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Preclinical study

Synergistic cytotoxicity of topoisomerase I inhibitors with alkylating agents and etoposide in human brain tumor cell lines

Anna J Janss, Avital Cnaan,[1] Huaqing Zhao,[1] Arkady Shpilsky,[1] Cindy Levow, Leslie Sutton[2] and Peter C Phillips

Divisions of Neurology and Oncology, [1]Division of Biostatistics, and [2]Division of Neurosurgery, Children's Hospital of Philadelphia, 34th and Civic Center Boulevard, Philadelphia, PA 19104, USA. Tel: (+1) 215 590-5170; Fax: (+1) 215 590-3709.

To evaluate potential synergistic interactions between topoisomerase I (Topo I) inhibitors, i.e. camptothecin (CPT) and topotecan (TPT), and chemotherapeutic agents known to be active in treatment of brain tumors, *in vitro* studies were conducted with human glioma and medulloblastoma cell lines. Tumor cells were exposed to CPT or TPT alone or in combination with cisplatin, 4-hydroperoxycyclophosphamide (4-HC), BCNU or etoposide (VP-16). Cytotoxicity was assessed by colony formation assays. Drug interactions were evaluated by means of a novel analytical model which permits statistical evaluation over a range of dose combination. Experimental results were corroborated by published models of drug interaction. Our findings indicate that *in vitro* cytotoxic interactions in brain tumor cells between Topo I inhibitors and alkylating agents or etoposide depend on drug dose, dose schedule and tumor cell line. Treatment of DAOY medulloblastoma cells with CPT and either cisplatin, 4-HC or VP-16 produced significant synergistic cytotoxicity over a wide range of dose combinations. When VP-16 was administered after CPT, synergy was reduced to a narrow range of dose combinations. For U251 glioma cells, incubation with CPT and cisplatin or 4HC also produced synergistic cytotoxicity over a broad range of dose combinations. By contrast, antagonistic interactions were observed after administration of CPT with BCNU or VP-16. Treatment of U251 cells with CPT and cisplatin produced synergistic or antagonistic cytotoxicity depending on the dose combination used. These findings support a role for pharmacokinetic analysis in multiagent phase II trials involving Topo I inhibitors and have important implications for clinical trial design strategies. [© 1998 Lippincott Williams & Wilkins.]

Key words: Brain tumor cell lines, camptothecin, cisplatin, etoposide, synergy, Topoisomerase I inhibitor.

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Correspondence to AJ Janss

Introduction

Topoisomerase I (Topo I) inhibitors represent an important class of antineoplastic agents with encouraging therapeutic profiles in preclinical and clinical studies. These drugs bind non-covalently to Topo I, stabilize the Topo I-DNA complex[1,2] and prevent repair of DNA single-strand breaks caused by Topo I. This action leads to an accumulation of DNA double-strand breaks and eventual cell death.[3] The clinical utility of camptothecin (CPT), the parent compound of this class of antineoplastic agents, was first documented in gastric cancer.[4] Development of new CPT analogs including topotecan (TPT), CPT-11 and others stimulated further laboratory and clinical investigations of this class of agents. Topotecan has shown clinical activity against a variety of solid tumors in adults including lung carcinoma, platinum-resistant ovarian cancer, and extensive stage small cell lung cancer in phase I and II clinical trials.[5–12] Phase II trials for recurrent solid tumor in children and are currrently ongoing (Children's Cancer Group; CCG-0952).[13,14]

Topo I inhibitors are attractive candidates for inclusion in brain tumor clinical trials because many are lipophilic, thereby facilitating delivery of drug across the blood-brain barrier.[15] They have a unique mechanism of cytotoxicity, are unlikely to compete with the activity of currently available agents[16] and they do not activate the multi-drug resistance complex.[17] Furthermore, preclinical studies in xenograft models indicate that Topo I inhibitors are extremely active against malignant gliomas and medulloblastomas.[18,19] *In vitro* studies report that Topo I inhibitors have synergistic cytotoxicity when combined with therapies known to be effective for brain tumor treatment, including radiation[20–24] and chemotherapy

with cisplatin or alkylating agents.[25-28] To identify combinations of Topo I inhibitors and chemotherapeutic agents with synergistic cytotoxicity for brain tumors, we conducted *in vitro* studies to quantify the cytotoxicity of CPT or TPT in combination with cisplatin, 4-hydroperoxycyclophosphamide (4-HC), BCNU or etoposide (VP-16) by use of clonogenic assays in human medulloblastoma and glioma cell lines. The agents tested with CPT or TPT in these cytotoxicity studies were chosen because of their well documented efficacy against human brain tumors.[3,19,29]

Investigation of drug combinations with synergistic cytotoxicity allows non-emperic development of multidrug regimens. However, identification of synergistic interactions may be confounded by a variety of factors including non-linear dose effects, dose schedule[27,30,31] or the mathematical models used to evaluate drug interactions.[32] Accordingly, we evaluated a range of drug-dose combinations and examined the effect of dose order on drug interaction for CPT and etoposide in the medulloblastoma cell line since this combination produced a substantial synergistic cytotoxicity. In this study, synergistic (i.e. supra-additive) cytotoxic effects were identified by use of a novel analytical model which, unlike several other models,[32-34] permits statistical analysis of the results over a range of dose combinations. We found significant synergistic interactions with combinations of CPT and cisplatin, 4-HC or VP-16. These interactions varied with the doses of drugs combined, the order of drug presentation and the cell line examined.

Methods and materials

Tumor cell lines

Two human brain tumor cell lines were used. DAOY medulloblastoma[35] and U251 malignant glioma were obtained from ATCC (Rockville, MD) Cells were maintained in Richter's zinc option medium (Gibco/BRL, Gaithersberg, MD) with 10% fetal calf serum (Sigma, St Louis, MO) and 50 000 units streptomycin per liter of medium. Cells were grown in monolayers at 37°C, at 5% CO_2 in 80–100% humidity and harvested as previously described.[19]

Drugs

TPT and CPT were obtained from SmithKline Beecham (Philadelphia, PA). VP-16 was purchased from Bristol-Myers Squibb (New York, NY), cisplatin and BCNU from Sigma, and 4-HC, which is the active metabolite of the pro-drug cyclophosphamide, was provided by Nova Pharmaceutical (Baltimore, MD). CPT, TPT and VP-16 were stored as 5 mM solutions in DMSO, and cisplatin as 5 mM solution in sterile water at −20°C. BCNU was dissolved in 100% ethanol and 4-HC in sterile water immediately prior to use. Dilutions of all drugs were prepared in zinc option medium immediately prior to use.

Drug treatment

Subconfluent cultures were trypsinized and plated as 400 cells per 2 ml of zinc option medium with 20% fetal calf serum in 3 mm plastic culture plates, as previously described.[19] Plates were incubated overnight to permit adherence of cells. For single-agent experiments, cells were exposed to serum-free medium (controls) or appropriate concentrations of CPT, TPT, cisplatin, 4-HC, VP-16 or BCNU for 1 h and then washed twice. Fresh zinc option medium with 20% fetal calf serum was added and the plates returned to the incubator. After 7 days, cells were stained with Coomassie blue and the colonies counted.

Drug combination experiments were carried out in a similar fashion. Cells were plated at 400 cells per dish and allowed to adhere. After 24 h they were exposed to either the medium alone or different concentrations of a combination of two drugs (e.g. 1 μM CPT + 10 μM cisplatin) for 1 h, then washed with Hank's solution, covered with fresh medium and returned to the incubator for 1 week. Cells were then stained and colonies counted. To evaluate the order of drug exposure cells were plated and allowed to adhere as described above. The cells were exposed to different concentrations of CPT for 1 h, rinsed twice with Hank's solution and then exposed to different concentrations of VP-16 for 1 h. The second drug was then rinsed from the plate with Hank's solution, and the plates incubated for 1 week, stained and the colonies counted as described above. These experiments were repeated reversing the order of drug exposure.

Experimental design

In each experiment, plating efficiency (control) was evaluated by treating six plates of cells with serum-free media. For each experiment, single drug or drug combination doses were tested on three plates. The effect of drug (or combination of drugs) at a given concentration(s) was computed as a survival fraction,

i.e. mean colony count for the three replicate plates treated with drug(s) divided by the mean colony count for six replicate plates treated with media. Each experiment was repeated in triplicate. Fractional cell kill was defined as 1.0 − fraction survival. Dose-response curves were plotted as mean fractional cell kill versus log dose. The following drug pairs were evaluated: in the DAOY medulloblastoma cell line, CPT + 4-HC, CPT + cisplatin, TPT + cisplatin and CPT + VP-16; in the U251 glioma cell line, CPT + BCNU, CPT + 4-HC, CPT + cisplatin, TPT + cisplatin and CPT + VP-16.

Analysis of drug interaction

The additivity model developed for this study defines additive cytotoxicity of a drug-dose combination as the sum of cell kill for each agent tested as a single drug, with a 90% confidence interval (CI) for this sum. Antagonistic cytotoxicity is defined as a drug interaction which caused less cytotoxicity than the sum of each agent used alone (i.e. less than an additivity). Synergy is identified when the effect of drugs combined is greater than the sum of the cytotoxicity for each agent used alone (i.e. greater than additivity). To test for synergistic cytotoxicity, the expected values derived from this model were compared to the combination results observed experimentally.

The ability to define a range of values for expected additive cytotoxicity of a pair of drugs at given doses distinguishes this model from others used to analyze drug interactions.[33,36] The treatment effect for each agent at a given dose was identified by means of single-agent dose-effect curves.[37,38] The cytotoxicity of single agents was further characterized by fitting dose-response curves to a linear model by means of regression analysis with transformations of the response (i.e. fractional cell kill) and the log of the dose. These transformations permit the calculation of 95% CI for single-agent dose-response curves and subsequent calculation of a 90% CI for the expected additive cytotoxicity. Transformation of dose-response curves to linear functions is a standard procedure in mathematical models of drug interactions.[33,36,39] Data for analysis of single-agent cytotoxicity included all doses up to the lowest dose producing 100% effect (fractional cell kill=1.0). ID_{50} and ID_{90} were calculated from fitted dose-response curves.

The results from data analysis using this model were compared with results using two published methods for analysis of drug interaction: isobolographic analysis at the 50% clonal survival (corresponding to 50% inhibition of colony formation)[25,33] and a method to estimate an additive drug interaction based on the linear-log dose-response curves of the single-agent experiments.[33] Isobologram additivity lines were constructed using the single-agent experiments to estimate the dose of each agent causing 50% cell kill.[33,36] Combination response curves (curvilinear isobols) were constructed from dose combinations which produced 50% cell kill experimentally and interpolation to estimate dose combinations not tested. The second method used for comparison was a strategy reported by Berenbaum[33] which computes the expected additive drug effect based on the linear-log dose-response curves of single agents in fixed drug combinations:

$$E(d) = \alpha + \beta \log d$$

where E is the expected drug effect, d is the drug tested, α is a constant derived from the linear portion of the dose-response curve and β is the slope of the curve when the log of drug effect is plotted against dose.

Results

Single agent cytotoxicity

One hour exposure of U251 glioblastoma or DAOY medulloblastoma cells to CPT, TPT, cisplatin, 4-HC and VP-16 produced a dose-dependent increase in cytotoxicity. The r^2 for the linearized functions for the single drug experiments ranged between 0.867 and 0.975. The ID_{50} (dose inhibiting 50% of colony formation) and ID_{90} (dose inhibiting 90% of colony formation) for each drug and each cell line are shown in Table 1. The dose of CPT resulting in maximum cell

Table 1. ID_{50} and ID_{90} for DAOY medulloblastoma and U251 glioma cells of each of the chemotherapeutic agents used in this study.

Cell line	Cytotoxic agent	ID_{50} (μM)	ID_{90} (μM)
DAOY	CPT	0.41	1.29
	TPT	0.36	3.46
	VP-16	4.97	9.08
	cisplatin	4.38	9.52
	4-HC	8.81	24.62
U251	CPT	0.17	0.94
	TPT	0.18	1.15
	VP-16	7.80	16.72
	cisplatin	9.98	25.48
	4-HC	12.35	18.93
	BCNU	10.18	29.50

kill was 8.28 μg in U251 glioma and 2.77 μg in DAOY medulloblastoma cells. The dose of TPT resulting in maximum cell kill was 24.3 μg in U251 glioma and 8.1 μg in DAOY medulloblastoma cells.

Drug combination cytotoxicity

Drug treatment combinations with U251 glioma and DAOY medulloblastoma cells, analyzed by the additivity model, show that the direction and degree of drug interactions (i.e. synergy, antagonism or additivity) is multifactorial and determined by drug-dose combinations and the treatment schedule. For U251 malignant glioma cells drug interactions ranged from significant antagonistic cytotoxicity to significant synergy. By contrast, DAOY medulloblastoma cell responses ranged from additivity to synergy. These results are summarized in Tables 2 and 3.

DAOY medulloblastoma cell line

Topo I inhibitors + alkylating agents. In DAOY medulloblastoma cells, CPT at 50 nM produced significant synergistic cytotoxicity with cisplatin over a range of doses easily achievable in the clinical setting (see Figure 1A). At higher doses of CPT the interaction was best characterized as additive. The interaction between CPT and 4-HC was comparable to that observed with cisplatin, i.e. synergism over a broad range of doses. In contrast, TPT showed synergistic cytotoxicity with cisplatin at only one dose combination (Figure 1B).

Topo I inhibitors + Topo II inhibitors. CPT produced synergistic cytotoxicity with VP-16 over a broad range of dose combinations (Figure 2). Treatment with CPT after VP16 did not alter the synergistic interaction and treatment with CPT before VP16

Table 2. Interaction of Topo I inhibitors and alkylating agent or VP-16 in DAOY medulloblastoma cell line

Cytotoxic agents		Significant synergy		Additivity	
Agent 1 (Topo I inhibitor)	Agent 2	Dose Topo I inhibitor (nM)	Dose range agent 2 (nM)	Dose Topo I inhibitor (nM)	Dose range agent 2 (nM)
CPT +	cisplatin	50	1000–5000	100	1000–2500
				250	1000–2500
TPT +	cisplatin	250	1000	100	1000–5000
				250	2500
				750	1000–2500
CPT +	4HC	50	1000–2500	50	5000–10000
				100	1000–5000
				250	1000–2500
CPT +	VP-16	50	500–2500	50	5000–10000
		100	500–2500	100	5000
				250	500–2500
				500	500–1000
CPT ←	VP-16	50	500–2500	50	5000–7500
		100	500–2500	100	5000
				250	500–2500
				500	500–1000
CPT →	VP-16	50	500–2500	50	5000–7500
		100	500–1000	100	2500–5000
				250	500–2500

The cytotoxic agents in a given combination treatment are listed in the left-most column. In most cases the agents were administered simultaneously, indicated by the plus sign. VP-16 and CPT were administered serially as well as simultaneously, and the order of administration is indicated in the left-most column by the arrow. The doses listed in the second column ('Significant synergy') are the doses of the two drugs in a given combination that resulted in cytotoxicity (average DAOY cell kill for three separate trials) greater than the upper 90% CI of the cell kill predicted by an additive drug interaction (see Materials and methods). The doses listed in the third column ('Additivity') are the doses of the two drugs in given combination that resulted in cytotoxicity (average DAOY cell kill for three separate trials) within the 90% CI of the cell kill predicted by an additive drug interaction. No dose combination in any of the drug combinations tested on the DAOY medulloblastoma cell line produced significant antagonistic cytotoxicity.

Table 3. Interaction of Topo I inhibitors and VP-16 or an alkylating agent in U251 glioma cell line

Cytotoxic agents		Significant synergy		Additivity		Significant antagonism	
Agent 1 (Topo I inhibitor)	Agent 2	Dose Topo I inhibitor (nM)	Dose range agent 2 (nM)	Dose Topo I inhibitor (nM)	Dose range agent 2 (nM)	Dose Topo I inhibitor (nM)	Dose range agent 2 (nM)
CPT +	cisplatin	25 50	500–10000 500–5000	50 250	10000 500–5000	1000	500–2500
TPT +	cisplatin			50 500 5000	5000–10000 50–5000 2500	5000	500–1000
CPT +	4-HC	25	1000–5000	25 250 2500	10000 1000–5000 1000–2500		
CPT +	BCNU			25 100	1000 1000	25 100 1500	5000–15000 5000–10000 1000
CPT +	VP16		100–1000	25 100	1000–10000 2500–5000	100 1500	10000 1000

The cytotoxic agents in a given combination treatment are listed in the left-most column. All agents listed in this table were administered simultaneously. Doses listed in the second column ('Significant synergy') are the doses of the two drugs in a given combination that resulted in cytotoxicity (average U251 cell kill for three seperate trials) greated than the upper 90% CI of the cell kill predicted by an additive drug interaction (see Materials and methods) on three separate trials. Doses listed in the third column ('additivity') are the doses of the two drugs given in combination that resulted in cytotoxicity (average U251 cell kill for three separate trials) within the 90% CI of the cell kill predicted by an additive drug interaction. Doses listed in the fourth column ('Significant antagonism') are the doses of the two agenst that given in combination produced cytotoxicity (average U251 cell kill for three separate trials) less than the lower 90% CI for the cell kill predicted by an additive drug interaction.

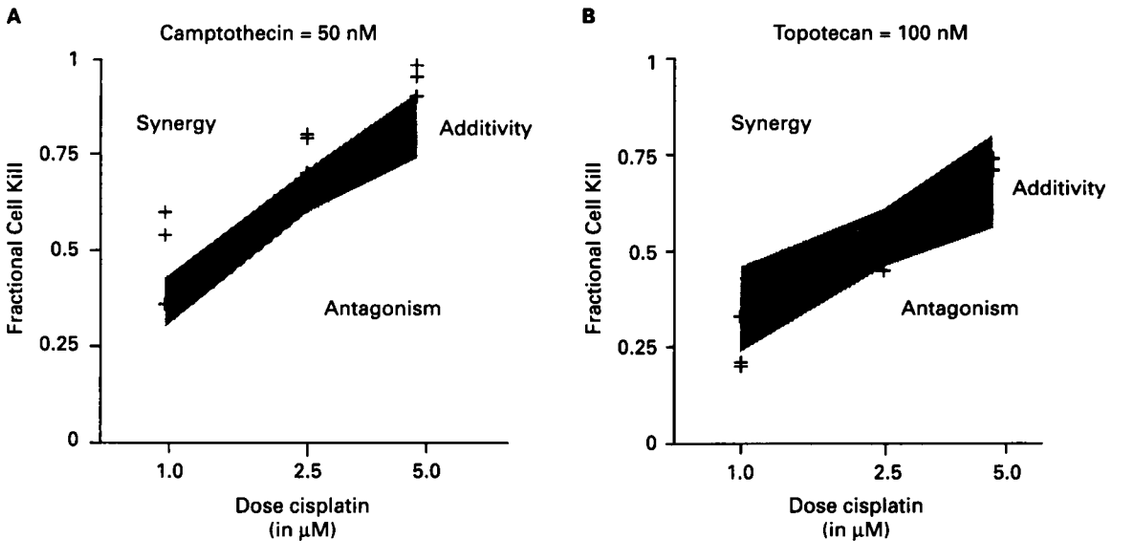

Figure 1. Differential interaction between cisplatin and the Topo I inhibitors, CPT and TPT, with simultaneous administration to DAOY medulloblastoma cells. (A) Dose–response curve of DAOY cells treated with simultaneous administration of 50 nM CPT and cisplatin. The 90% CI for predicted additive drug interaction (see Materials and methods) is shown as the shaded area and observed cytotoxicity of dose combinations tested as points (+). Most observed cytotoxicity (six of 12 points) and the mean of three trials for all dose combinations tested was above the 90% CI, indicating a synergystic cytotoxicity for these dose combinations. (B) Dose–response curve of DAOY cells treated with simultaneous administration of 100 nM TPT and cisplatin. Most observations (seven of 12 points) fell within the 90% CI for predicted addictive cytotoxicity and the mean of three trials for each dose combination tested fell within the shaded area, indicating addictive cytotoxicity for these dose combinations.

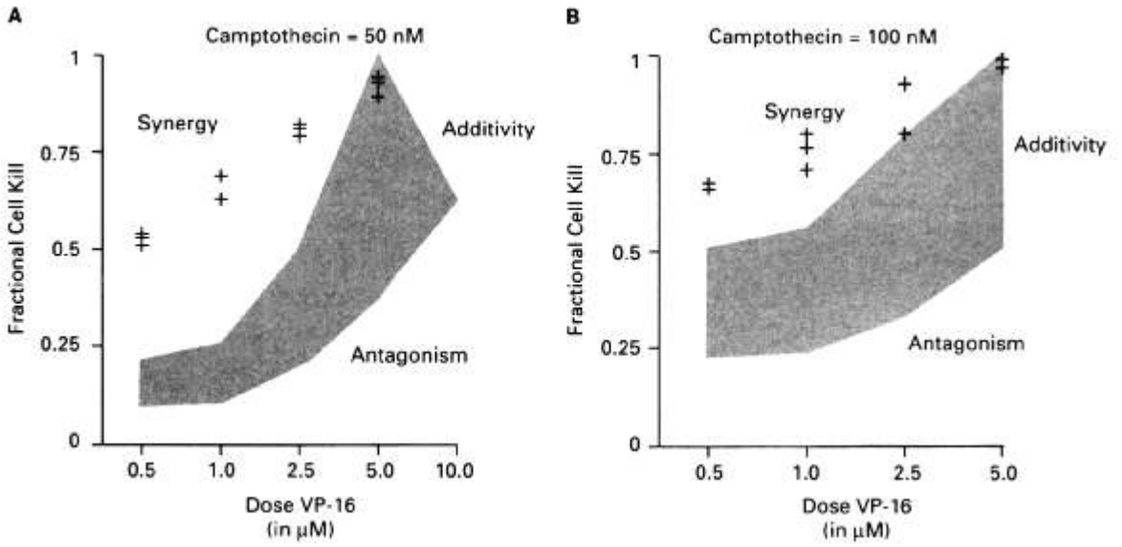

Figure 2. Synergistic cytotoxic effect of simultaneous administration of CPT and VP-16 to DAOY medulloblastoma cells. (A) Dose–response curve of DAOY cells treated with simultaneous administration of 50 nM CPT and VP-16. The 90% CI for predicted additive cytotoxicity (see Materials and methods) is shown as the shaded area and observed cytotoxicity of dose combinations tested as points (+). Most (nine of 12 points) observed cytotoxicity plotted as points on the graph (+) were above the 90% CI, indicating a synergystic cytotoxicity for the corresponding dose combinations. The remaining three observations (dose combination: camptothecin 50 nM+VP-16 5 μM) were within the 90% CI, indicating additive cytotoxicity for this dose combination. (B) Dose–response curve of DAOY cells treated with simultaneous administration of 100 nM CPT and VP-16. Again, most (eight of 12) observations (+) fell above the upper 90% CI for additive drug interaction, indicating synergystic interaction for the corresponding dose combinations. The observed cytotoxicity for the dose combination of CPT 100 nM and VP-16 5 μM was within the 90% CI, indicating additive cytotoxicity for this dose combination.

reduced the number of tested dose combinations producing significant synergy.

U251 malignant glioma cell line

Topo I inhibitors + Alkylating agents. The combination of CPT and cisplatin in U251 cells produced opposite interactive effects in a dose-dependent fashion. CPT at 50 and 100 nM produced significant synergy with cisplatin. An intermediate dose of 250 nM CPT produced additive cytotoxicity with cisplatin while a higher dose of CPT, 1000 nM, produced significant antagonism with cisplatin. The interaction between CPT and 4-HC in U251 cells was comparable to that observed with cisplatin. There was significant synergy with CPT 25 nM and 4-HC over a broad range of doses. Additive interactions were observed with CPT at higher doses. No significant antagonism was observed between CPT and 4-HC at any of the dose combinations tested. In contrast, the majority of dose combinations of TPT and cisplatin produced additive interactions. TPT at a high concentration, 5000 nM, produced significant antagonism with cisplatin and no significant synergy was observed for any of the dose combinations tested. By contrast with all other alkylating agents, the majority of dose combinations of CPT and BCNU exhibited significant antagonism (Figure 3).

Topo I inhibitors + Topo II inhibitors. Treatment with CPT and VP-16 in U251 cells produced additive cytotoxicity for the majority of dose combinations tested (Figure 4). Significant synergy was observed at only one dose combination.

Comparison of analysis models

Evaluation of cytotoxicity data by means of isobolographic analysis or by estimates of additivity based on log-linear dose–response curves were comparable to those obtained using the additivity model detailed above. To illustrate the concordance of results using the different methods, complete analyses are presented for three drug combinations, one with synergistic cytotoxicity (CPT + VP-16 in DAOY cells), one with additive cytotoxicity (CPT + VP-16 in U251 cells) and one with antagonistic cytotoxicity (CPT + BCNU in U251 cells).

Synergistic cytotoxicity of CPT and VP-16 in DAOY medulloblastoma cells, as determined using

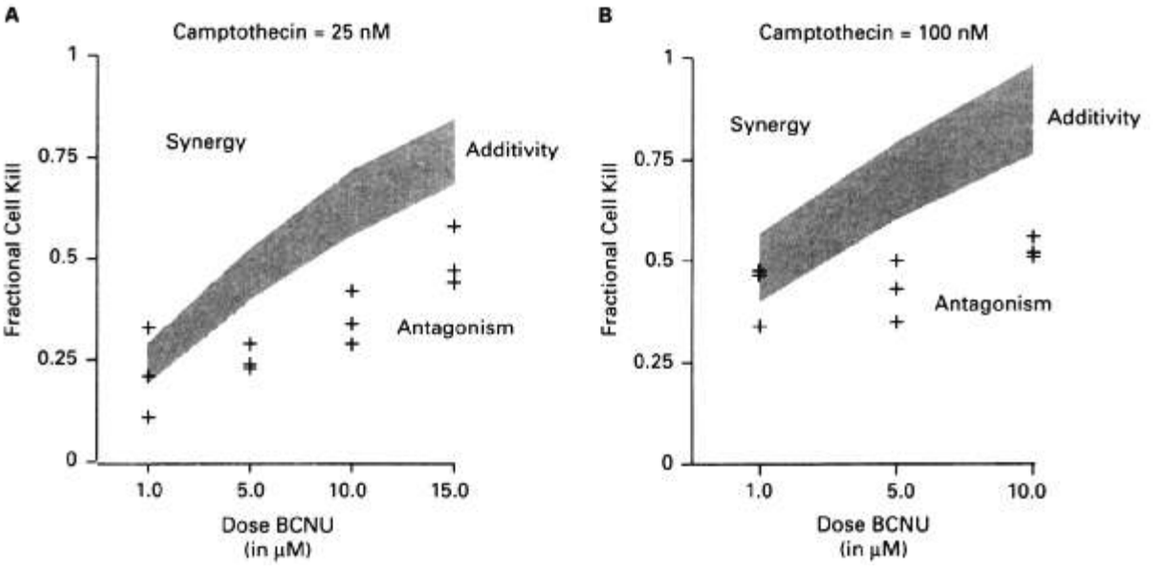

Figure 3. Antagonistic cytotoxic effects of simultaneous administration of CPT and BCNU to U251 glioma cells. (A) Dose–response curve of U251 cells treated with simultaneous administration of 25 nM CPT and BCNU. The 90% CI for predicted additive cytotoxicity (see Materials and methods) is shown as the shaded area and observed cytotoxicity of dose combinations tested as points (+). Most (10 of 12) observations and the mean of three trials for each of the three dose combinations tested were below the 90% CI, indicating antagonistic cytotoxicity for the corresponding dose combinations. (B) Dose–response curve of U251 cells treated with simultaneous administraiton of 100 nM CPT and BCNU. Most (seven of nine) observations (+) and the mean of three trials for each of the three dose combinations tested fell below the lower 90% CI for cytotoxicity predicted by additive drug interaciton, indicating antagonistic interaction for these dose combinations.

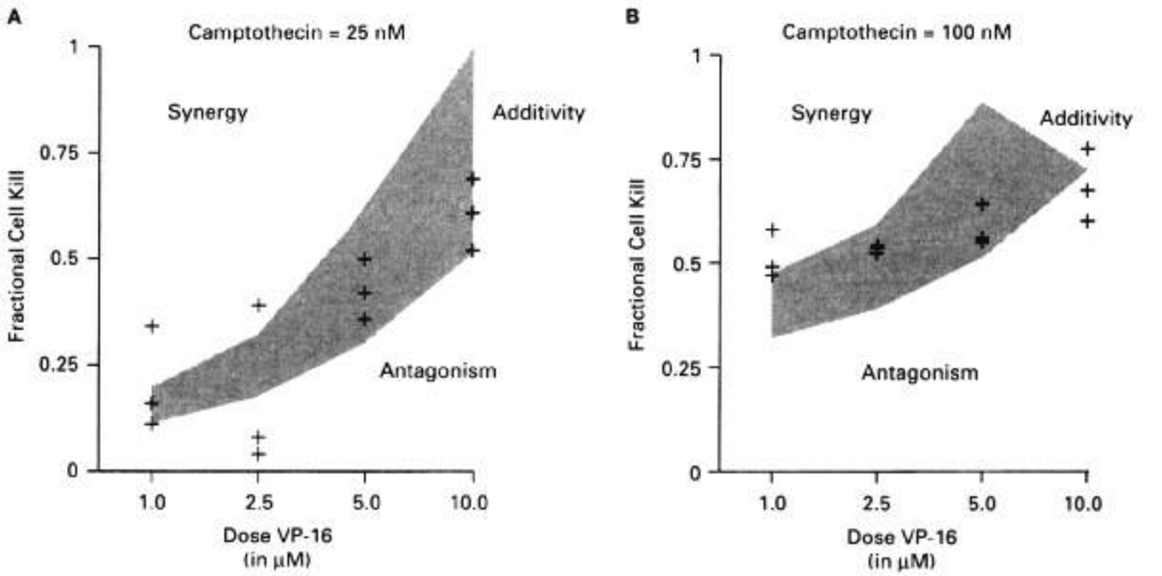

Figure 4. Additive cytotoxic effect of simultaneous administration of CPT and VP-16 to U251 glioma cells. (A) Dose–response curve of U251 cells treated with simultaneous administration of 25 nM CPT and VP-16. The 90% CI for predicted additive cytotoxicity (see Materials and methods) is shown as the shaded area and observed cytotoxicity of dose combinations tested as points (+). Most observed cytotoxicity (eight of 12 points) and the mean of the three trials for all four dose combinations were within the shaded 90% CI, indicating additive cytotoxicity for the dose combinations. (B) Dose–response curve of U251 cells treated with simultaneous administration of 100 nM CPT and VP-16. Most (nine of 12) observations (+) fell within the 90% CI for additive cytotoxicity. Two observations were below the lower 90% CI and one above the upper 90% CI.

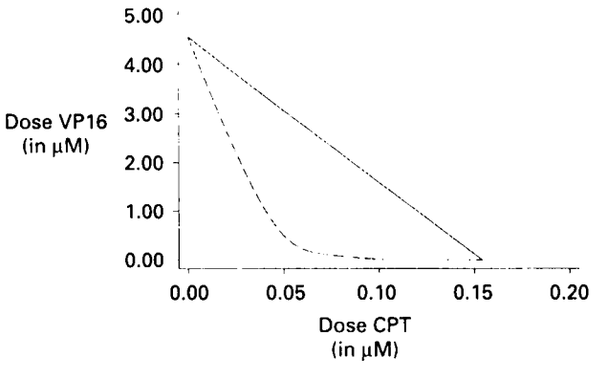

Figure 5. Isobologram for 50% clonogenic survival for DAOY cells treated with CPT and VP-16. The dose of VP-16 (in nM) is plotted along the ordinate and dose of CPT (in nM) on the abscissa. The straight line (——) indicates the dose combination of these two drugs which produced 50% clonogenic survival predicted by an additive interaction as described for the isobol method (see Materials and methods). The curvilinear line (- - - -) indicates the dose combination of these two drugs which produces 50% clonogenic survival as calculated from dose combinations which produced 50% DAOY cell kill when tested experimentally. The position of the experimental isobol beneath the isobol for additivity indicates that CPT and VP-16 have synergistic cytotoxicity at 50% clonogenic survival.

Table 4. Comparison of additive cytotoxicity estimated by a linear-log dose–response curve of CPT and VP-16 in DAOY medulloblastoma cells to observed values

CPT (nM)	VP-16 (nM)	Observed values (mean)	Expected additive cytotoxicity	Observed minus expected
50	500	0.53	0.34	0.19
50	1000	0.65	0.37	0.28
50	2500	0.81	0.47	0.34
50	5000	0.92	0.58	0.34
50	10000	1.00	0.73	0.27
100	500	0.67	0.45	0.22
100	1000	0.76	0.47	0.29
100	2500	0.88	0.54	0.34
100	5000	0.98	0.63	0.35
250	500	0.69	0.60	0.09
250	1000	0.78	0.61	0.17
250	2500	0.89	0.65	0.24
500	500	0.80	0.72	0.08
500	1000	0.87	0.72	0.15

Columns 1 and 2 indicate the dose combination for each comparison. Column 3 is cytotoxicity (fractional DAOY cell kill) observed for each dose combination presented as mean of three separate trials. Column 4 is additive cytotoxicity (fractional DAOY cell kill) estimated by an equation reported by Berenbaum for a linear-log dose–response curve (ref 33, p 109, equation 19). Column 5 is the difference between observed values (column 3) and expected additive cytotoxicity (column 4). A positive value in column 5 indicates greater toxicity than predicted (synergy), a negative value less toxicity than predicted (antagonism) and zero indicates additivity. Note that all the values in column 5 are positive values, indicating a synergistic drug interaction for all dose combinations tested.

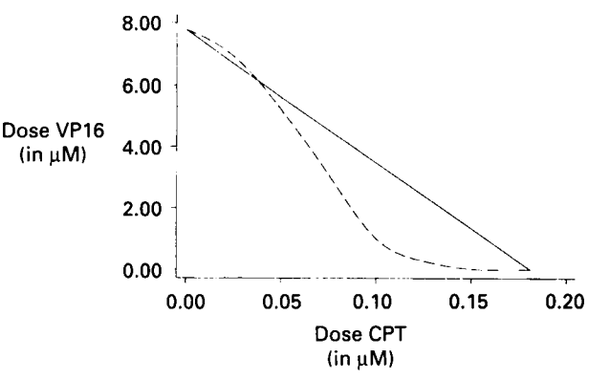

Figure 6. Isobologram for 50% clonogenic survival for U251 cells treated with CPT and VP-16. The dose of VP-16 (in nM) is plotted along the ordinate and dose of CPT (in nM) along the abscissa. The straight line (———) indicates the dose combination of these two drugs which produced 50% clonogenic survival predicted by an additive interaction as described for the isobol method (see Materials and methods). The curvilinear line (- - - -) indicates the dose combination of these two drugs which produces 50% clonogenic survival as calculated from dose combinations which produced 50% U251 cell kill when tested experimentally. The position of the experimental isobol closely approximating the isobol for additivity indicates that CPT and VP-16 have either a slight synergistic, antagonistic or additive cytotoxicity at 50% clonogenic survival, depending on the dose combination tested.

the additivity model, is illustrated in Figure 2. Isobolographic analysis of the same data (Figure 5) confirmed synergistic cytotoxicity at doses of CPT and VP-16 which produced 50% cell kill. Table 4 shows additivity computed for the combination of CPT and VP-16 in DAOY cells using the log-linear equation.[33] According to this analysis, all dose combinations tested were synergistic. However, for nearly 40% of the dose combinations tested the differences between observed and estimated additive cytotoxicity were small (less than 0.2) and this method does not permit an estimate of statistical significance. Simultaneous exposure of U251 cells to CPT and VP-16 produced additive cytotoxicity according to the model of additivity (Figure 4). Isobolographic analysis of this data for 50% clonogenic survival (Figure 6) and estimates of additive cell kill computed with the log-linear equation (Table 5) produced comparable results. Antagonistic cytotoxicity was observed following simultaneous exposure of U251 glioma cells to CPT and BCNU (Figure 3). Isobolographic analysis at 50% clonogenic survival (Figure 7) and comparison of estimated additive cytotoxicity using the log-linear equation

with observed cytotoxicity (Table 6) also indicated antagonistic interactions between CPT and BCNU.

Table 5. Comparison of additive cytotoxicity estimated by a linear-log dose–response curve of CPT and VP-16 in U251 glioma cells to observed values

CPT (nM)	VP-16 (nM)	Observed values (mean)	Expected additive cytotoxicity	Observed minus expected
25	1000	0.20	0.29	0.09
25	2500	0.17	0.36	−0.19
25	5000	0.43	0.45	−0.02
25	10000	0.61	0.58	0.03
100	1000	0.51	0.44	0.07
100	2500	0.53	0.48	0.05
100	5000	0.58	0.53	0.05
100	10000	0.68	0.62	0.06
1500	1000	0.71	0.78	−0.07

Columns 1 and 2 indicate the dose combination for each comparison. Column 3 is the cytotoxicity (fractional DAOY cell kill) observed for each dose combination presented as the mean of three separate trials. Column 4 is the additive cytotoxicity (fractional DAOY cell kill) estimated by an equation reported by Berenbaum for a linear-log dose–response curve (ref 33, p 108, equation 19). Column 5 is the difference between observed values (column 3) and cytotoxicity expected for an additive drug interaction (column 4). A positive value in column 5 indicates synergy, a negative value antagonism and value of zero indicates additivity. Note that all the values in column 5 are very close to zero.

Table 6. Comparison of additive cytotoxicity estimated by a linear-log dose–response curve of CPT and BCNU in U251 glioma cells to observed values

CPT (nM)	BCNU (nM)	Observed values (mean)	Expected additive cytotoxicity	Observed minus expected
25	1000	0.22	0.29	−0.07
25	5000	0.25	0.45	−0.20
25	10000	0.35	0.59	−0.24
25	15000	0.50	0.69	−0.19
100	1000	0.42	0.44	−0.02
100	5000	0.43	0.54	−0.11
100	10000	0.53	0.63	−0.10
1500	1000	0.67	0.78	−0.11

Columns 1 and 2 indicate the dose combination for each comparison. Column 3 is the cytotoxicity (fractional DAOY cell kill) observed for each dose combination presented as the mean of three separate trials. Column 4 is the additive cytotoxicity (fractional DAOY cell kill) estimated by an equation reported by Berenbaum for a linear-log dose–response curve (ref 33, p 108, equation 19). Column 5 is the difference between observed values (column 3) and cytotoxicity expected for an additive drug interaction (column 4). A positive value in column 5 indicates synergy, a negative value antagonism and value of zero indicates additivity. Note that all the values in column 5 are negative, indicating an antagonistic drug interaction for all the dose combinations tested.

Discussion

Cytotoxic interactions between Topo I inhibitors and alkylating/platinating agents are tumor cell line and drug specific as well as dose and schedule dependent. In this study, substantial *and* significant synergistic cytotoxicity was identified for combinations of CPT with cisplatin, 4-HC and VP-16 in DAOY medulloblastoma cells, and for CPT with cisplatin and 4-HC in U251 malignant glioma cells. The interactions between CPT and VP-16 were schedule dependent since presentation of VP-16 after CPT produced synergistic cytotoxicity at fewer dose combinations. Antagonistic cytotoxicity was observed when CPT and BCNU were administered simultaneously to glioma cells over a wide range of dose combinations. The finding that CPT, but not TPT, had a synergistic cytotoxic effect on medulloblastoma and glioma cells indicated that drug interactions of a parent compound do not reliably predict the pattern of drug interactions for its analogs.

Interactions between Topo I inhibitors and alkylating/platinating agents have been addressed in a limited number of studies, none of which have included brain tumors. Synergistic cytotoxicity between CPT analogs and cisplatin has been reported by several investigators using a variety of cancer cell lines and prolonged

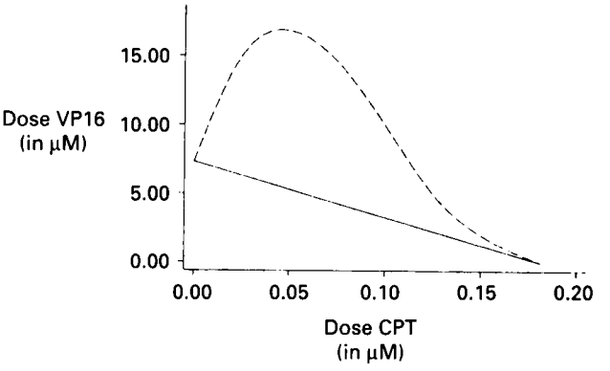

Figure 7. Isobologram for 50% clonogenic survival for U251 cells treated with CPT and BCNU. The dose of BCNU (in nM) is plotted along the ordinate and the dose of CPT (in nM) along the abscissa. The straight line (——) indicates the dose combinations of these two drugs which produced 50% clongenic survival predicted by an additive interaction as described for the isobol method (see Materials and methods). The curvilinear line (- - - -) indicates the dose combination of these two drugs which produces 50% clongenic as calculated from dose combinations which produced 50% U251 cell kill when tested experimentally. The position of the concave experimental isobol (- - - -) above the isobol for additivity indicates an antagonistic cytotoxic interaction at 50% clonogenic survival.

exposure times. Chou *et al.* reported that TPT and cisplatin produced synergistic cytotoxicity for human teratocarcinoma cell lines (833K and 833K/64CP10P) when administered simultaneously for 4 days.[25] Cytotoxicity was quantified by protein measurement and evaluation of drug interaction by the combination index-isobologram method, a model based upon the median-effect principle.[34] Methodological differences (e.g. distinct cell lines, treatment schedules, cytotoxicity assays and analysis of drug interaction) are likely responsible for the disparity between our findings, that there is minimal synergistic cytotoxicity between TPT and cisplatin, and those of Chou *et al.* Fukuda *et al.* documented synergistic killing of small cell lung cancer cells, SBC-3, for combined cisplatin and Topo I inhibitors, CPT-11 and SN38 (the active metabolite of CPT-11).[26] These investigators treated cells for 72 h, quantified cytotoxicity using the tetrazolium dye (MTT) assay and evaluated drug interaction using a modified isobolographic analysis. Pei *et al.* documented synergistic cytotoxicity between cisplatin and SN38 for small cell, but not non-small cell lung cancer cells using the same drug exposure time and method for evaluating drug interaction as Fukuda *et al.*[28] These reports illustrate the schedule dependence and cell line specificity of drug interactions.

There are currently no published reports of interactions between Topo I inhibitors and the alkylating agent 4-HC, and although Cheng *et al.* studied the schedule dependence of combined TPT and BCNU drug interactions in Chinese hamster V79 cells,[27] they did not rigorously evaluate whether these interactions were synergistic or additive.

Synergistic, additive and antagonistic interactions have been observed between Topo I inhibitors and VP-16 in non-brain tumor cell lines; Pei *et al.* reported supra-additive cell kill with simultaneous administration of SN38 and VP-16 in human small cell and non-small cell lung cancer cell lines.[28] However, simultaneous administration of CPT and VP-16 produced antagonistic cytotoxic effects in human leukemia cells,[40] colon carcinoma cells[30] and Chinese hamster ovary cells.[41] Chou *et al.* report that the combination of the Topo I inhibitor, TPT and VP-16 produced additive cytotoxicity of human teratocarcinoma cells,[25] while Stahl *et al.* reported additive cytotoxicity with simultaneous or sequential combination of VP-16 and CPT in human gastric and lung cancer cell lines.[42] The schedule dependence of the interaction between Topo I and Topo II antagonists reported above has been noted by other investigators. For example, antagonism of CPT and VP-16 can be overcome by sequential administration of CPT and VP-16 in human colon cancer cells.[30]

Analysis of drug interactions is controversial.[32,33] Some authors define synergy as an effect greater than would be expected from either drug based on their potency as single agents.[43–45] The goal of this work was to identify drug combinations with *supra*-additive cytotoxic effects.[25,32–34,36,46] Such combinations would permit use of lower drug doses, thereby reducing toxicity and the opportunity for development of resistance[34] or increase tumor death without augmenting toxicity.[32] Techniques used to identify drug combinations with supra-additive cytotoxicity have been described in the literature, but their limitations motivated our effort to identify an alternative analysis. Problems with various techniques include: (i) inability to perform statistical analysis on results (e.g. isobolographic analysis[33,36,39]), (ii) restriction of comparisons to a fixed level of cell kill (e.g. isobolographic analysis or median effect[25,34,47]) and (iii) restriction of the comparisons to fixed doses (e.g. fixed ratio analysis[32]).

The model of additivity presented in this report permits: (i) direct comparison of experimentally obtained results to the expected additive interaction (Figures 1–4), (ii) identification of dose combinations with synergistic interaction over a range of cytotoxicity rather than at a single fixed level and (iii) objective identification of dose combinations producing synergistic or antagonistic cytotoxicity at 0.1 significance level. Additionally, two previously reported methods were used to evaluate drug interactions in this study: an isobologram was constructed for each drug combination at a response level of 50% clonogenic survival and the data were analyzed using a method for estimating additive drug interaction reported by Berenbaum.[33] The constancy of the results, despite the use of a different analytical method, lends validity to the conclusions of the report, as well as to the use of the model of additivity.

Different techniques for analysis of drug interaction applied to the same data set may yield distinct conclusions,[33] so it is crucial in analyzing drug interactions to choose a statistical method that is appropriate for the experimental design and to understand the constrains and assumptions underlying the method chosen.[48] The principal assumptions of the additivity model reported here are that: (i) an additive drug interaction can be defined mathematically as a sum, (ii) transformation of single-agent dose-response curves to a linear function accurately represents the pharmacological behavior of the agent and (iii) observations outside of the 90% CI around this sum represent *significant* synergy or antagonism. The first assumption is one shared by most models of additivity,[25,33,34,36] including the method calculation

based on log-linear dose-response curves used in this report[33] and median-effect analysis published by Chou and Talalay.[34] The second assumption is justified because the regression coefficients of the single-agent dose-response curves confirmed that the transformations closely approximated experimentally obtained data and is an assumption used in other models of additivity.[33,34] Finally, the 90% CI used to define a range of expected additivity in this model defines a significance level of 0.10. Although this is an arbitrary definition of significance, neither of the two analyses used for comparison in this report include statistical methods,[25,33,36] making designation of what constitutes a significant or substantial deviation from the estimated additive effect subjective. Other models, such as the median-effect model, incorporate statistical analysis of the data, but do not define a level of significance.[25,34]

Conclusions

The results of the present study have implications for the role of CPT in brain tumor therapy, indicating that combination of these agents with etoposide or cisplatin may increase the effectiveness of medulloblastoma treatment, and combinations of CPT with cisplatin or cyclophosphamide may increase the effectiveness of glioma therapy. Although the use of CPT has been limited clinically due to toxicity,[3,17] use of lower, thus less toxic doses, may produce significant tumor kill when combined with an agent which exhibits synergistic cytotoxicity. These studies illustrate the importance of identifying the most efficacious dose combinations. The results of these experiments also indicate that simultaneous administration of CPT and BCNU should be avoided in designing treatment protocols for malignant gliomas. Xenograft studies will be needed to determine if the synergistic cytotoxic effect of these drug combinations observed *in vitro* persists *in vivo*.

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