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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Supplementary Materials

Materials and Methods

Example of a work through of the equation for method 1 and 2

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).

Here is an example for the calculation of the equation for method 1 and 2.

■ 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:

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

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

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

For example, for a CBD (0.000001 M) and BCNU (0.00003 M) combination on PDGF-GBM cell proliferation produces the effect of 54.28% of the Vehicle group.

$C_{BCNU+CBD}$ is the concentration of BCNU in the combination which is 0.00003 M;

C_{BCNU} is the concentration of BCNU alone when producing the same effect as in the combination (54.28%). This value is determined by interpolating the unknown x value (BCNU concentration) when y value is set as 54.28% from the BCNU dose response curve on PDGF-GBM cell proliferation using GraphPad Prism. C_{BCNU} is 0.0000407 M when it produces 54.28% effect alone;

$FIC_{BCNU} = C_{BCNU+CBD} / C_{BCNU} = 0.00003\ M / 0.0000407\ M = 0.737$

$C_{CBD+BCNU}$ is the concentration of CBD in the combination, which is 0.000001 M;

C_{CBD} is the concentration of CBD alone when producing the same effect as in the combination (54.28%). This value is determined by interpolating the unknown x value (CBD concentration) when y value is set as 54.28% from the CBD dose response curve on PDGF-GBM cell proliferation using GraphPad Prism. C_{CBD} is 0.00000309 M when it produce 54.28% effect alone;

$FIC_{CBD} = C_{CBD+BCNU} / C_{CBD}$ = 0.000001 M / 0.00000309 M = 0.324

Thus, for this combination, $\sum FIC = FIC_{BCNU} + FIC_{CBD}$ = 0.737 + 0.324 = 1.061, which is in the range of 0.5 to 4 counting as additivity.

After calculation of the FIC indices from all the combinations on the checkerboard assay, 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.

■ 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:

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

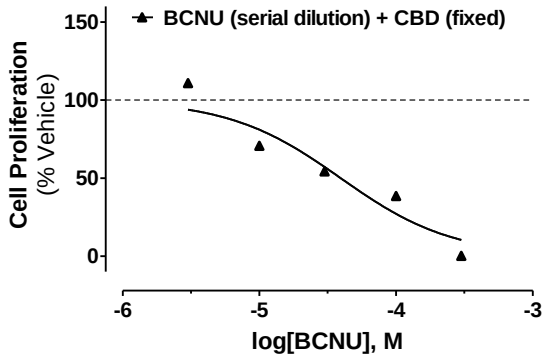
$FIC\ (B) = C_{B\_fixed} / IC_{50B}$

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

For example, in one column of the checkerboard of the CBD/BCNU combination on PDGF-GBM cell proliferation where the CBD concentration is fixed at 0.000001 M, the result obtained from the experiment is as follow:

CBD fixed at 0.000001 M	
BCNU (M)	**Cell proliferation (% Vehicle)**
0.000003	110.8722
0.00001	70.66937
0.00003	54.27992
0.0001	38.53955
0.0003	0.121704

We first plotted the dose response curve of the inhibitory effects of the combinations (containing a serial half log10 dilution of BCNU in the presence of the fixed concentration of CBD at 0.000001 M) against the concentration of BCNU used in the combinations.



The IC_{50} of BCNU (in the presence of CBD at 0.000001 M) was then calculated using GraphPad Prism, and the result is 0.00003931 M. Therefore, the combination of CBD (at the fixed concentration of 0.000001 M) and the IC_{50} of BCNU (0.00003931 M) produce a half maximal inhibition. Thus, $IC_{50\_BCNU+CBD}$ is 0.00003931 M. IC_{50_BCNU} is the half maximal inhibitory concentration of BCNU alone on the PDGF-GBM cell proliferation, which is 0.0000481 M.

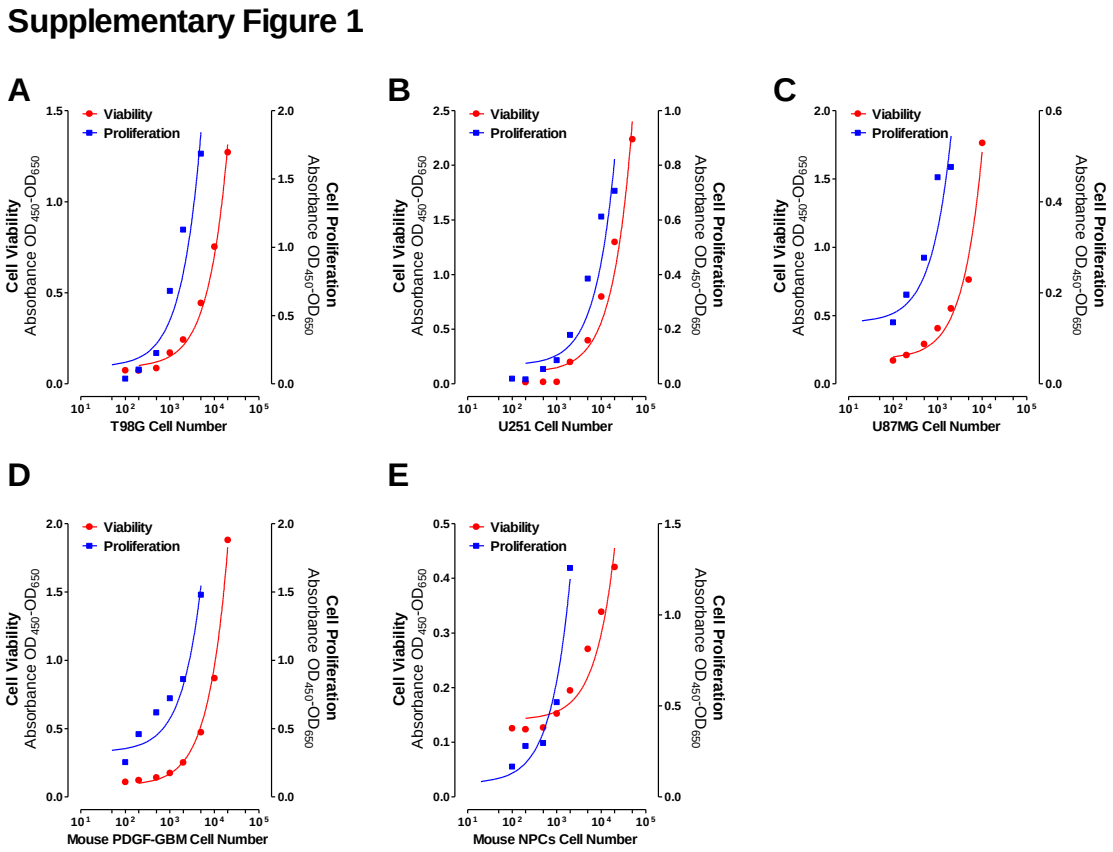
$FIC_{BCNU} = IC_{50\_BCNU+CBD} / IC_{50\_BCNU} = 0.00003931\ M / 0.0000481\ M = 0.817$

Since the concentration of CBD in all the combinations in this column of the checkerboard assay is fixed at 0.000001 M, thus C_{CBD_fixed} is 0.000001 M. IC_{50_CBD} is the half maximal inhibitory concentration of CBD alone on the PDGF-GBM cell proliferation, which is 0.00000314 M.

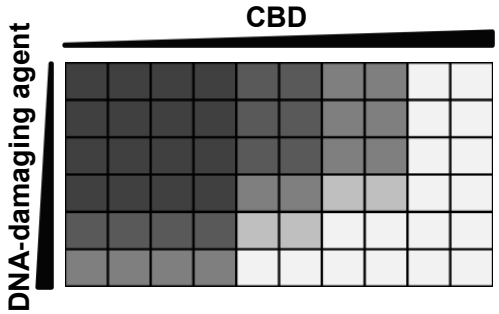
$FIC_{CBD} = C_{CBD\_fixed} / IC_{50\_CBD} = 0.000001\ M / 0.00000314\ M = 0.318$

Thus, for this column, $\sum FIC = FIC_{BCNU} + FIC_{CBD} = 0.817 + 0.318 = 1.135$, which is in the range of 0.5 to 4. Thus, the FIC index based on the combinations that produced half maximal inhibition of this column suggests additivity.

Supplementary Fig. 1: Optimization of the cell number for the viability and proliferation assay for each glioma cell type tested over 72 hrs time period. The viability (wst-1) and proliferation (BrdU) assays show high correlation to the cell number of (A) T98G, (B) U251, (C) U87MG, (D) primary mouse PDGF-GBM cells, and (E) control primary mouse NPCs.



Supplementary Fig. 2: Illustration of checkerboard assay. The interaction between CBD and DNA-damaging agents was tested using the checkerboard assay. Thus, serial half log_{10} dilutions of CBD (0 and 1 -100 µM) were combined with TMZ (0 and 1 µM - 1mM), BCNU (0 and 3 µM - 1mM) or CDDP (0 and 0.1 - 100 µM). All combinations were performed in duplicate and repeated three times.



Supplementary Table 1: Half maximal inhibitory concentration (IC_{50}, M) of CBD, TMZ, BCNU and CDDP on cell proliferation and viability. Data are expressed as IC_{50s} of best-fit values generated from the dose response curves by GraphPad Prism (n = 5-9 per experiment).

Abbreviations: CBD, cannabidiol; TMZ, temozolomide; BCNU, carmustine; CDDP, cisplatin.

	CBD	TMZ	BCNU	Cisplatin
Proliferation				
Human cell lines				
T98G	5.08E-06	1.14E-03	4.75E-04	2.52E-05
U251	8.54E-06	9.56E-04	2.05E-04	6.76E-06
U87MG	6.59E-06	6.04E-04	6.61E-04	2.35E-06
Mouse primary cells				
PDGF-GBM	3.14E-06	5.26E-04	4.81E-05	2.54E-06
NPCs	3.07E-06	4.57E-04	3.41E-05	3.17E-06
Viability				
Human cell lines				
T98G	6.75E-06	1.51E-03	2.93E-04	7.78E-06
U251	9.21E-06	4.45E-04	1.45E-04	1.07E-05
U87MG	5.13E-06	9.51E-04	5.10E-04	6.94E-05
Mouse primary cells				
PDGF-GBM	4.08E-06	5.15E-05	9.41E-06	8.29E-07
NPCs	3.19E-06	4.49E-05	1.01E-05	5.93E-07

Supplementary Table 2: Hillslopes of the dose response curves of CBD, TMZ, BCNU and CDDP on cell proliferation and viability. Data are expressed as hillslope of best-fit values generated from the dose response curves by GraphPad Prism (n = 5-9 per experiment). Abbreviations: CBD, cannabidiol; TMZ, temozolomide; BCNU, carmustine; CDDP, cisplatin.

	CBD	TMZ	BCNU	Cisplatin
Proliferation				
Human cell lines				
T98G	-4.23	-3.25	-1.56	-9.13
U251	-14.30	-1.79	-1.85	-11.14
U87MG	-5.76	-2.16	-1.05	-1.62
Mouse primary cells				
PDGF-GBM	-11.26	-1.03	-1.00	-1.04
NPCs	-3.18	-1.01	-1.10	-1.93
Viability				
Human cell lines				
T98G	-2.75	-1.86	-0.50	-0.60
U251	-14.90	-1.26	-0.79	-3.74
U87MG	-3.90	-3.31	-1.00	-1.29
Mouse primary cells				
PDGF-GBM	-5.04	-1.08	-1.41	-0.84
NPCs	-11.05	-0.98	-1.02	-0.60

Supplementary Table 3: Maximal efficacies of CBD, TMZ, BCNU and CDDP on cell proliferation and viability. Data are expressed as mean ± SEM of maximal % inhibition (at minimal maximal inhibitory concentrations) (n = 5-9 per experiment). Abbreviations: CBD, cannabidiol; TMZ, temozolomide; BCNU, carmustine; CDDP, cisplatin.

	CBD	TMZ	BCNU	Cisplatin
Proliferation				
Human cell lines				
T98G	99.93 ± 0.25 (30 µM)	23.26 ± 2.39 (1 mM)	92.27 ± 4.35 (1 mM)	96.14 ± 0.69 (300 µM)
U251	99.66 ± 0.31 (30 µM)	62.20 ± 6.56 (1 mM)	94.27 ± 2.23 (1 mM)	97.93 ± 0.42 (100 µM)
U87MG	97.47 ± 1.35 (30 µM)	56.11 ± 10.20 (1 mM)	63.86 ± 5.62 (1 mM)	97.76 ± 0.14 (300 µM)
Mouse primary cells				
PDGF-GBM	97.92 ± 1.04 (10 µM)	35.34 ± 8.50 (300 µM)	92.84 ± 2.39 (300 µM)	94.52 ± 1.55 (100 µM)
NPCs	97.75 ± 0.66 (10 µM)	30.77 ± 8.43 (300 µM)	94.15 ± 2.45 (300 µM)	98.48 ± 3.97 (100 µM)
Viability				
Human cell lines				
T98G	100.00 ± 0.44 (30 µM)	48.66 ± 7.15 (1 mM)	92.06 ± 2.56 (1 mM)	100.00 ± 0.55 (300 µM)
U251	100.00 ± 1.41 (30 µM)	96.03 ± 0.29 (1 mM)	93.04 ± 4.83 (1 mM)	90.34 ± 2.82 (100 µM)
U87MG	100.00 ± 0.80 (30 µM)	52.31 ± 7.98 (1 mM)	90.05 ± 1.68 (1 mM)	77.48 ± 13.09 (300 µM)
Mouse primary cells				
PDGF-GBM	97.50 ± 0.93 (10 µM)	86.54 ± 7.94 (300 µM)	97.04 ± 4.54 (300 µM)	94.69 ± 3.20 (10 µM)
NPCs	94.19 ± 2.59 (10 µM)	76.12 ± 6.54 (300 µM)	93.47 ± 3.26 (300 µM)	93.04 ± 5.91 (10 µM)