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Title: Quantitative analyses of synergistic responses between cannabidiol and DNA-damaging agents on the proliferation and viability of glioblastoma and neural progenitor cells in culture

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ANOVA	analysis of variance
Akt	protein kinase B

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BCNU	carmustine
BrdU	5-Bromo-2'-deoxy-uridine
CBD	cannabidiol
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
CDDP	cisplatin
CMT	combined modality therapy
χ^2 analysis	Chi-square analysis
CNS	central nervous system
CO_2	carbon dioxide
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
ERK	extracellular signal–regulated kinase
FBS	fetal Bovine Serum
FGF	fibroblast growth factor
FIC	fractional inhibitory concentration
GBM	glioblastoma multiforme
GPCRs	G protein–coupled receptors
GPR55	G protein-coupled receptor 55
HEK	Human Embryonic Kidney
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
IC_{50}	half maximal inhibitory concentration

IACUC	Institutional Animal Care and Use Committee
NIDA	National Institute on Drug Abuse
NPCs	neural progenitor cells
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGF-GBM cells	PDGFA-Ink4a-arf$^{-/-}$ GBM cells
PPARγ	peroxisome proliferator-activated receptor-γ
RCAS	replication competent ALV LTR with a splice acceptor
TCGA	the Cancer Genome Atlas
TMZ	temozolomide
TRPV	transient receptor potential cation channel subfamily V member
WST-1	water-soluble tetrazolium salt 1

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Abstract

Evidence suggests that the non-psychotropic *cannabis*-derived compound, cannabidiol (CBD), has anti-neoplastic activity in multiple types of cancers, including glioblastoma multiforme (GBM). DNA-damaging agents remain the main standard of care treatment available for patients diagnosed with GBM. Here we studied the anti-proliferative and cell-killing activity of CBD alone and in combination with DNA-damaging agents (temozolomide, carmustine or cisplatin) in several human GBM cell lines and in mouse primary GBM cells in cultures. This activity was also studied in mouse neural progenitor cells (NPCs) in culture to assess for potential central nervous system (CNS) toxicity. We found that CBD induced a dose-dependent reduction of both proliferation and viability of all cells with similar potencies, suggesting no preferential activity for cancer cells. Hill plot analysis indicates an allosteric mechanism of action triggered by CBD in all cells. Co-treatment regiments combining CBD and DNA-damaging agents produced synergistic anti-proliferating and cell-killing responses over a limited range of concentrations in all human GBM cell lines and mouse GBM cells as well as in mouse NPCs. Remarkably, antagonistic responses occurred at low concentrations in select human GBM cell lines and in mouse GBM cells. Our study suggests limited synergistic activity when combining CBD and DNA-damaging agents in treating GBM cells, along with little-to-no therapeutic window when considering NPCs.

Introduction

Standard of care treatment for glioblastoma multiforme (GBM, the predominant and devastating subtype of gliomas that develops in human adults) extends the median survival of patients from approximately 12 months to only 15-17 months (Stupp et al., 2005; Adamson et al., 2009; Omuro and DeAngelis, 2013; Ostrom et al., 2014). Patients diagnosed with GBM undergo surgical resection followed by radiotherapy and chemotherapy. The most commonly prescribed chemotherapeutics are the DNA-damaging agents temozolomide (TMZ, Temodar®) and carmustine (BCNU), both of which have limited value at curbing GBM pathogenesis as they exhibit poor efficacy at stopping cell proliferation and eliminating tumor mass by killing them (Adamson et al., 2009). Importantly, high dose of DNA-damaging agents are required to treat patients diagnosed with GBM and result in significant debilitating side-effects due to their poor cancer-selectivity and ensuing detrimental effects on dividing cells (Adamson et al., 2009). The poor prognoses associated with GBM along with the lack of safe standard of care available to treat and ultimately cure this disease advocates for an urgent need to develop much improved medicines to treat this devastating type of cancer (Brem et al., 1995; Westphal et al., 2003; Stupp et al., 2005; Adamson et al., 2009; Omuro and DeAngelis, 2013).

The genetic profiling of human glioma tissues by The Cancer Genome Atlas (TCGA) revealed a remarkable heterogeneity in driver mutations and gene amplification that led to the classification of GBM into three subtypes – proneural, mesenchymal and classical (Verhaak et al., 2010; Dunn et al., 2012; Ozawa et al., 2014). Several genetic mouse models of GBM have revealed how driver mutations participate in its pathogenesis (Zhu et al., 2009; Halliday et al., 2014; Leder et al., 2014; Ozawa et al., 2014) and a recent study indicated that amplification of platelet-derived growth factor (PDGF) signaling is associated with the proneural subtype of

GBM and occurs early during GBM pathogenesis (Ozawa et al., 2014). Thus, the genetic makeup of GBM tumors needs to be considered when developing novel therapeutic strategies to treat and ultimately cure this cancer.

It has been shown that cannabidiol (CBD) exhibits anti-neoplastic activity in multiple GBM cell lines in culture and in xenograft mouse models (Massi et al., 2004; Vaccani et al., 2005; Massi et al., 2006; Massi et al., 2008; Marcu et al., 2010; Torres et al., 2011; Nabissi et al., 2013; Solinas et al., 2013; Soroceanu et al., 2013). This anti-neoplastic activity is mediated through plasma membrane-associated receptors, including G protein-coupled receptor 55 (GPR55) and transient receptor potential cation channel subfamily V member (TRPV) 1/2, and involves the production of reactive oxygen and induction of autophagy and apoptosis (Bisogno et al., 2001; Ligresti et al., 2006; Massi et al., 2006; Ford et al., 2010; Ramer et al., 2010; Yamada et al., 2010; Pineiro et al., 2011; Anavi-Goffer et al., 2012). Interestingly, a recent study reported that the anti-neoplastic activity of CBD synergizes with that of TMZ and BCNU, suggesting that combination treatment regimens (Combined Modality Therapy) could provide greater benefit to patients diagnosed with GBM when considering CBD; however, this study reported activity at a single concentration of CBD and in one human GBM cell line (U87MG) (Nabissi et al., 2013). Thus, a more detailed and quantitative evaluation of the combined responses induced by CBD and DNA-damaging agents in multiple cell culture models is still required to better understand the therapeutic potential of CBD in treating patients diagnosed with GBM.

Here we studied the anti-proliferative and cell-killing activities of CBD alone and combined with three DNA-damaging agents [TMZ, BCNU, and cisplatin (Platinol®, CDDP)] in three human GBM cell lines (e.g., T98G, U251 and U87MG), as well as in primary cells derived from a genetically-engineered mouse model of GBM that carries amplified PDGF signaling (PDGF-

GBM cells). Mouse neural progenitor cells (NPCs) in culture were used to assess central nervous system (CNS) toxicity. The drug interactions between treatments were analyzed using checkerboard and fractional inhibitory concentration (FIC) allowing for quantitative and statistical evaluations of each combination.

Materials and Methods

Reagents

Cannabidiol (CBD, PubChem CID: 644019) was obtained from National Institute on Drug Abuse (NIDA) Drug Supply Service (Bethesda, MD, USA). Temozolomide (TMZ, PubChem CID: 5394, catalog #: 2706) and cisplatin (CDDP, PubChem CID: 441203, catalog #: 2251) were obtained from Tocris Bioscience (Ellisville, MO, USA). Carmustine (BCNU, PubChem CID: 2578, catalog #: C0400), dimethyl sulfoxide (DMSO), trypsin-EDTA solution (catalog #: T3924), and laminin (catalog #: 11243217001) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CBD and TMZ were dissolved in DMSO; BCNU was dissolved in ethanol; CDDP was dissolved in PBS. HyClone® Dulbecco's Modified Eagles Medium (DMEM with L-Glutamine, Sodium Pyruvate, catalog #: SH30243.01), PhenoRed-free DMEM (with High Glucose, Modified, without PhenoRed, without L-Glutamine, with Sodium Pyruvate, catalog #: SH30604.01), and fetal Bovine Serum (FBS, catalog #: SH30088) were obtained from GE Healthcare Life Sciences (Logan, UT, USA). Gibco® Dulbecco's phosphate-buffered saline (PBS, catalog #: 14040141), Penicillin-Streptomycin (catalog #: 15140122), Sodium Bicarbonate 7.5% solution (catalog #: 25080094), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, catalog #: 15630106), human recombinant fibroblast growth factor (bFGF, catalog #: 13256029) and epidermal growth factor (EGF, catalog #: PHG0314) were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). NeuroCult Neural Stem Cell Basal Medium (mouse, catalog #: 05700), NeuroCult Neural Stem Cell Proliferation Supplements (mouse, catalog #: 05701), heparin Solution (0.2 %, catalog #: 07980), and accutase (catalog #: 07920) were purchased from StemCell Technologies (Vancouver, BC, Canada).

Generation of the mouse PDGF-GBM cells and NPCs

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All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Fred Hutchinson Cancer Research Center. Mouse GBM cells harboring amplified PDGF signaling (PDGF-GBM) were generated by injecting replication competent ALV LTR with a splice acceptor (RCAS)-PDGFA viruses into *Nestin (N)/tv-a;Ink4a-arf*$^{-/-}$*;Pten*$^{fl/fl}$ mice, as previously described (Ozawa et al., 2014). Animals were sacrificed with Carbon dioxide (CO_2) at age of 72 days when presented brain tumor symptoms (i.e., cranial swelling and lethargy) and PDGFA-Ink4a-arf$^{-/-}$ GBM cells (PDGF-GBM cells) were harvested by mechanical dissociation of the tumors that had developed in mouse brain. Mouse neural progenitor cells (NPCs) were generated by the mechanical dissociation of the new born pups forebrains of *N/tv-a;Ink4a-arf*$^{-/-}$*;Pten*$^{fl/fl}$ mice. Mouse PDGF-GBM cells and NPCs were initially maintained as floating neurosphere cultures, and starting at passage 3 and 5 (PDGF-GBM cells and NPCs, respectively) cultured as adherent monolayers on laminin-coated dishes.

Cell culture

T98G, U251, and U87MG cells were expanded in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01 M HEPES, and 0.075 % Sodium Bicarbonate. For cell proliferation and viability assays, cells were switched to DMEM (PhenoRed free) supplemented with 3% FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin when seeded onto 96-well plates.

Mouse PDGF-GBM cells and NPCs were expended in mouse NeuroCult neural stem cell basal medium supplemented with NeuroCult neural stem cell proliferation supplements, 2 µg/ml heparin, 20 ng/ml EGF, 10 ng/ml FGF, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere (37 ^{o}C and 5 % CO_2). Cells at passages of 4-6 were used in the following experiment.

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Drug Treatments

Each cell type was treated with CBD, TMZ, BCNU, or CDDP at concentrations ranging from $10^{-7.5}$ to 10^{-3} M in half $\log_{10}$ steps and proliferation and viability were measured 72 hrs after treatment. All dose response experiments were conducted in triplicate and repeated for at least three times. Data analyses was performed using GraphPad Prism and each experiment considered as n = 1.

Cell proliferation and viability assays

Cell proliferation was measured using the 5-Bromo-2'-deoxy-uridine (BrdU) cell proliferation ELISA (colorimetric) kit (Roche, Indianapolis, IN, USA) following manufacturer's protocol. The cell density per well for each cells type was selected in the logarithmic growth range (Supplementary Fig. S1). Thus, cells were seeded in 96-wells plates overnight, and then cultured with compounds and BrdU labeling agents for 72 hrs. BrdU incorporated in newly synthesized cellular DNA was measured using anti-BrdU antibodies (conjugated with peroxidase) and detected by the subsequent substrate reaction. Sulfuric acid was used to stop the substrate reaction and the absorbance at 450 nm was quantified using a multi-well spectrometer (Packard SpectraCount).

Cell viability was measured using the water-soluble tetrazolium salt 1 (WST-1) reagent (Roche, Indianapolis, IN, USA) following manufacturer's protocol. The cell density per well for each cell type was selected in the logarithmic growth range (Supplementary Fig. S1). Thus, cells were seeded into 96-well plates, treated with compounds 24 hrs later and cell viability measured 72 hrs later with WST-1 by adding 10 µl of reagent and measuring absorbance at 450nm after an hour using a multi-well spectrometer (Packard SpectraCount).

Checkerboard assay and data analyses

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The interaction between CBD and DNA-damaging agents was tested using the checkerboard assay (Supplementary Fig. S2). Thus, serial half $\log_{10}$ dilutions of CBD (0.3 - 100 µM) were combined with TMZ (1 µM - 1mM), BCNU (3 µM - 1mM) or CDDP (0.1 - 100 µM). Drugs for combination were prepared separately and added into the cell separately, but at the same time. All combinations were performed in duplicate and repeated three times. Data analyses was performed using GraphPad Prism and each experiment considered as n = 1.

Different analytical methods of the checkerboard assay may lead to differences in the interpretations (Bonapace et al., 2002). Thus, two methods were used to analyze the results from the checkerboard assay to provide a better understanding of these interactions. Method #1 focuses on the percentage of occurrence of synergy, additivity and antagonism observed in all the tested concentrations of each individual combination, whereas Method #2 focuses on the examination of the interactions of compounds based on their efficacies (when producing half maximal inhibition effects).

■ Method #1: Full-plate fractional inhibitory concentration (FIC). Full-plate FIC was calculated over all the combinations tested for drug A and drug B on a checkerboard assay. For each combination, the formula is as follows:

$$FIC\ (A) = C_{A+B} / C_A$$

$$FIC\ (B) = C_{B+A} / C_B$$

$$\sum FIC = FIC\ (A) + FIC\ (B)$$

C_{A+B} is the concentration of drug A in the presence of drug B and C_A is the concentration of drug A alone when producing the same effect as in the combination, and *vice versa* for C_{B+A} and C_B. The drug A (or B) alone concentration was calculated by determining the concentration (x) for the effect of the combination (y) from the dose response curve of drug A (or B) using

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GraphPad Prism. FIC index (∑FIC) was then calculated to determine the synergic interactions for each combination of drug A and drug B on a checkerboard assay. Synergy was defined as ∑FIC < 0.5, antagonism was defined as ∑FIC > 4, and additivity was defined when ∑FIC was in the range of 0.5 to 4, as previously described (Orhan et al., 2005). The percentage of occurrences of either synergy, additivity or antagonism that occurred throughout all the combination of drug A and drug B tested on a checkerboard assay was then calculated (Bonapace et al., 2002).

■ Method #2: Efficacy FIC index. This index was calculated based on the combinations that produced half maximal inhibition. Thus, to identify the combinations that produced half maximal effects, we fixed the concentration of drug A and plotted the dose response curve of the inhibitory effects of the combinations (containing a serial half log10 dilution of drug B in the presence of the fixed concentration of drug A) against the concentration of drug B used in the combinations. The IC_{50} of drug B (in the presence of the fixed concentration of drug A) was then calculated using GraphPad Prism. Therefore, the combination of the fixed concentration of drug A and the IC_{50} of drug B produce a half maximal inhibition. The efficacy FIC was calculated following the formula:

$$FIC\ (A) = IC_{50A+B} / IC_{50A}$$

$$FIC\ (B) = C_{B\_fixed} / IC_{50B}$$

$$\sum FIC = FIC\ (A) + FIC\ (B)$$

IC_{50A+B} is the half maximal inhibitory concentration of drug A in the presence of drug B, IC_{50A} is the half maximal inhibitory concentration of drug A alone, C_{B_fixed} is the fixed concentration of drug B, and IC_{50B} is the half maximal inhibitory concentration of drug B alone. Synergy was defined as ∑FIC < 0.5, antagonism was defined as ∑FIC > 4, and additivity was defined when ∑FIC was in the range of 0.5 to 4, as previously described (Orhan et al., 2005).

Statistical analyses

Dose-responses for each compound were analyzed using GraphPad Prism Version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). Chi-square (χ^2) analysis was used to identify the source of significant differences (e.g., differences among cell type for each individual drug combination; differences among all the drug combinations in each cell type) from checkerboard results obtained from method #1. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test or student t test was used to identify the source of significant differences in the FIC indices from checkerboard results obtained from method #2. Statistical analyses were performed using GraphPad Prism Version 5.01. $P < 0.05$ was considered statistically significant.

Results

Anti-neoplastic activity of CBD

The anti-neoplastic activity of CBD was assessed by measuring both its anti-proliferative and cell-killing activities in three human GBM cell lines (T98G, U251 and U87MG) and in mouse PDGF-GBM cells in primary culture. Its potential CNS toxicity was assessed by measuring the same readouts in mouse NPCs in culture. Thus, cells were treated with half $\log_{10}$ concentrations of CBD and three days later changes in cell proliferation and cell viability were measured by quantifying BrdU incorporation and mitochondrial activity with WST-1, respectively. We found that CBD inhibited cell proliferation and reduced cell viability of all cells with similar potencies (cell proliferation IC_{50s}: 3.1 - 8.5 μM and cell viability IC_{50s}: 3.2 - 9.2 μM) (Fig. 1 and supplementary table 1). All dose-responses were remarkably steep and hill-plot calculations indicate an allosteric mechanism of action for each response (supplementary table 2). Regarding the efficacy of this activity, we found that all cell types were greatly sensitive to both the anti-proliferative and cell-killing activities of CBD (maximal efficacy = 94.19% - 100%). Specifically, for both anti-proliferative response and cell-killing activity, mouse PDGF-GBM cells and NPCs were the most sensitive (i.e., lower dose required for activity), whereas the three human GBM cell lines were the least sensitive (i.e., higher dose required for activity) (Supplementary table 3). Our results confirm previous reports on the anti-neoplastic activity of CBD in GBM cells in culture and extend them by now emphasizing an allosteric mechanism of action that profoundly reduces both cell proliferation and viability with similar potency and efficacy in all human GBM cell lines (irrespective of their genetic makeup), as well as in mouse PDGF-GBM cells and NPCs.

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Anti-neoplastic activity of DNA-damaging agents

The anti-neoplastic activity of TMZ, BCNU and CDDP was assessed using the same experimental design and readouts as with CBD. We found that these agents inhibited cell proliferation and reduced cell viability of all cell types but with remarkably different potencies and efficacies (Fig. 2A-C, 3A-C, and supplementary table 1-3). Here, three sets of responses are worth noting. First, TMZ exhibited limited anti-proliferative activity in all cells and limited cell-killing activity in the three human GBM cell lines. By contrast, the cell-killing activity of TMZ started at 30 μM in both mouse PDGF-GBM cells and NPCs (Fig. 2A and 3A). Second, mouse PDGF-GBM cells and NPCs were remarkably more sensitive to BCNU (IC_{50}: 34.1 - 48.1 μM) than the three human GBM cell lines (IC_{50s}: 205 - 661 μM) (Fig. 2B and 3B). Third, mouse PDGF-GBM cells and NPCs, as well as U87MG cells, were more sensitive to CDDP (IC_{50s}: 2.4 - 3.2 μM) than two of the human glioma cell lines T98G and U251 (IC_{50s}: 6.8 - 25.2 μM, respectively) (Fig. 2C and 3C). Hill plot calculations of all dose-responses indicated single-order non-allosteric responses triggered by DNA-damaging agents (Supplementary table 2). Our results show that mouse PDGF-GBM cells and NPCs are in general more sensitive than human GBM cell lines to these DNA-damaging agents.

Interactions between CBD and DNA-damaging agents on cell proliferation

Combined Modality Therapy uses optimal low-doses of two agents to achieve maximal anti-neoplastic effects while reducing potential side-effects (Kummar et al., 2010). Here we performed quantitative and statistical analyses of the interactions between CBD and DNA-damaging agents on cell proliferation as measured by quantifying BrdU incorporation and performing checkerboard analyses.

We first studied the interactions between CBD and DNA-damaging agents over full dose-responses [CBD (0.3 - 100 μM) combined with either TMZ (1 μM - 1mM), BCNU (3 μM - 1mM) or CDDP (0.1 - 100 μM)] and analyzed all possible combinations with the checkerboard method #1. Based on these results, we then calculated the percentage of occurrence of synergistic, additive or antagonistic responses when evaluating all combinations. Fig. 2D shows that the vast majority of CBD/DNA-damaging agent combinations resulted in additive anti-proliferative responses in all cells. No significant difference in the frequency distribution of drug interactive effects was found among the three human GBM cell lines ($P > 0.44$) or between mouse PDGF-GBM cells and NPCs ($P > 0.30$) for each drug combination, suggesting similar responses in these groups of cells.

Four sets of interactions are worth emphasizing. First, low concentrations of CBD (1 μM) and TMZ (10 μM) exhibit a synergistic anti-proliferative response in T98G cells (Fig. 2D). Second, synergistic responses occur between low concentrations of CBD (0.3 - 10 μM) and BCNU (300 μM) in three GBM cells (T98G, U251 and mouse PDGF-GBM cells) but also in mouse NPCs (Fig. 2D). Third, co-treatment with CBD and BCNU leads to all possible interactions (synergistic, additive, and antagonistic) depending on the concentrations in both mouse PDGF-GBM cells and NPCs (Fig. 2D). Forth, low concentrations of CBD (1 - 3 μM) and CDDP (100 μM) have antagonistic anti-proliferative responses in all GBM cells, but not in mouse NPCs (Fig. 2D).

We then analyzed the interactions between CBD and DNA-damaging agents based on their efficacies by using checkerboard method #2 and calculated the FIC response of the combinations producing half maximal inhibition effects. Fig. 4A and Table 1 show that all half maximal inhibitory combinations produced additive effects in all cells as indicated by their FIC indices

that are between 0.5 and 4. Thus, the combinations of CBD and DNA-damaging agents principally resulted in additive anti-proliferative responses.

Interactions between CBD and DNA damaging agents on cell viability

The interaction between CBD and DNA-damaging agents on cell viability was assessed using the same experimental design but measuring cell viability with WST-1. Fig. 3D shows that the vast majority of combinations between CBD and DNA-damaging agent resulted in additive cell-killing activity in all cells. Similar to the effects on cell proliferation, no significant difference in the frequency distribution of drug interactive effects was observed among the three human GBM cell lines ($P > 0.14$) for each drug combination, suggesting an overall similar response in these cells. However, differences between mouse PDGF-GBM cells and NPCs were observed for drug combination of CBD/TMZ ($P < 0.001$) and CBD/CDDP ($P < 0.05$).

Five sets of interactions are worth noting. First, synergistic cell-killing responses occur when combining low concentrations of CBD (1 - 10 μM) with either TMZ (30 μM) or BCNU (30 μM - 1 mM) in T98G cells, as well as with BCNU (10 - 100 μM) in U87MG cells (Fig. 3D). Second, combinations of CBD and BCNU at concentrations that produce a synergistic anti-proliferation responses of mouse PDGF-GBM cells and NPCs (Fig. 2D) resulted in merely additive reduction in cell viability (Fig. 3D). Third, combinations of CBD and BCNU produced antagonistic cell-killing responses in mouse PDGF-GBM cells and NPCs. Fourth, the vast majority of concentrations combining CBD and TMZ lead to antagonistic cell-killing responses in mouse PDGF-GBM cells, but not in NPCs ($P < 0.05$). Fifth, combinations of CBD and CDDP antagonized their cell killing activity in all GBM cell lines (Fig. 3D).

Analyses using method #2 indicated additive cell-killing responses for most combinations and clear antagonist killing responses between CBD/CDDP in T98G and U251 cells (Fig. 4B and Table 2). Analyses of the cell-killing activity of CBD and TMZ also showed profound antagonistic responses in mouse PDGF-GBM cells, consistent with the results obtained with method #1 (76.9 %) (Fig. 4B and Table 2). These results extend the result obtained with method #1 by showing that a vast majority of cell-killing activity measured when combining CBD and DNA-damaging agents are additive, with several notable antagonist responses.

Discussion

We evaluated the anti-neoplastic activity of CBD alone and in combination with DNA-damaging agents in human GBM cell lines and mouse PDGF-GBM cells in culture, and assessed the potential CNS toxicity of this treatment with mouse NPCs. We found that CBD both inhibits cell proliferation and kills all cells tested here with similar low micromolar potencies, suggesting that the molecular mechanism of action triggered by CBD that leads to the reduction of the proliferation and viability of all cells is not affected by their genetic makeup. Thus, it appears that CBD triggers a mechanism of action that does not preferentially target cancer cells and will likely be associated with NPCs toxicity. Analyses of all dose-responses triggered by CBD indicated a cooperative/allosteric mechanism of action. Together, these results suggest significant challenges when considering the poor therapeutic index and steep dose-response of CBD used as a single agent to treat patients diagnosed with GBM.

Several studies have shown that the biological activity of CBD is concentration-dependent and likely mediated through multiple plasma membrane-associated receptors (Pertwee, 2008). At nanomolar concentrations, CBD binds to GPR55 as determined by *in vitro* radioligand binding assays (Ryberg et al., 2007). Accordingly, CBD modulates GPR55 activity as show by 1 - 3 µM CBD that blocks the L-α-lysophosphatidylinositol-induced GPR55-mediated migration and polarization of breast cancer cells (Ford et al., 2010), as well as inhibits the proliferation of prostate cancer cells (Pineiro et al., 2011). The pharmacological activity of CBD at GPR55 appears complex and likely cell-dependent as suggested by one study reporting that this compound promotes cell proliferation via ERK pathway in glioma cells (Andradas et al., 2011). One to ten micromolar concentrations of CBD bind CB_1 and CB_2 receptors as measured by radioligand binding assays (Petitet et al., 1998; Thomas et al., 2007; Pertwee, 2008).

Accordingly, 1-10 μM CBD triggers apoptosis through CB_2 receptors in leukemia cells (McKallip et al., 2006) and10 μM CBD suppresses glioma cell proliferation and this response is partially blocked by a CB_2 antagonist (Massi et al., 2004). Micromolar concentrations of CBD modulate the activity of several plasma membrane-associated ion channel receptors, including TRPV1 receptors with an EC_{50} of 3 μM (Bisogno et al., 2001; Iannotti et al., 2014), TRPV2 receptors when applied at 10 - 30 μM (Qin et al., 2008; Nabissi et al., 2013) and serotonin 1A (5-HT_{1A}) receptor with an EC_{50} of 32 μM (Russo et al., 2005). CBD at 100 nM also modulates that activity of intracellular transcription factors such as peroxisome proliferator-activated receptor-γ (PPARγ) (Esposito et al., 2011). It is important to emphasize that the lipophilic nature of CBD triggers biological responses that are independent of protein-mediated mechanisms, including rapid changes in both membrane lipid raft and cholesterol metabolism when applied at 5 - 20 μM (Ligresti et al., 2006; Rimmerman et al., 2011; Rimmerman et al., 2013). Additional examples of the protein-independent mechanism of CBD include increase in oxidative stress resulting in apoptosis, DNA damage and autophagy in breast cancer cells (Shrivastava et al., 2011) and in glioma cells (Bisogno et al., 2001; Massi et al., 2004; Solinas et al., 2013; Soroceanu et al., 2013) when this compound is applied at 5 - 40 μM. Thus, there is a wide range of protein-dependent and protein-independent biological activities induced by CBD applied in the micromolar range. Although the identification of the molecular target(s) and signaling step(s) that mediates the inhibition of cell proliferation and cell-killing activity of CBD reported here is beyond the scope of this work, the allosteric response of CBD that we measured in the 1-10 μM range in all cells suggests the involvement of a single class of proteins that mediates this activity.

Allosteric modulation plays an important role in the signal transduction mechanism of many proteins by favoring protein confirmations that enhance (i.e., positive allosteric modulator,

PAM) or reduce (i.e., negative allosteric modulator, NAM) activities, and thus represents an active area of research for the development of new therapeutics (Changeux and Edelstein, 2005; Melancon et al., 2012). CBD at a concentration range of 0.1-5 μM is a NAM of CB_1 receptors signaling when studied in Human Embryonic Kidney 293 (HEK293) cells (Laprairie et al., 2015). CBD at 3 - 30 μM affects TRPV1 and at 1 - 30 μM affects GPR55 activities (Bisogno et al., 2001; Ford et al., 2010; Pineiro et al., 2011; Iannotti et al., 2014), both of which contain allosteric regulatory sites (Pineiro et al., 2011; Anavi-Goffer et al., 2012; Cao et al., 2013; Maione et al., 2013). At 100 μM, CBD acts as a PAM of opioid mu receptors (Kathmann et al., 2006) but these high concentrations suggest that this target is unlikely to mediate the bioactivity of CBD. Thus, the 1-10 μM anti-neoplastic activity of CBD reported here could be mediated through at least 3 plasma membrane targets (GPR55, CB_1 and VRPV1) that are known to both interact with CBD in this range of concentrations and have allosteric regulatory sites. Whether any of these targets mediate the response studied here still needs to be determined.

A common strategy to improve the therapeutic index of certain drugs is combined modality therapy (CMT) whereby treatment with reduced concentrations of two drugs known to be associated with side-effects will result in enhancing overall therapeutic efficacy while reducing the incidence of side-effects (Kummar et al., 2010). A recent study reported that 10 μM CBD exhibits a synergistic GBM-killing activity when combined with either 400 μM TMZ or 200 μM BCNU and using U87MG cell as model system (Nabissi et al., 2013). While we reproduced this result, a more quantitative and unbiased analysis of the interaction between these modalities using two independent methods of calculations (method #1 analyzes all concentrations and method #2 analyzes the efficacy-related drug interactions) indicate that only a limited range of concentrations lead to synergistic responses. In fact, the predominant range of concentrations that

we tested resulted in additive responses and a significant number of combinations resulted in antagonism. Thus, our analyses of the combined anti-neoplastic activity of CBD and DNA-damaging agents suggests little improvement in their respective therapeutic indices and in some cases loss in therapeutic efficacy by antagonism.

We identified several interactions between CBD and DNA-damaging agents occurring at select concentrations and in certain GBM cells that provide insights into the anti-neoplastic activity of these compounds. In mouse PDGF-GBM cells, we found CBD/TMZ combinations antagonizes their anti-proliferative response while triggering an additive cell-killing response. This result suggests a clear dichotomy in the anti-proliferative and cell-killing mechanism of action triggered when combining CBD and TMZ in mouse PDGF-GBM cells. In U87MG cells, combining CBD and DNA-damaging agents induced synergistic cell killing, but only additive inhibition of cell proliferation. This result suggests that the cell-killing produced by these combinations do not rely on their ability to simply reduce cell proliferation and are likely independent. Thus, our results provide examples where drug treatments exhibit dissociated and even opposite responses on cell proliferation and viability of GBM cells.

Several studies have shown that regimented treatment of glioma xenograft models with CBD significantly reduces tumor growth (Massi et al., 2004; Massi et al., 2008; Torres et al., 2011; Soroceanu et al., 2013). CBD is currently being tested in human clinical trials for the treatment of GBM patients and the vast majority of this patient population will likely be also treated with standard care DNA-damaging modalities (Saklani and Kutty, 2008). Our study provides a quantitative and unbiased evaluation of the anti-neoplastic activity of CBD alone and in combination with DNA-damaging agents in cell culture models of GBM that suggests caution

when considering this phytocannabinoid for the treatment of patients diagnosed with GBM and treated with standard of care DNA-damaging agents.

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Authorship Contributions

Participated in research design: Deng and Stella

Conducted experiments: Deng, Ng, and Ozawa

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Wrote or contributed to the writing of the manuscript: Deng, Ozawa, and Stella

References

Adamson C, Kanu OO, Mehta AI, Di C, Lin N, Mattox AK and Bigner DD (2009) Glioblastoma multiforme: a review of where we have been and where we are going. *Expert opinion on investigational drugs* **18**:1061-1083.

Anavi-Goffer S, Baillie G, Irving AJ, Gertsch J, Greig IR, Pertwee RG and Ross RA (2012) Modulation of L-alpha-lysophosphatidylinositol/GPR55 mitogen-activated protein kinase (MAPK) signaling by cannabinoids. *The Journal of biological chemistry* **287**:91-104.

Andradas C, Caffarel MM, Perez-Gomez E, Salazar M, Lorente M, Velasco G, Guzman M and Sanchez C (2011) The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. *Oncogene* **30**:245-252.

Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R and Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *British journal of pharmacology* **134**:845-852.

Bonapace CR, Bosso JA, Friedrich LV and White RL (2002) Comparison of methods of interpretation of checkerboard synergy testing. *Diagnostic microbiology and infectious disease* **44**:363-366.

Brem H, Piantadosi S, Burger PC, Walker M, Selker R, Vick NA, Black K, Sisti M, Brem S, Mohr G and et al. (1995) Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-brain Tumor Treatment Group. *Lancet* **345**:1008-1012.

Cao E, Liao M, Cheng Y and Julius D (2013) TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* **504**:113-118.

Changeux JP and Edelstein SJ (2005) Allosteric mechanisms of signal transduction. *Science* **308**:1424-1428.

Dunn GP, Rinne ML, Wykosky J, Genovese G, Quayle SN, Dunn IF, Agarwalla PK, Chheda MG, Campos B, Wang A, Brennan C, Ligon KL, Furnari F, Cavenee WK, Depinho RA, Chin L and Hahn WC (2012) Emerging insights into the molecular and cellular basis of glioblastoma. *Genes & development* **26**:756-784.

Esposito G, Scuderi C, Valenza M, Togna GI, Latina V, De Filippis D, Cipriano M, Carratu MR, Iuvone T and Steardo L (2011) Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement. *PloS one* **6**:e28668.

Ford LA, Roelofs AJ, Anavi-Goffer S, Mowat L, Simpson DG, Irving AJ, Rogers MJ, Rajnicek AM and Ross RA (2010) A role for L-alpha-lysophosphatidylinositol and GPR55 in the modulation of migration, orientation and polarization of human breast cancer cells. *British journal of pharmacology* **160**:762-771.

Halliday J, Helmy K, Pattwell SS, Pitter KL, LaPlant Q, Ozawa T and Holland EC (2014) In vivo radiation response of proneural glioma characterized by protective p53 transcriptional program and proneural-mesenchymal shift. *Proceedings of the National Academy of Sciences of the United States of America* **111**:5248-5253.

Iannotti FA, Hill CL, Leo A, Alhusaini A, Soubrane C, Mazzarella E, Russo E, Whalley BJ, Di Marzo V and Stephens GJ (2014) Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS chemical neuroscience* **5**:1131-1141.

Kathmann M, Flau K, Redmer A, Trankle C and Schlicker E (2006) Cannabidiol is an allosteric modulator at mu- and delta-opioid receptors. *Naunyn-Schmiedeberg's archives of pharmacology* **372**:354-361.

Kummar S, Chen HX, Wright J, Holbeck S, Millin MD, Tomaszewski J, Zweibel J, Collins J and Doroshow JH (2010) Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements. *Nature reviews Drug discovery* **9**:843-856.

Laprairie RB, Bagher AM, Kelly ME and Denovan-Wright EM (2015) Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British journal of pharmacology* **172**:4790-4805.

Leder K, Pitter K, Laplant Q, Hambardzumyan D, Ross BD, Chan TA, Holland EC and Michor F (2014) Mathematical modeling of PDGF-driven glioblastoma reveals optimized radiation dosing schedules. *Cell* **156**:603-616.

Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M and Di Marzo V (2006) Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *The Journal of pharmacology and experimental therapeutics* **318**:1375-1387.

Maione S, Costa B and Di Marzo V (2013) Endocannabinoids: a unique opportunity to develop multitarget analgesics. *Pain* **154 Suppl 1**:S87-93.

Marcu JP, Christian RT, Lau D, Zielinski AJ, Horowitz MP, Lee J, Pakdel A, Allison J, Limbad C, Moore DH, Yount GL, Desprez PY and McAllister SD (2010) Cannabidiol enhances the inhibitory effects of delta9-tetrahydrocannabinol on human glioblastoma cell proliferation and survival. *Molecular cancer therapeutics* **9**:180-189.

Massi P, Vaccani A, Bianchessi S, Costa B, Macchi P and Parolaro D (2006) The non-psychoactive cannabidiol triggers caspase activation and oxidative stress in human glioma cells. *Cellular and molecular life sciences : CMLS* **63**:2057-2066.

Massi P, Vaccani A, Ceruti S, Colombo A, Abbracchio MP and Parolaro D (2004) Antitumor effects of cannabidiol, a nonpsychoactive cannabinoid, on human glioma cell lines. *The Journal of pharmacology and experimental therapeutics* **308**:838-845.

Massi P, Valenti M, Vaccani A, Gasperi V, Perletti G, Marras E, Fezza F, Maccarrone M and Parolaro D (2008) 5-Lipoxygenase and anandamide hydrolase (FAAH) mediate the antitumor activity of cannabidiol, a non-psychoactive cannabinoid. *Journal of neurochemistry* **104**:1091-1100.

McKallip RJ, Jia W, Schlomer J, Warren JW, Nagarkatti PS and Nagarkatti M (2006) Cannabidiol-induced apoptosis in human leukemia cells: A novel role of cannabidiol in the regulation of p22phox and Nox4 expression. *Molecular pharmacology* **70**:897-908.

Melancon BJ, Hopkins CR, Wood MR, Emmitte KA, Niswender CM, Christopoulos A, Conn PJ and Lindsley CW (2012) Allosteric modulation of seven transmembrane spanning receptors: theory, practice, and opportunities for central nervous system drug discovery. *Journal of medicinal chemistry* **55**:1445-1464.

Nabissi M, Morelli MB, Santoni M and Santoni G (2013) Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents. *Carcinogenesis* **34**:48-57.

Omuro A and DeAngelis LM (2013) Glioblastoma and other malignant gliomas: a clinical review. *Jama* **310**:1842-1850.

Orhan G, Bayram A, Zer Y and Balci I (2005) Synergy tests by E test and checkerboard methods of antimicrobial combinations against Brucella melitensis. *Journal of clinical microbiology* **43**:140-143.

Ostrom QT, Gittleman H, Liao P, Rouse C, Chen Y, Dowling J, Wolinsky Y, Kruchko C and Barnholtz-Sloan J (2014) CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2007-2011. *Neuro-oncology* **16 Suppl 4**:iv1-63.

Ozawa T, Riester M, Cheng YK, Huse JT, Squatrito M, Helmy K, Charles N, Michor F and Holland EC (2014) Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer cell* **26**:288-300.

Pertwee RG (2008) The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *British journal of pharmacology* **153**:199-215.

Petitet F, Jeantaud B, Reibaud M, Imperato A and Dubroeucq MC (1998) Complex pharmacology of natural cannabinoids: evidence for partial agonist activity of delta9-tetrahydrocannabinol and antagonist activity of cannabidiol on rat brain cannabinoid receptors. *Life sciences* **63**:PL1-6.

Pineiro R, Maffucci T and Falasca M (2011) The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene* **30**:142-152.

Qin N, Neeper MP, Liu Y, Hutchinson TL, Lubin ML and Flores CM (2008) TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**:6231-6238.

Ramer R, Merkord J, Rohde H and Hinz B (2010) Cannabidiol inhibits cancer cell invasion via upregulation of tissue inhibitor of matrix metalloproteinases-1. *Biochemical pharmacology* **79**:955-966.

Rimmerman N, Ben-Hail D, Porat Z, Juknat A, Kozela E, Daniels MP, Connelly PS, Leishman E, Bradshaw HB, Shoshan-Barmatz V and Vogel Z (2013) Direct modulation of the outer mitochondrial membrane channel, voltage-dependent anion channel 1 (VDAC1) by cannabidiol: a novel mechanism for cannabinoid-induced cell death. *Cell death & disease* **4**:e949.

Rimmerman N, Juknat A, Kozela E, Levy R, Bradshaw HB and Vogel Z (2011) The non-psychoactive plant cannabinoid, cannabidiol affects cholesterol metabolism-related genes in microglial cells. *Cellular and molecular neurobiology* **31**:921-930.

Russo EB, Burnett A, Hall B and Parker KK (2005) Agonistic properties of cannabidiol at 5-HT1a receptors. *Neurochemical research* **30**:1037-1043.

Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T and Greasley PJ (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *British journal of pharmacology* **152**:1092-1101.

Saklani A and Kutty SK (2008) Plant-derived compounds in clinical trials. *Drug discovery today* **13**:161-171.

Shrivastava A, Kuzontkoski PM, Groopman JE and Prasad A (2011) Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy. *Molecular cancer therapeutics* **10**:1161-1172.

Solinas M, Massi P, Cinquina V, Valenti M, Bolognini D, Gariboldi M, Monti E, Rubino T and Parolaro D (2013) Cannabidiol, a non-psychoactive cannabinoid compound, inhibits

proliferation and invasion in U87-MG and T98G glioma cells through a multitarget effect. *PloS one* **8**:e76918.

Soroceanu L, Murase R, Limbad C, Singer E, Allison J, Adrados I, Kawamura R, Pakdel A, Fukuyo Y, Nguyen D, Khan S, Arauz R, Yount GL, Moore DH, Desprez PY and McAllister SD (2013) Id-1 is a key transcriptional regulator of glioblastoma aggressiveness and a novel therapeutic target. *Cancer research* **73**:1559-1569.

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, European Organisation for R, Treatment of Cancer Brain T, Radiotherapy G and National Cancer Institute of Canada Clinical Trials G (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine* **352**:987-996.

Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA and Pertwee RG (2007) Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *British journal of pharmacology* **150**:613-623.

Torres S, Lorente M, Rodriguez-Fornes F, Hernandez-Tiedra S, Salazar M, Garcia-Taboada E, Barcia J, Guzman M and Velasco G (2011) A combined preclinical therapy of cannabinoids and temozolomide against glioma. *Molecular cancer therapeutics* **10**:90-103.

Vaccani A, Massi P, Colombo A, Rubino T and Parolaro D (2005) Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism. *British journal of pharmacology* **144**:1032-1036.

Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM, Hayes DN and Cancer Genome Atlas Research N (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell* **17**:98-110.

Westphal M, Hilt DC, Bortey E, Delavault P, Olivares R, Warnke PC, Whittle IR, Jaaskelainen J and Ram Z (2003) A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. *Neuro-oncology* **5**:79-88.

Yamada T, Ueda T, Shibata Y, Ikegami Y, Saito M, Ishida Y, Ugawa S, Kohri K and Shimada S (2010) TRPV2 activation induces apoptotic cell death in human T24 bladder cancer cells: a potential therapeutic target for bladder cancer. *Urology* **76**:509 e501-507.

Zhu H, Acquaviva J, Ramachandran P, Boskovitz A, Woolfenden S, Pfannl R, Bronson RT, Chen JW, Weissleder R, Housman DE and Charest A (2009) Oncogenic EGFR signaling cooperates with loss of tumor suppressor gene functions in gliomagenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106**:2712-2716.

Footnotes

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Figure Legends

Fig. 1. CBD inhibits the cell proliferation and viability of GBM cells and NPCs. Dose-response of CBD on inhibiting (A) proliferation and (B) viability of (red) human GBM cell lines (T98G, U251 and U87MG) and (blue) mouse PDGF-GBM cells and NPCs at 72hrs time-point. Data are expressed as mean ± SEM (n = 5-9 independent experiments, each in triplicate). Vehicle: 0.1% DMSO.

Fig. 2. DNA-damaging agents on the proliferation of GBM cells and NPCs: Single and CBD combination responses. (A-C) Dose response of (A) TMZ, (B) BCNU, and (C) CDDP on inhibiting proliferation of (red) human GBM cells (T98G, U251 and U87MG) and (blue) mouse PDGF-GBM cells and NPCs at 72hrs time-point. Data are expressed as mean ± SEM (n = 5-9 independent experiments, each in triplicate). Vehicle: (A) 0.1% DMSO, (B) 0.1% ethanol, or (C) 0.1% PBS. (D) Interactions between CBD and DNA-damaging agents on inhibiting proliferation (Method #1)**.** Percentage of synergy (white), additivity (grey), and antagonism (black) occurred in the checkerboard assay. Synergy, $\sum FIC < 0.5$; antagonism, $\sum FIC > 4$; additivity, $0.5 < \sum FIC < 4$. Data are expressed as percentage of occurrence calculated using the mean $\sum FIC$ from 3 independent experiments, each in duplicate. Vehicle: 0.05% DMSO (Vehicle in lieu of CBD) + 0.05% DMSO, ethanol or PBS (Vehicle in lieu of TMZ, BCNU or CDDP, respectively).

Fig. 3. DNA-damaging agents on the cell viability of GBM cells and NPCs: Single and CBD combination. (A-C) Dose response of (A) TMZ, (B) BCNU, and (C) CDDP on inhibiting viability of (red) human GBM cells (T98G, U251 and U87MG) and (blue) mouse PDGF-GBM cells and NPCs at 72hrs time-point. Data are expressed as mean ± SEM (n = 5 independent

experiments, each in triplicate). Vehicle: (A) 0.1% DMSO, (B) 0.1% ethanol, or (C) 0.1% PBS (D) Interactions between CBD and DNA-damaging agents on inhibiting viability (Method #1)**.** Percentage of synergy (white), additivity (grey), and antagonism (black) occurred in the checkerboard assay. Synergy, $\sum FIC < 0.5$; antagonism, $\sum FIC > 4$; additivity, $0.5 < \sum FIC < 4$. Data are expressed as percentage of occurrence calculated using the mean $\sum FIC$ from 3 independent experiments, each in duplicate. Vehicle: 0.05% DMSO (Vehicle in lieu of CBD) + 0.05% DMSO, ethanol or PBS (Vehicle in lieu of TMZ, BCNU or CDDP, respectively).

Fig. 4. Efficacy FIC analyses on interactive responses between CBD and DNA-damaging agents on GBM cells and NPCs (method #2). Efficacy FIC indices calculated from the combinations producing half maximal inhibitory effects on (A) proliferation and (B) viability when DNA-damaging agents were combined with CBD at fixed concentration of 1μM. Synergy (white), FIC index ($\sum FIC$) < 0.5; additivity (grey), $0.5 < \sum FIC < 4$; antagonism (black), $\sum FIC > 4$. Data are expressed as mean ± SEM (n = 3 independent experiments, each in duplicate). Vehicle: 0.05% DMSO (Vehicle in lieu of CBD) + 0.05% DMSO, ethanol or PBS (Vehicle in lieu of TMZ, BCNU or CDDP, respectively).

Table 1. Quantitative efficacy FIC analyses of the interactive responses between CBD and DNA-damaging agents on cell proliferation of GBM cells and NPCs (method #2). When DNA-damaging agents were combined with CBD at fixed concentration of 1 or 3 μM, IC_{50s} of the DNA-damaging agents and the efficacy FIC indices were calculated from the combinations producing half maximal inhibitory effects on inhibiting cell proliferation of human GBM cell lines, primary mouse PDGF-GBM and NPCs. Synergy, FIC index (∑FIC) < 0.5; additivity, 0.5 < ∑FIC < 4; antagonism, ∑FIC > 4. Data are expressed as mean ± SEM (n = 3 independent experiments, each in duplicate). Vehicle: 0.05% DMSO (Vehicle in lieu of CBD) + 0.05% DMSO, ethanol or PBS (Vehicle in lieu of TMZ, BCNU or CDDP, respectively).

		TMZ		BCNU		CDDP	
	CBD (M)	**IC_{50} (M)**	**FIC index**	**IC_{50} (M)**	**FIC index**	**IC_{50} (M)**	**FIC index**
Human cell lines							
T98G	1.00E-06	3.71E-03 ± 2.14E-03	3.46 ± 1.88	4.42E-04 ± 4.19E-05	1.13 ± 0.09	3.99E-05 ± 2.02E-05	1.78 ± 0.80
	3.00E-06	1.25E-03 ± 2.25E-04	1.69 ± 0.20	3.61E-04 ± 1.08E-04	1.35 ± 0.23	3.74E-05 ± 1.10E-05	2.08 ± 0.44
U251	1.00E-06	1.01E-03 ± 4.08E-07	1.17 ± 0.00	1.84E-04 ± 7.38E-05	1.01 ± 0.36	1.43E-05 ± 3.35E-06	2.23 ± 0.49
	3.00E-06	1.07E-03 ± 4.08E-06	1.47 ± 0.00	1.93E-04 ± 4.85E-05	1.29 ± 0.24	2.39E-05 ± 5.07E-06	3.89 ± 0.75
U87MG	1.00E-06	5.73E-04 ± 1.53E-04	1.10 ± 0.25	4.65E-04 ± 1.38E-04	0.86 ± 0.21	2.19E-06 ± 4.26E-07	1.08 ± 0.18

	3.00E-06	2.45E-04 ± 5.75E-05	0.86 ± 0.10	2.16E-04 ± 8.03E-05	0.78 ± 0.12	2.73E-06 ± 8.56E-07	1.62 ± 0.36
Mouse primary cells							
PDGF-GBM	1.00E-06	6.66E-04 ± 1.85E-04	1.59 ± 0.35	7.78E-05 ± 3.14E-05	1.40 ± 0.71	4.88E-06 ± 1.82E-06	2.24 ± 0.72
	3.00E-06	1.44E-04 ± 1.39E-05	1.23 ± 0.03	6.22E-05 ± 1.65E-05	2.25 ± 0.34	2.85E-06 ± 1.73E-06	2.08 ± 0.68
NPCs	1.00E-06	1.57E-03 ± 1.03E-03	0.66 ± 0.27	8.39E-05 ± 3.50E-05	2.78 ± 1.03	5.85E-06 ± 9.08E-07	2.17 ± 0.29
	3.00E-06	1.09E-05 ± 5.39E-07	1.00 ± 0.00	5.43E-05 ± 5.33E-05	2.57 ± 1.56	1.98E-06 ± 1.01E-06	1.60 ± 0.32

Table 2. Quantitative efficacy FIC analyses of the interactive responses between CBD and DNA-damaging agents on cell viability of GBM cells and NPCs (method #2). When DNA-damaging agents were combined with CBD at fixed concentration of 1 or 3 μM, IC_{50s} of the DNA-damaging agents and the efficacy FIC indices were calculated from the combinations producing half maximal inhibitory effects on inhibiting cell viability of human GBM cell lines, primary mouse PDGF-GBM and NPCs. Synergy, FIC index (∑FIC) < 0.5; additivity, 0.5 < ∑FIC < 4; antagonism, ∑FIC > 4. Data are expressed as mean ± SEM (n = 3 independent experiments, each in duplicate). **P* < 0.05, mouse PDGF-GBM cells versus mouse NPCs. Vehicle: 0.05% DMSO (Vehicle in lieu of CBD) + 0.05% DMSO, ethanol or PBS (Vehicle in lieu of TMZ, BCNU or CDDP, respectively).

		TMZ		BCNU		CDDP	
	CBD (M)	**IC50 (M)**	**FIC index**	**IC50 (M)**	**FIC index**	**IC50 (M)**	**FIC index**
Human cell lines							
T98G	1.00E-06	9.19E-04 ± 7.50E-05	0.76 ± 0.05	2.81E-04 ± 8.78E-05	1.10 ± 0.30	4.34E-05 ± 1.84E-05	5.73 ± 2.36
	3.00E-06	6.83E-04 ± 1.55E-04	0.90 ± 0.10	1.94E-04 ± 1.08E-04	1.09 ± 0.37	3.92E-05 ± 2.12E-05	5.48 ± 2.73
U251	1.00E-06	7.78E-04 ± 8.00E-05	1.86 ± 0.18	1.66E-04 ± 4.25E-05	1.26 ± 0.29	4.42E-05 ± 2.22E-05	4.24 ± 2.08
	3.00E-06	8.99E-04 ± 8.54E-07	2.34 ± 0.00	2.40E-04 ± 2.75E-05	1.99 ± 0.19	5.99E-05 ± 7.51E-06	5.93 ± 0.70
U87MG	1.00E-06	9.17E-04 ± 1.79E-04	1.16 ± 0.19	5.86E-04 ± 8.58E-05	1.34 ± 0.17	2.48E-04 ± 5.15E-05	3.76 ± 0.74

	3.00E-06	1.52E-03 ± 1.16E-03	2.19 ± 1.22	5.01E-04 ± 1.46E-04	1.57 ± 0.29	1.75E-04 ± 9.19E-06	3.11 ± 0.13
Mouse primary cells							
PDGF-GBM	1.00E-06	5.43E-04 ± 9.26E-05	10.78 ± 1.80*	1.78E-05 ± 7.04E-07	2.14 ± 0.07	2.05E-06 ± 3.97E-07	2.72 ± 0.48
	3.00E-06	1.20E-04 ± 6.86E-05	3.06 ± 1.33	1.47E-05 ± 4.44E-06	2.29 ± 0.47	3.14E-06 ± 5.68E-07	4.53 ± 0.69
NPCs	1.00E-06	1.24E-04 ± 4.55E-05	3.07 ± 1.01	3.29E-05 ± 1.81E-05	3.58 ± 1.79	8.32E-07 ± 1.56E-07	1.72 ± 0.26
	3.00E-06	4.99E-05 ± 4.84E-05	2.05 ± 1.08	8.71E-06 ± 4.49E-06	1.80 ± 0.45	6.00E-07 ± 2.74E-07	1.95 ± 0.46

Figure 1

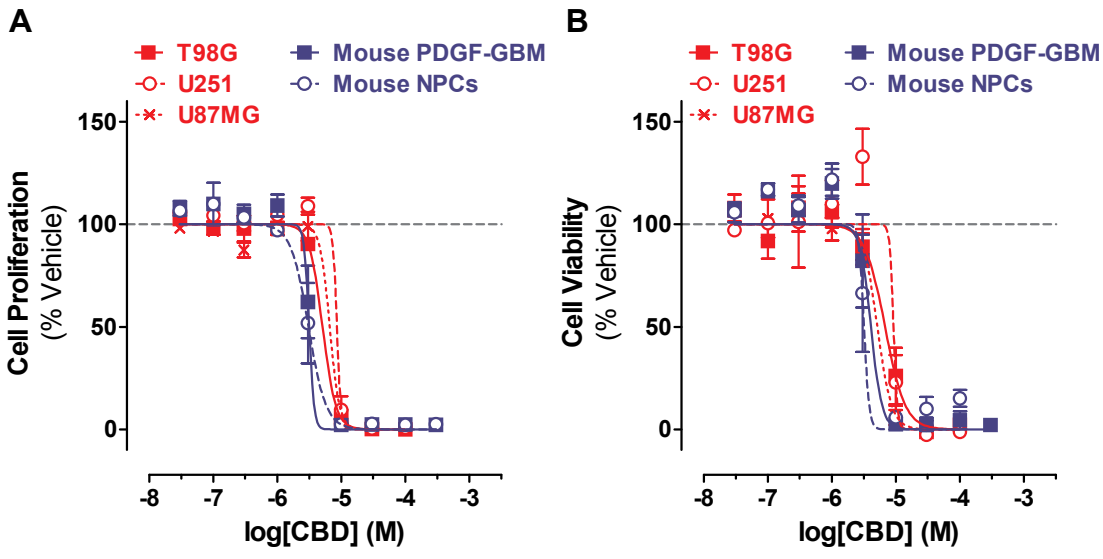

Figure 2

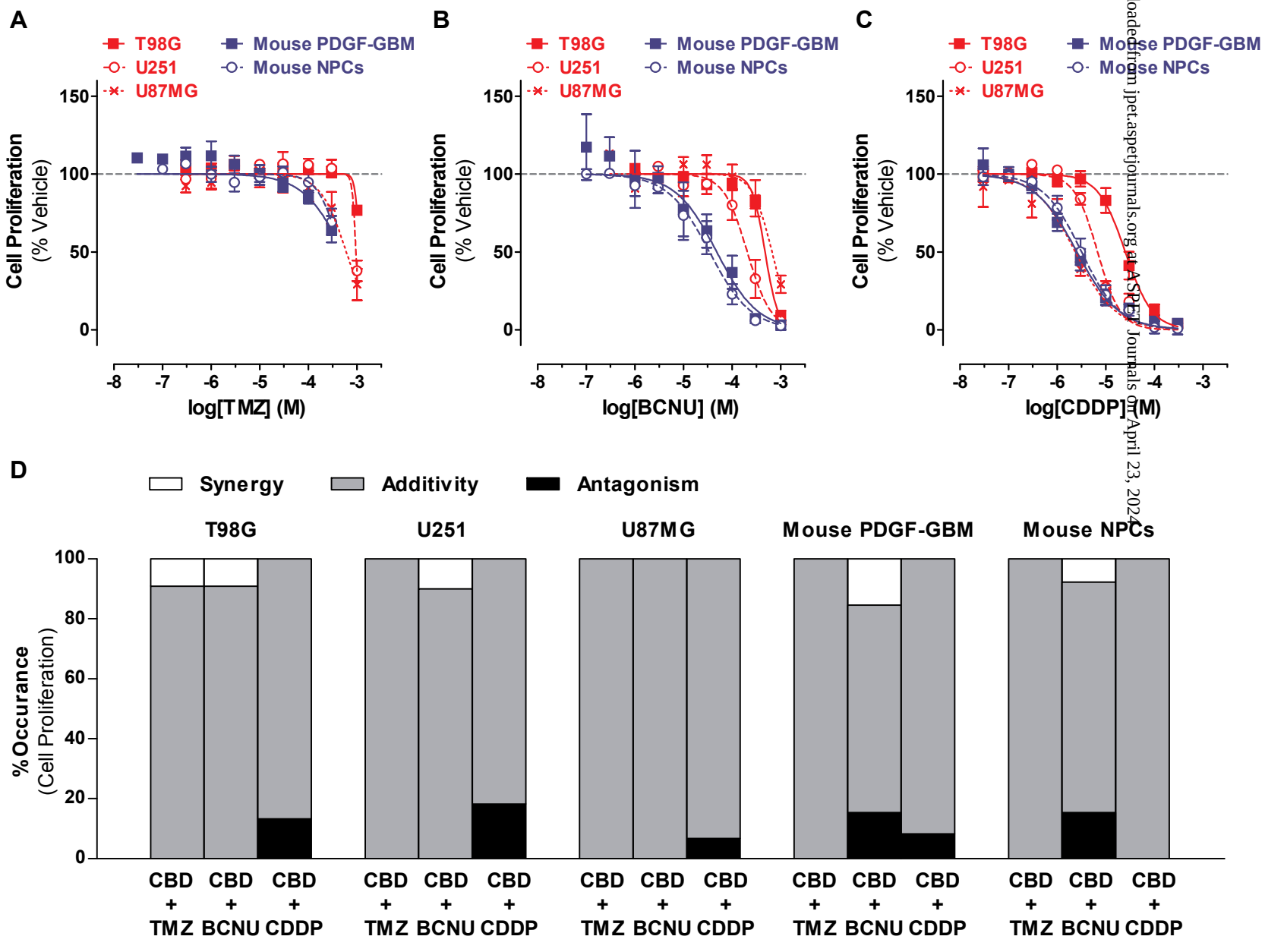

Figure 3

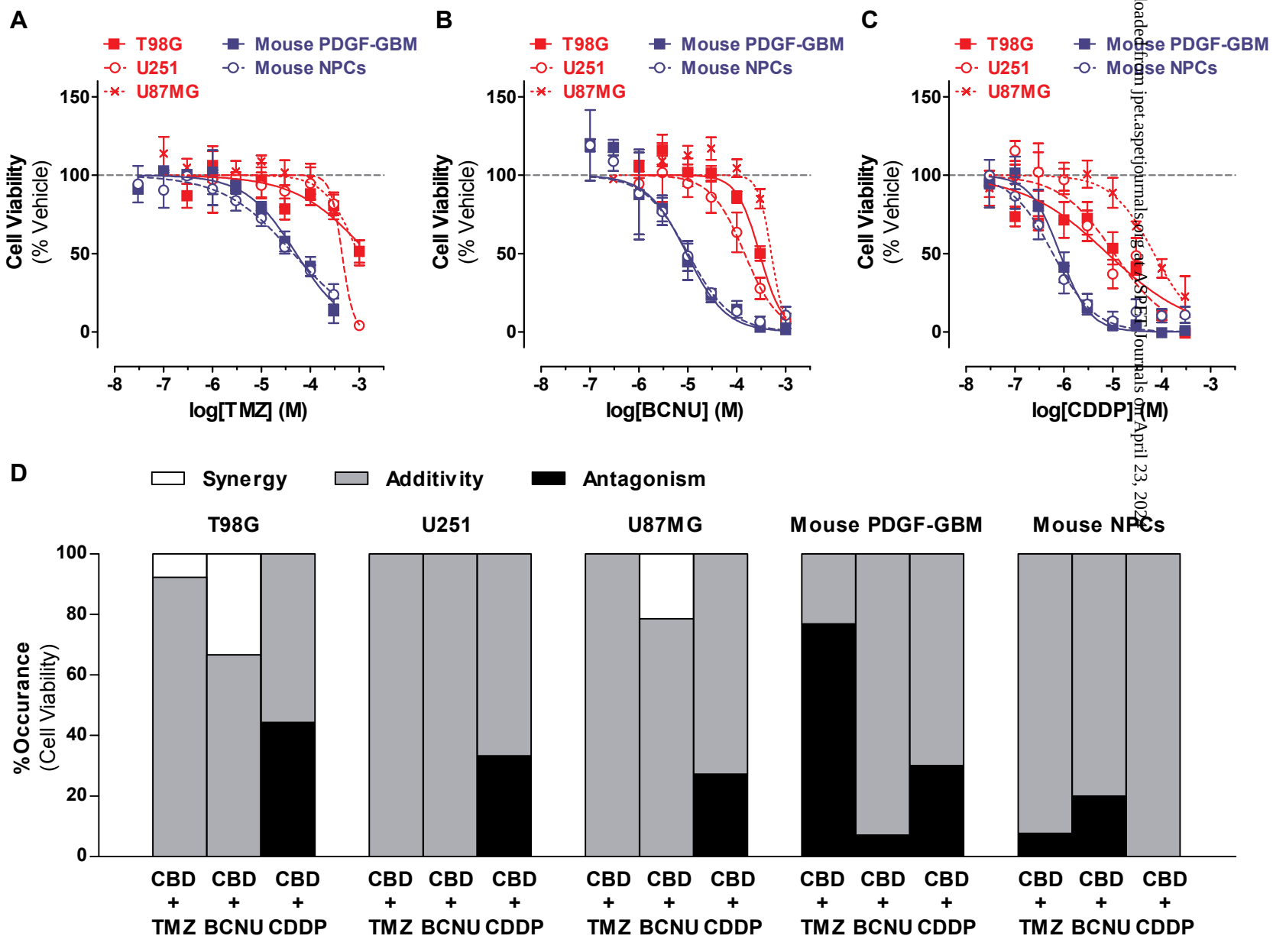

Figure 4