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

Liting Deng, Lindsay Ng, Tatsuya Ozawa and Nephi Stella*

LD, LN, and NS, Department of Pharmacology, Department of Psychiatry and Behavioral Sciences, University of Washington School of Medicine, Seattle, WA, USA; TO, Department of Neurosurgery and Alvord Brain Tumor Center, University of Washington, Division of Human Biology and Solid Tumor Translational Research, Fred Hutchinson Cancer Research Center, Seattle, WA, USA, current address: Division of Brain Tumor Translational Research, National Cancer Center Research Institute, Tokyo, Japan
a) Running title (51 characters): Cannabidiol and DNA-damaging agents on glioblastoma

b) Corresponding author:

Dr. Nephi Stella
HSC BB1504A
1959 NE Pacific St
Seattle, WA 98195-7280
Tel: 206-221-5220
Fax: 206-543-9520
E-mail: nstella@uw.edu

c) Number of text pages: 39 pages

  Number of tables: 2
  Number of figures: 4
  Number of references: 51
  Number of Supplementary Materials: 1
  Number of words in the Abstract: 211
  Number of words in the Introduction: 660
  Number of words in the Discussion: 1,276

d) Non-standard abbreviations

  ANOVA                analysis of variance
  Akt                  protein kinase B
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BCNU</td>
<td>carmustine</td>
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<tr>
<td>BrdU</td>
<td>5-Bromo-2’-deoxy-uridine</td>
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<tr>
<td>CBD</td>
<td>cannabidiol</td>
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<tr>
<td>CB₁</td>
<td>cannabinoid receptor 1</td>
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<td>CDDP</td>
<td>cisplatin</td>
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<td>CMT</td>
<td>combined modality therapy</td>
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<td>$\chi^2$ analysis</td>
<td>Chi-square analysis</td>
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<td>carbon dioxide</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
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<td>epidermal growth factor</td>
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<td>ERK</td>
<td>extracellular signal–regulated kinase</td>
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<td>fetal Bovine Serum</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FIC</td>
<td>fractional inhibitory concentration</td>
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<tr>
<td>GBM</td>
<td>glioblastoma multiforme</td>
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<tr>
<td>GPCRs</td>
<td>G protein–coupled receptors</td>
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<td>GPR55</td>
<td>G protein-coupled receptor 55</td>
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<td>HEK</td>
<td>Human Embryonic Kidney</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
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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

e) Recommended Section: Chemotherapy, Antibiotics, and Gene Therapy
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
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 µg/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 ºC and 5 % CO$_2$). Cells at passages of 4-6 were used in the following experiment.
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
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:

\[
\text{FIC (A)} = \frac{C_{A+B}}{C_A}
\]

\[
\text{FIC (B)} = \frac{C_{B+A}}{C_B}
\]

\[
\sum \text{FIC} = \text{FIC (A)} + \text{FIC (B)}
\]

\(C_{A+B}\) 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 \text{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...
GraphPad Prism. FIC index ($\sum$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 $\sum$FIC < 0.5, antagonism was defined as $\sum$FIC > 4, and additivity was defined when $\sum$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:

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

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

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

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 $\sum$FIC < 0.5, antagonism was defined as $\sum$FIC > 4, and additivity was defined when $\sum$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 ($\chi^2$) 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\textsubscript{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\textsubscript{50s}: 3.1 - 8.5 µM and cell viability IC\textsubscript{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.
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$_{50}$s: 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$_{50}$s: 2.4 - 3.2 µM) than two of the human glioma cell lines T98G and U251 (IC$_{50}$s: 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 CB₁ and CB₂ 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\textsubscript{2} receptors in leukemia cells (McKallip et al., 2006) and 10 µM CBD suppresses glioma cell proliferation and this response is partially blocked by a CB\textsubscript{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 \textit{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\textsubscript{1A}) receptor with an \textit{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-\gamma (PPAR\gamma) (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 CB₁ 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, CB₁ 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.

Acknowledgments

The authors wish to thank Dr. Eric C. Holland (University of Washington, Fred Hutchinson Cancer Research Center) for helpful discussions and providing expert inputs on the manuscript.

Authorship Contributions

Participated in research design: Deng and Stella

Conducted experiments: Deng, Ng, and Ozawa

Performed data analysis: Deng

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.


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


Footnotes

This work was supported by National Institute of Health National Institute on Drug Abuse [Grants DA026430]. All authors state no conflict of interests.
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, $\Sigma FIC < 0.5$; antagonism, $\Sigma FIC > 4$; additivity, $0.5 < \Sigma FIC < 4$. Data are expressed as percentage of occurrence calculated using the mean $\Sigma FIC$ 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_{50} 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 (ΣFIC) < 0.5; additivity, 0.5 < ΣFIC < 4; antagonism, ΣFIC > 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).

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**Mouse primary cells**

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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, IC₅₀s 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).

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|                | 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-06 ± 2.74E-07 | 1.95 ± 0.46 |
Figure 1

A

Cell Proliferation (% Vehicle)

log[CBD] (M)

B

Cell Viability (% Vehicle)

log[CBD] (M)

-8 -7 -6 -5 -4 -3

T98G - Mouse PDGF-GBM
U251 - Mouse NPCs
U87MG

-8 -7 -6 -5 -4 -3

T98G - Mouse PDGF-GBM
U251 - Mouse NPCs
U87MG
Figure 3

A

B

C

D

Synergy

Additivity

Antagonism

T98G

U251

U87MG

Mouse PDGF-GBM

Mouse NPCss

Cell Viability (% Vehicle)

log[TMZ] (M)

log[BCNU] (M)

log[CDDP] (M)

% Occurrence (Cell Viability)

CBD + TMZ

CBD + BCNU

CBD + CDDP

CBD + TMZ

CBD + BCNU

CBD + CDDP

CBD + TMZ

CBD + BCNU

CBD + CDDP

CBD + TMZ

CBD + BCNU

CBD + CDDP

CBD + TMZ

CBD + BCNU

CBD + CDDP