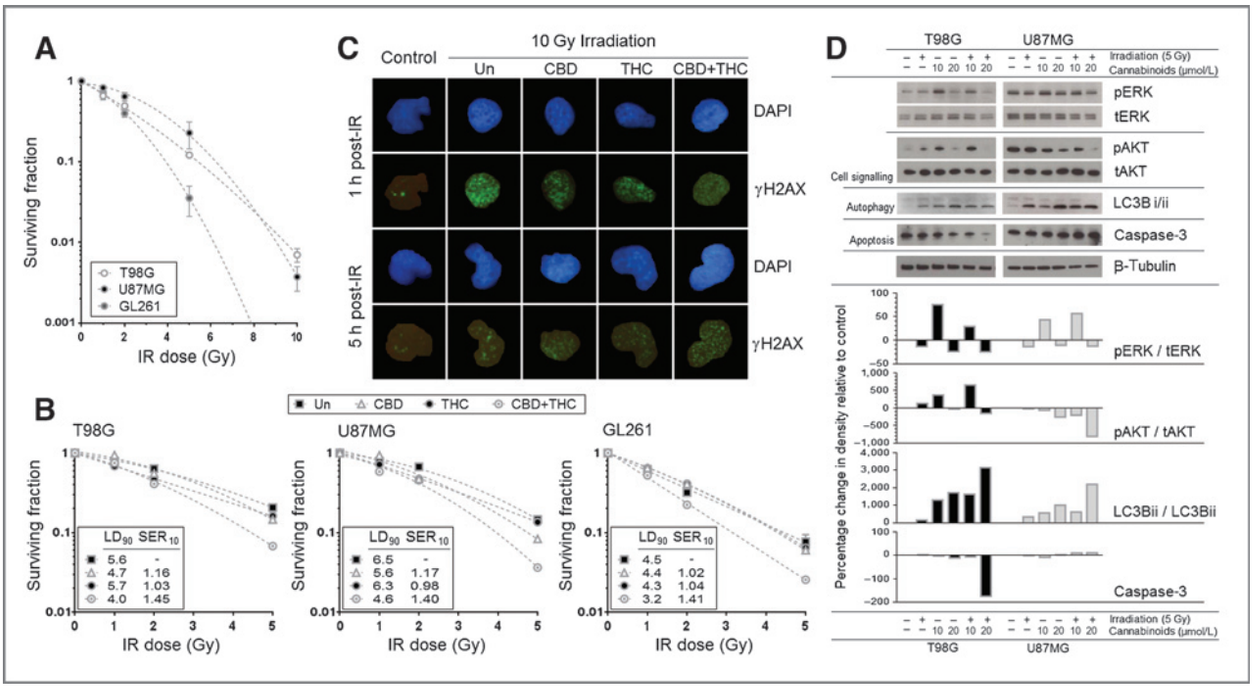

Figure 3. Effect of CBD, THC, and irradiation on colony-forming efficiency. T98G, U87MG, and GL261 were cultured with the pure forms of CBD and THC individually or concomitantly for 4 hours, before irradiation (<10 Gy). Clonogenic potential was then assessed. Irradiation was active in the cell lines, GL261 being most radiosensitive (A). Using either CBD or THC singly before irradiation did not alter the fraction of surviving cells (B); however, preculturing cells with the combination of CBD and THC enhanced the activity of irradiation, as the dose required to kill 90% (LD_{90}) of cells was increased. For example, in T98G cells, LD_{90} was altered from 5.6 Gy to 4.0 Gy (~SER_{10} of 1.45; B). Irradiating cells (10 Gy) resulted in an increase in γ-H2AX foci, a marker of DNA double-strand breaks. Foci persisted for a longer duration in cells pretreated with CBD and THC when compared with irradiated cells that had no pretreatment (C). Western blotting was performed to assess the effects of these combinations on markers of cell signaling, apoptosis, and autophagy (D).

A combination of CBD and THC enhances the effects of radiation

For gliomas that are accessible to it, surgery is the principal treatment. This is commonly supported by radiotherapy and/or chemotherapy, and so for this reason we assessed the benefit of combining the cannabinoids with γ-irradiation. We assessed whether the cannabinoids can prime cells to irradiation and more specifically studied whether in this instance they should be used individually or with each other. Methodologically, cells were pretreated for 4 hours with a single 20 μmol/L dose of CBD-P or THC-P, or with a combination of CBD-P and THC-P (equimolar dose of 10 μmol/L of both), before being subjected to ionizing radiation. Treated cells were then reseeded into plates, and colony-forming efficiency was assessed by enumerating colonies after 14 days using methylene blue.

Results showed that all three cell lines tested were radiosensitive, with a surviving fraction at 5 Gy of 0.12 ± 0.016, 0.22 ± 0.11, and 0.035 ± 0.025 for T98G, U87MG, and GL261, respectively (Fig. 3A). Following pretreatment with single agent CBD or THC, there was no significant difference in colony numbers, but there was, however, a trend toward a smaller surviving fraction when CBD and THC were used in combination (Fig. 3B). For example, in U87MG cells, the extrapolated LD_{90} for radiation alone was 6.5 Gy, and 5.6 Gy and 6.3 Gy when used in combination with CBD or THC. However, this was reduced to 4.6 Gy when cells were pretreated with CBD and THC concomitantly before irradiation, giving an SER_{10} of 1.4 (Fig. 3B). The effect of the cannabinoids on the repair of DNA double-strand breaks caused by irradiation was assessed using γ-H2AX staining, whereby γ-H2AX foci indicate individual sites of damage. 10 Gy caused a substantial increase in the number of these foci, present 1 hour after irradiation, and this increase was unaffected by pretreatment with the cannabinoids. Five hours after irradiation, a large proportion of the foci were repaired and fluorescence intensity in the irradiation alone cells decreased. In the cannabinoid pretreated cells, however, fewer foci were repaired and fluorescence intensity remained higher, particularly in those cells pretreated with a combination of CBD and THC (Fig. 3C). These results suggested that cannabinoids slowed the repair of double-strand breaks and that DNA damage persisted in these cells.

To further understand the mechanisms of the benefit of combining cannabinoids with irradiation, we examined the markers of general signaling pathways as well as

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markers of apoptosis and autophagy in cells exposed to CBD and THC with or without irradiation for 4 hours, as these have been previously implicated in cannabinoid action. Generally, our results showed that irradiation enhanced the actions of the cannabinoids. Specifically, the cannabinoids, when used at higher concentrations, decreased pAKT and pERK levels, and these were reduced further when combined with irradiation. Similarly, cannabinoids induced autophagy in the cells as indicated by a dose-dependent increase in the ratio of LC3Bii:LC3Bi, which again was further enhanced in the irradiation combination group. This pattern was not replicated by the markers of apoptosis; however, the only time that cleavage of caspase-3 was seen was when the higher dose of cannabinoids was combined with irradiation (Fig. 3D).

CBD, THC, and radiotherapy is a superior treatment regimen in mice

The efficacy of a treatment regimen consisting of CBD, THC, and radiotherapy was subsequently assessed in a murine model. The growth of the GL261 glioma tumor orthotopically implanted in C57BL/6 mice was tracked by MRI technology, and the effects on it of either pure CBD and THC together, irradiation, or the combination of both treatment modalities were determined (Fig. 4A). Of the mice inoculated with tumor cells, 80% developed tumors. Tumors in control mice were visible around day 9 and reached their permissible volumes by day 21, at which time all animals were sacrificed. Animals were exposed to suboptimal doses of each of the treatments, which allowed a window through which any improved combinatorial activity could be seen. Results

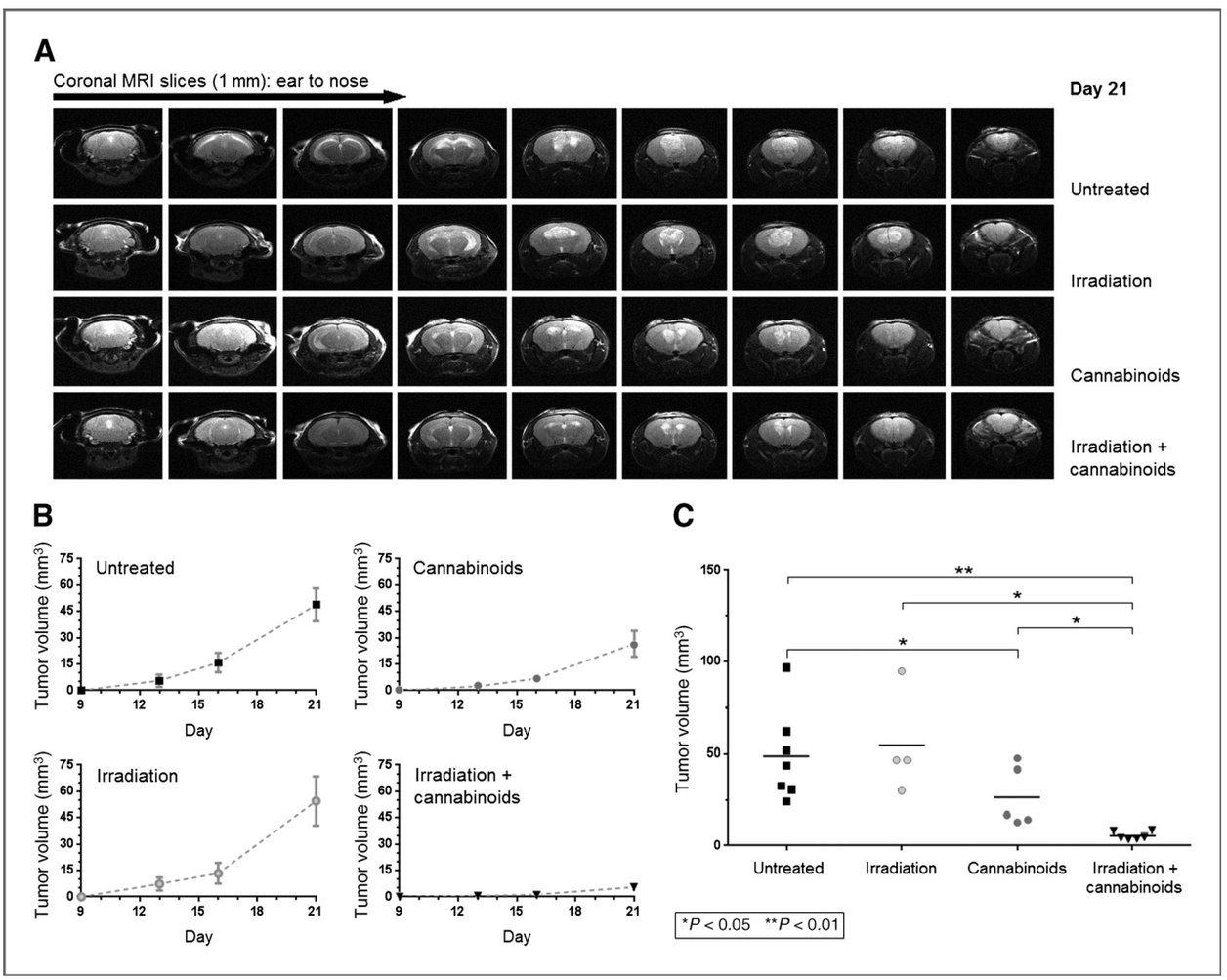

Figure 4. Effect of CBD+THC and irradiation in an orthotopic murine model. GL261 cells were injected intracranially into C57BL/6 mice, and the effect on tumor growth of pure CBD and pure THC together, irradiation, or a combination of the two modalities was assessed. MRI scans were performed on days 9, 13, 16, and 21. Representative images from MRI are shown in A, which shows the presence of tumor masses within the brain. The rate of tumor growth was much slower in the group of mice treated with CBD, THC, and irradiation (B). Furthermore, the final tumor sizes were significantly smaller in this group compared with the other three groups (C). *P* values were established from paired *t* tests following determinations of differences between variable and control groups using one-way ANOVA.

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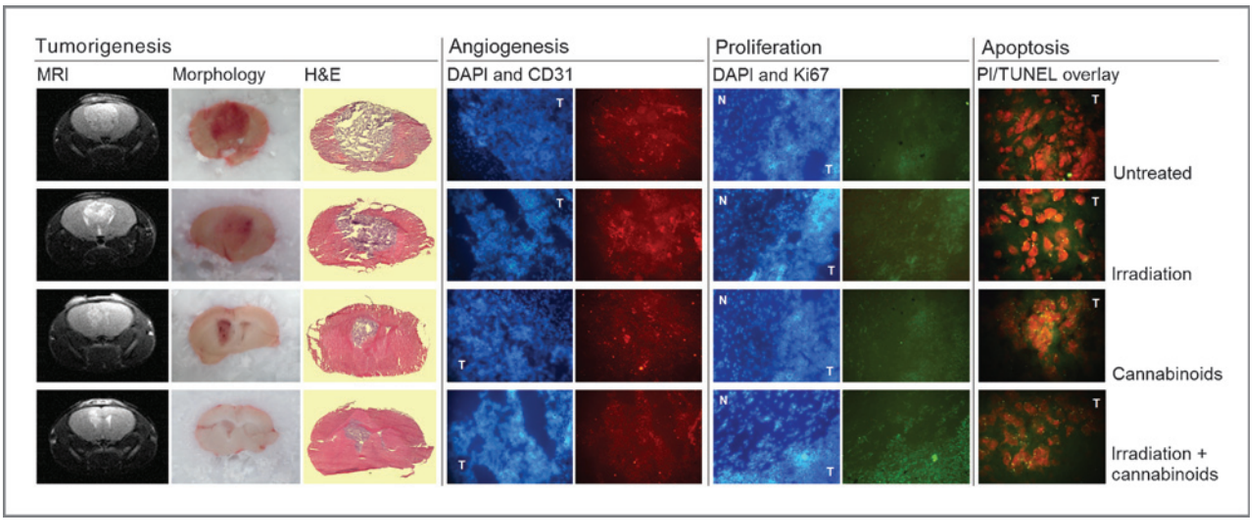

Figure 5. Effect of CBD+THC and irradiation on tumor morphology. Sections of the brains removed from mice treated with CBD+THC, irradiation and CBD+THC with irradiation had tumor masses that were distinguishable *in situ*. Images, where T denotes tumor region and N denotes normal tissue, were: MRI images obtained on day 21; morphology of whole-brain tissue, images of a coronal slice of the brain in mountant; composite H&E; angiogenesis: DAPI (blue) and CD31 (red); proliferation: DAPI (blue) and Ki67 (green); apoptosis: overlay of propidium iodide (PI; red) and TUNEL (green).

showed that 4 Gy irradiation had no dramatic effect on tumor growth, whereas CBD and THC administered together reduced tumor progression (Fig. 4A and B). Combining the cannabinoids with irradiation impeded even further the rate at which tumor growth progressed, which was virtually stagnant throughout the experiment course (Fig. 4B); and correspondingly, tumor sizes on the final day of the study were significantly smaller in this cohort of animals compared with any of the others (Fig. 4C). The ensuing *ex vivo* analyses of the brains resected from the mice showed that *in situ* the tumor masses were denser in the two groups that had been treated with the cannabinoids, and furthermore, that these tumors also appeared less sanguine. Subsequent immunohistochemical analysis confirmed this: expression of the vascularization marker CD31 was lower in the cannabinoid treated groups, particularly in the combination treated samples. Expression of the proliferation marker Ki67 appeared similar in all samples (Fig. 5). TUNEL staining was positive in all sections, but generally higher where cannabinoids were used, and specifically when combined with irradiation. Mean staining scores for TUNEL were 0/+, +, +, and +/++ for controls, IR, cannabinoids, and the combination groups, respectively (Fig. 5).

Discussion

There is ever-increasing evidence supporting a role for cannabinoids in cancer therapy, and so the current study was undertaken to explore this further. Of particular interest is the impact that cannabinoids have on signaling pathways that underlie cell fate. This impact allows scope for exciting combination strategies, specifically, the effect of combining these agents with existing therapeutic modalities, which presents an attractive novel avenue for research. In this study, we have concentrated on combining cannabinoids with radiotherapy as this is the primary form of treatment for patients with glioma following surgical resection. The principal aim of the current study, therefore, was to assess the efficacy of CBD and THC in combination with radiotherapy, to determine the potential clinical benefits of this combination in improving radiotherapy outcome. The most significant finding of this study has been the dramatic reduction in tumor growth *in vivo* when cannabinoids and ionizing radiation were used together.

CBD and THC were tested in this study as they have previously been shown to be effective in glioma both *in vitro* and *in vivo*, and display multiple mechanisms of action. Both cannabinoids can reduce cell numbers by inhibiting cell-cycle progression and cell growth as well as by triggering apoptosis and engaging autophagy (19), and are also antiangiogenic and antimigratory (15). The two compounds have also been combined in a preparation that is currently licensed to treat multiple sclerosis, which is now undergoing trials in patients with glioma. In the first part of the study, we assessed the individual activities of CBD and THC in two human glioma cell lines, and confirmed that they both cause dose-dependent reductions in cell numbers in these lines. These reductions were associated with mild increases in apoptosis and no clear alterations to the DNA profiles of the cells. This suggested the cannabinoids were both cytotoxic and cytostatic in nature under our *in vitro* test conditions.

In the preparation of the compounds used in the current study, the cannabinoids were extracted and purified from the cannabis plant. Depending on the level of purification, this process can result in CBD and THC preparations that contain small amounts of other

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minor cannabinoids and other plant products. These cannabinoids can all bind to a super-family of G protein–coupled receptors, which includes cannabinoid receptors 1 and 2, whose activation feeds into a number of intracellular signaling programs. These interactions can thus determine the extent to which cellular functions such as proliferation are engaged. The level of these interactions can vary, as the individual cannabinoids possess their own different affinities for the each of the receptors (20). Unlike THC, which has high affinities for both cannabinoid receptors (21), CBD and the majority of these minor cannabinoids exhibit little, if any, affinity for either receptor (20, 22). It is important to note, however, that they can still antagonize those cannabinoids that do engage receptors.

At first, we tested and compared the activities of the pure forms of CBD and THC with the less refined BDS forms. Whereas pure CBD and THC contained less than 5% impurities, the BDS-forms contained only approximately 65% (w/w) of each respective agent. The impurities in both BDS compounds were made up of a number of other cannabinoids, a number of which possess their own bioactivities. Our results showed that generally, pure CBD was more efficacious than the BDS-form; for example, in T98G cells, the IC_{50} at 48 hours was 8.4 ± 2.9 μmol/L versus 16 ± 4.7 μmol/L for the P and BDS forms, respectively ($P < 0.01$). Although the reduced activity of the CBD-BDS could be in part attributed to the lower amount of CBD within the formulation, dose–response experiments suggested this not to be the case entirely. Conversely, THC appeared to be more active in the BDS-form, which consisted of approximately 35% other non-THC cannabinoids. These data reiterate the complexity of the interactions between the cannabinoids, and suggest that overall action depends upon the type and amount of each one present. We and others have shown that activities of distinct cannabinoids are dependent upon the mixtures within which they are present. For example, CBD can specifically enhance the action of THC in glioma cells (23), and can also combine with other minor cannabinoids in a synergistic fashion (11).

It is unclear whether or not cannabinoid activity is entirely dependent upon the levels of their cognate receptors; overall cannabinoid activity does not always correlate with the specific binding affinities of the range of compounds (24). The confusion surrounding the mode of action of the cannabinoids stems from the intrinsic heterogeneity of the proteins impacted upon by these compounds. The fact that these proteins are inextricably linked (25) results in a "snowball" effect, where the cause and effect can become uncoupled and unstable. Consequently, depending upon the cell type, drug concentration, and treatment schedule/timing, cannabinoid activities have been shown to be both dependent and independent of receptors. However, what remains consistent is that the activities of cannabinoids involve modifications to key intracellular signaling cascades (15, 26, 27). MAPK is a major signaling pathway that underpins a number of important cellular processes that support cancer survival, growth, and development, and was therefore assessed in the current study. We showed that culturing cells with lower concentrations of CBD and THC could cause an increase in pERK, whereas higher concentrations may reduce it. This was in agreement with the work of others that suggested the effects of cannabinoids on MAPK was both dose-dependent and cell line specific (15, 23). In addition to MAPK, the PI3-kinase/AKT pathway was also considered as it has also been linked to cannabinoid action and is an important determinant of cell fate (28). Our results showed modest reductions in pAKT, which was most apparent at the higher concentrations of CBD. The ER-stress pathway is also an important mechanism through which cannabinoids can exert their effects. Markers of this include the stress genes p8 and TRB3, which have been shown to be increased in response to accumulation of *de novo* synthesized ceramide. Lacking good commercial antibodies to assess the effects on these proteins directly, we opted to use a downstream general marker of autophagy as a read out of effects on this pathway, namely LC3B. Our results showed this marker to be increased. However, parallel gene expression analysis by microarray showed these specific stress genes to be upregulated after treatment (data not shown).

The only cannabinoid medicine licensed for human use is made up of CBD and THC at a 1:1 ratio; we therefore, explored the effect that combining these two cannabinoids had on cell number by using median-effect analysis. This method uses established algorithms to generate CI values that signify the nature of the drug–drug interactions. Drugs were combined in every permutation of the P and BDS forms at a 1:1 ratio to mimic the clinical preparation, and results showed nonantagonistic interactions between them all in the three cell lines as CI values <1. In addition, there was no impact on the nature of the combination effect depending on the different cannabinoid forms combined. We, therefore, used the pure form of each cannabinoid in our subsequent combination studies to allow for a more precise understanding of cannabinoid action. The results of our median effect combination modeling suggested CBD and THC combined favorably. For example, to achieve 50% cell kill in U87MG cells, approximately 14 μmol/L of CBD or approximately 19 μmol/L of THC would be needed if used individually. However, if they were used in combination, the concentrations required to achieve the same magnitude of cell kill would be approximately 7 μmol/L for each (combination IC_{50}, 14 μmol/L). This nonantagonistic interaction of CBD and THC, therefore, suggested that combining cannabinoids with other agents that share common pathways may be a viable strategy in a clinical setting; this may also include more conventional chemotherapies (4, 11). Indeed, recent studies have highlighted that CBD and THC can combine successfully with other chemotherapy, namely temozolomide, to reduce the progression of glioma cells in a xenograft model (13, 14).

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Having confirmed that CBD and THC could be combined with no significant loss of activity, we introduced radiation into the schedule and explored the value of this "triple combination." A combination such as this would more closely resemble what would happen clinically, and would be a concept that was supported by recent evidence in murine models that showed CBD and THC could be combined successfully with the alkylating agent temozolomide (13). An initial dose–response experiment was carried out to determine the radiosensitivity of the individual cell lines. Methodologically, the ability of cells to survive an irradiation insult and continue to form colonies was used as our read-out. To assess the effect of the cannabinoids on this process, cells were pretreated with CBD and/or THC for 4 hours before irradiation, and the impact of this on the ability of cells to react to the subsequent radiation assault was measured. SER_{10} values indicated that CBD and THC, when used together, enhanced the radiosensitivity of the cell lines. Indeed, although the staining for γ-H2AX foci was used principally as a way of confirming irradiation induced DNA damage, the prolonged presence of these foci when cannabinoids were also used implies a delay in their repair. The consequence of this is not yet clear, but may suggest that the cannabinoids are interfering with DNA damage repair, which may, therefore, improve the efficacy of ionizing radiation. In addition, using irradiation treatment alongside the cannabinoids appeared to enhance their capacity to induce autophagy and apoptosis.

In support of the *in vitro* clonogenicity data, we next examined the effect of combining CBD and THC with irradiation in an orthotopic murine model for glioma. This is as an area where there is a notable lack of information, as previous studies investigating the action of cannabinoids in glioma have only been carried out in xenograft models (13, 14). Our hypothesis was that cannabinoids could support the cell killing effect of irradiation by engaging and/or priming apoptosis and autophagy mechanisms required for successful cell death. Dramatic responses were seen when the cannabinoids and irradiation were used together, where tumor growth was slowed considerably, and as a consequence, resultant tumor size significantly reduced. Subsequent immunohistochemistry showed that areas of tumor cells were easily discernible from normal tissue, with untreated and radiation treated tumors also being visually hematic in appearance. All tumor cells stained positive for Ki67, the intensities of which were not altered remarkably following any of the treatments. This was disappointing but not surprising as a limitation of Ki67 staining is its inability to specifically distinguish rates of proliferation (29) Nonetheless, as our *in vitro* data suggested that cannabinoids hinder cell growth, this antiproliferative effect may have accounted for the smaller tumor sizes in the animals treated with cannabinoids even though histologically they were Ki67 positive. In addition, the impressive reductions in tumor volumes in mice treated with both cannabinoids and irradiation could not be explained by increased apoptosis in the combination regimen. Specifically, TUNEL staining indicated the presence of apoptosis in the tumor masses; however, there was no significant difference between the cannabinoids alone and the combination groups. Taken together our data could not clearly demonstrate an increase in apoptosis as being the underlying mechanism of the effect *in vivo*, but suggested the probability of it being one of many.

The histology also highlighted a change in the cellular density and coloration of the tumors *in situ*. In addition to a reduction in tumor volume, treatment with cannabinoid and irradiation caused a lessening in the reddish color. In addition to its role in determining tumor cell survival and growth, MAPK signaling can influence tumorigenesis (30), and through this, some cannabinoids have also been shown to be antiangiogenic and antiinvasive (31). Our data support this, as the lessening of the red, bloody hue of the tumor mass in the brain sections also correlated with a reduction in the endothelial maker of angiogenesis/neovascularization CD31 (32). It is intriguing to speculate at this time, that in addition to hindering tumor growth through an antiproliferative effect, the treatment was also reducing cancer development by inhibiting tumor angiogenesis. There is a discernible scarcity in our understanding of this observation because of the novelty in these works, and consequently, further work is required to define the precise mechanism of action of this treatment, which is an area in which we continue to investigate. Moreover, these data dovetail attractively with the work of Velasco's group (Complutense University, Madrid, Spain) who have shown the combination of CBD and THC with temozolomide, the chemotherapy currently used to treat patients with glioma is therapeutically attractive (13). Supplementary studies are ongoing that explore the exciting potential of a treatment strategy/regimen comprising irradiation, temozolomide, and cannabinoids.

In summary, these data add further support to the concept that cannabinoids both alone and in combination with each other, possess anticancer properties. We have reaffirmed the effects that CBD and THC have on central intracellular signaling pathways responsible for maintaining cell growth and survival, and have shown that using these two cannabinoids concomitantly resulted in mild synergism, which was also able to enhance the cytotoxic effect of irradiation. Astonishing results were observed *in vivo*, where the triple combination of CBD, THC, and irradiation significantly inhibited tumor progression in an orthotopic syngeneic model. It is now important to expand this study to understand the biologic basis of this enhancement, particularly the suggestion that neovascularization may be impeded. Similarly, we have previously shown that the schedule with which cannabinoids are administered is crucial, and so these questions are being addressed in our ongoing work. Ultimately,

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these studies will provide us with a better understanding of how best these compounds should be used most effectively and how a combination of cannabinoids and irradiation can be used to improve the efficacy of glioma treatment.

Disclosure of Potential Conflicts of Interest

W.M. Liu received a commercial research grant from GW Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: K.A. Scott, W.M. Liu
Development of methodology: K.A. Scott, W.M. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Scott, W.M. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.A. Scott, A.G. Dalgleish, W.M. Liu
Writing, review, and/or revision of the manuscript: K.A. Scott, A.G. Dalgleish, W.M. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Scott
Study supervision: W.M. Liu

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