Drug ratio–dependent antitumor activity of irinotecan and cisplatin combinations *in vitro* and *in vivo*

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Abstract

Irinotecan and cisplatin are two established anticancer drugs, which together constitute an effective combination for treating small-cell lung cancer. We investigated whether the efficacy of this combination could be improved by controlling drug ratios following *in vivo* administration. Irinotecan and cisplatin combinations were evaluated systematically for drug ratio–dependent synergy *in vitro* using a panel of 20 tumor cell lines. *In vitro* screening informatics on drug ratio–dependent cytotoxicity identified a consistently antagonistic region between irinotecan/cisplatin molar ratios of 1:2 to 4:1, which was bordered by two synergistic regions. Liposomal co-formulations of these two agents were developed that exhibited plasma drug half-lives of 6 hours and maintained a fixed drug ratio for more than 24 hours. Drug ratio–dependent antitumor activity was shown *in vivo* for these liposome formulations, and irinotecan/cisplatin ratios between 5:1 and 10:1 were identified as therapeutically optimal. The relationship between irinotecan/cisplatin ratio and *in vivo* efficacy was consistent with *in vitro* drug ratio dependency results. Superior antitumor activity was observed for the liposome-encapsulated 7:1 molar ratio of irinotecan/cisplatin (designated CPX-571) compared with the free-drug cocktail in all models tested. Further efficacy studies in a range of human tumor xenografts, including an irinotecan-resistant model, showed that both liposomal agents contributed to the overall efficacy in a manner consistent with *in vivo* synergy. These results show the ability of drug delivery technology to enhance the therapeutic activity of irinotecan/cisplatin combination treatment by maintaining synergistic ratios *in vivo*. CPX-571, a fixed-ratio formulation of irinotecan and cisplatin, is a promising candidate for clinical development. [Mol Cancer Ther 2009;8(8):2266–75]

Introduction

Small-cell lung cancer (SCLC) is characterized by rapid disease progression, low cure rates, and a high incidence of mortality. There are an estimated 32,000 new cases per year in the United States, representing 15% of all lung cancers (1). Patients receive multimodality treatment including a chemotherapy regimen of etoposide/cisplatin (EP) and chest radiation therapy, which has remained the standard of care for 25 years (2). SCLC is initially responsive to both chemotherapy and radiotherapy; however, the vast majority of patients relapse. Although the current standard of care represents the most effective drug therapy for patients, novel treatments are warranted to improve survival rates.

Irinotecan has emerged as a promising drug candidate in the treatment of SCLC. Phase II clinical evaluation of irinotecan in SCLC patients showed a 50% objective response rate in chemotherapy-naïve patients and a 47% objective response rate in patients previously treated with cisplatin (3). These values are higher than the 35% response rate observed for patients receiving etoposide monotherapy (4). Based on these studies, it was anticipated that a combination of irinotecan and cisplatin may be superior to etoposide/cisplatin chemotherapy, the standard of care for extensive-stage SCLC.

The combination of irinotecan and cisplatin has shown synergy or supra-additive effects when exposed to cultured human tumor cells (5, 6), human xenograft tumor models (5, 7), and cancer cells freshly isolated from colorectal patients (8). This information provided the scientific rationale for testing this combination in a clinical setting. A phase III study by the Japan Clinical Oncology Group in 2002 showed that the irinotecan and cisplatin chemotherapy regimen (IP) in SCLC patients was significantly more efficacious than the etoposide and cisplatin chemotherapy regimen (EP), leading to the adoption of IP as standard of care in Japan and other Asian countries (9). In 2006, a North American follow-up study compared IP and EP protocols and showed that IP was equally effective and potentially less toxic (10). A recent phase III trial by the Southwest Oncology Group was designed to confirm the Japanese results by using the same dose and schedule; however, this trial concluded that IP had the same response rate, progression-free survival, and overall survival profiles as EP (11). The lack of survival benefit with the irinotecan/cisplatin combination is somewhat surprising considering

the superiority of irinotecan over etoposide as a monotherapy in SCLC patients.

We hypothesized that the failure to show a therapeutic benefit with irinotecan/cisplatin over the etoposide/cisplatin combination may be attributed, in part, to the occurrence of drug ratio dependency in one or both combinations. Recent studies by our laboratory and others have shown that the therapeutic activity of drug combinations is often drug ratio dependent, where synergistic or antagonistic interactions occur when tumors are exposed to different drug/drug ratios (12–15). Thus, the full benefit of combination chemotherapy may not be realized *in vivo* unless the ratios of the drugs are carefully controlled and maintained to avoid exposure of tumor cells to antagonistic drug ratios. In the studies presented here, we evaluated the combination of irinotecan/cisplatin for drug ratio–dependent synergy *in vitro* and used liposome nanotechnology to maintain drug ratios *in vivo* and deliver synergistic ratios to tumor sites (13, 16). The efficacy of this dual-drug liposome formulation was compared with the free-drug cocktail as well as individual liposome-encapsulated drugs in a variety of human xenograft tumor models.

Materials and Methods

Reagents

Phospholipids were purchased from Lipoid LLC. [³H] Cholesteryl hexadecyl ether was purchased from Perkin-Elmer Life Sciences. Lyophilized cisplatin was purchased from Polymed, and irinotecan hydrochloride trihydrate was obtained from ScinoPharm. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other chemicals were obtained from Sigma Chemical Company.

Cell Culture

All tumor cell lines used in the study were purchased from American Type Culture Collection. An irinotecan-resistant HCT-116 human colon carcinoma line with 10-fold resistance compared with the parental strain was obtained by exposing cells to 1.0 μmol/L irinotecan, followed by clonal selection and confirmation of resistance by IC_{50} analysis as described below. Cells were propagated in their recommended tissue culture media supplemented with 10% FCS and 2 mmol/L L-glutamine at 37°C in a humidified atmosphere with 5% CO_2.

Cell Proliferation Assays

The sensitivity of a panel of tumor cells to individual drugs and the drug combination was determined using a cytotoxicity assay previously described (13, 14). Briefly, cells were plated in triplicate in 96-well, flat-bottomed microplates and incubated for 24 h in medium, followed by addition of the test agents. Following a 72-h incubation, the relative effect on the presence of viable cells was determined by MTT assay (17). Due to the wide range of ratios analyzed and differences in IC_{50} values, the data sets were truncated at the two ends of the sigmoidal curve where the MTT assay is unresponsive at low drug concentrations and again at high cytotoxicity where there is a low MTT

signal. Synergy of the two drugs over a range of drug ratios and concentrations was analyzed by the median-effect algorithm (18, 19). The occurrence of ratio-dependent synergy was determined by plotting the combination index (CI < 1, synergy; CI ~ 1, additivity; and CI > 1, antagonism) versus the fraction of cells affected (Fa), which indirectly reflects the drug concentration.

Liposome Preparation

The required amount of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine/1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine/1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol/cholesterol (35:35:20:10 molar ratio) was dissolved in dichloromethane/methanol/water (93:5:2, v/v/v). Radiolabeled liposomes were prepared by adding [³H]cholesteryl hexadecyl ether, a nonexchangeable, nonmetabolizable liposome tracer. Liposome preparation details are described in Supplementary Data. For cisplatin encapsulation, the drug was dissolved at a concentration of 7.5 mg/mL in 150 mmol/L NaCl and mixed with the liposome solution for 1 h at 60°C in the presence of ethanol to enhance drug uptake. Subsequently, the cisplatin liposomes were buffer exchanged against 150 mmol/L NaCl to remove ethanol and unencapsulated drug. For irinotecan encapsulation, liposomes and irinotecan were mixed at a 0.22:1 drug-to-lipid molar ratio at 41°C for 1 h. The liposomes were subsequently buffer exchanged against 300 mmol/L sucrose, 20 mmol/L phosphate (pH 7). To generate CPX-571, liposomal cisplatin and liposomal irinotecan formulations were mixed together at a 7:1 molar drug ratio and stored at –20°C. Following storage, the drug encapsulation was ~98%. Liposome pharmacokinetics and tumor accumulation data are included in Supplementary Data.

Experimental Tumor Models

Female CD-1 nude (Charles River) and *Foxn*1 nude mice (Harlan) were used in these studies. All procedures involving experimental mice were conducted according to the guidelines of the Canadian Council of Animal Care. Maximum tolerated dose (MTD) values were defined as survival in the absence of significant tumor burden with ≤15% body weight loss nadir lasting ≤2 d. Animals were terminated (CO_2 asphyxiation) when signs of toxicity or tumor-related illness were observed. Tumor cells, consisting of human SCLC cell line H69 (1×10^7 in 50% growth factor–reduced Matrigel; BD Bioscience), human non–small cell lung cancer (NSCLC) H460 (2×10^6), human NSCLC H1299 (2×10^6), human colon carcinoma HT29 (2×10^6), or human pancreatic carcinoma Capan-1 (2×10^6), were implanted s.c. in the right flank of mice. Tumor volumes were measured using calipers, and the values calculated according to the equation $L \times W^2/2$.

Statistical Analysis

A standard one-way ANOVA was used to determine statistically significant differences from the mean. Survival curves were computed using the Kaplan-Meier method. Treatment groups were analyzed by Microsoft Excel statistics software and compared using a two-sample log-rank test. $P < 0.05$ was considered significant for all statistical tests.

Results

In vitro Examination of Irinotecan/Cisplatin Combinations for Drug Ratio–Dependent Synergy

High-throughput screening using MTT cytotoxicity assays was done for multiple fixed-ratio combinations of irinotecan and cisplatin diluted over a range of concentrations within the complete cell viability dose-response curve for 20 tumor cell lines. We chose to focus our *in vitro* evaluations on irinotecan, rather than its potent metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38), because this reflects the situation experienced for liposomal delivery of irinotecan *in vivo* where extravasation and accumulation of liposomes in tumors provide a local infusion reservoir of irinotecan. In addition, in contrast to irinotecan, SN-38 is extremely membrane permeable and was not amenable to stable retention in liposomal delivery systems after injection.

Median-effect analysis of dose-response curves was used to calculate the combination index (CI) for each tested irinotecan/cisplatin ratio at a 0.8 fraction of affected cells (80% cell growth inhibition) to determine whether synergy depended on the drug ratio (18, 19). This mathematical algorithm generates CI values <0.9 when drug-drug interactions are synergistic, 0.9–1.1 when additive, and >1.1 when antagonistic. The results are presented as a synergy "heat map" (Table 1) where CI values reflecting synergy, additivity, and antagonism are color coded as green, yellow, and red, respectively. The results identify two distinct regions of synergy separated by a zone of antagonism between irinotecan/cisplatin molar ratios of 1:2 and 4:1. This transition between synergy and antagonism was striking given that an irinotecan/cisplatin molar ratio of 2:1 produced antagonism in 12 of 20 cell lines (maximum CI value of 2.57), whereas increasing the ratio to 8:1 resulted in synergy in 17 of 20 cell lines (minimum CI value of 0.18; see Table 1) and no antagonism. Synergy also predominated at irinotecan/cisplatin molar ratios <1:2. For example, 13 of 20 cell lines exhibited synergy at a drug ratio of 1:4 and antagonism was observed in only 4 of 20 tumor lines. It should be noted that similar relationships were obtained for other therapeutically relevant fraction of affected cell levels (e.g., Fa = 0.5).

Irinotecan and Cisplatin Liposome Formulations That Maintain Drug Ratios *In vivo*

We next investigated whether the drug ratio–dependent synergy observed *in vitro* could be manifested *in vivo* by delivering irinotecan/cisplatin as a fixed-ratio formulation using nanoscale liposomal delivery vehicles. Irinotecan and cisplatin were formulated into individual liposomes with identical membrane compositions to alleviate drug instability when the two agents were co-encapsulated. By combining individual liposomal agents, the internal buffer composition was tailored for each individual drug, thereby increasing drug stability and providing additional flexibility in coordinating drug release kinetics. In a recent study

Table 1. *In vitro* synergy heat map of irinotecan/cisplatin ratios for a panel of 20 human tumor cell lines for CI values at Fa = 0.8

Cell lines screened	Tumor	CI at Fa = 0.8												
		1:64	1:32	1:16	1:8	1:4	1:2	1:1	2:1	4:1	8:1	16:1	32:1	64:1
LCC6	Breast	0.69	0.58	0.71	0.89	0.83	1.01	1.57	1.03	0.85	0.63	0.52	0.54	0.76
MCF-7	Breast	0.71	0.67	0.86	0.88	0.95	1.15	0.71	0.82	0.70	1.00	0.87	0.83	0.88
MB 231	Breast	1.02	1.01	0.90	0.90	0.70	0.87	1.54	1.34	0.90	1.10	0.76	1.40	0.61
HCT-116	Colon	0.38	0.36	0.37	0.33	0.62	0.84	1.50	1.73	9.40	0.71	0.76	0.82	1.06
Colon-26	Colon	1.22	1.67	1.40	1.16	1.00	1.21	1.04	1.38	1.10	1.03	1.31	1.25	0.82
HT-29	Colon	1.05	0.88	0.90	0.86	1.21	1.09	1.57	1.17	0.90	0.76	0.82	0.97	1.05
A549	Lung	0.67	0.61	0.67	0.56	0.44	0.39	0.44	1.65	1.31	0.49	0.37	0.41	0.49
H460	Lung	0.92	0.81	0.84	0.80	0.75	0.73	0.94	1.14	2.03	0.53	0.36	0.80	0.48
H322	Lung	0.47	0.52	0.68	0.96	0.57	1.28	1.24	0.95	0.73	0.54	0.44	0.52	0.54
H1299	Lung	1.07	1.08	1.12	1.09	0.76	0.98	3.01	2.57	1.83	0.65	0.64	1.49	0.82
H522	Lung	1.15	0.62	1.09	0.84	0.82	0.92	2.26	1.80	0.89	0.48	0.39	0.73	0.47
Ovcar-3	Ovarian	1.33	1.35	1.08	1.10	0.80	0.78	0.89	0.89	1.84	0.53	0.28	0.33	0.35
Ovcar-5	Ovarian	1.29	1.64	1.55	1.34	1.30	1.42	1.51	1.30	1.15	0.80	1.05	0.99	1.17
SK-OV-3	Ovarian	1.33	1.26	1.30	1.51	1.49	1.70	1.75	1.28	1.17	0.55	0.70	0.79	0.76
IGROV-1	Ovarian	1.16	1.16	1.16	1.00	0.95	0.95	1.23	1.20	1.16	0.77	0.78	0.71	0.75
A2780	Ovarian	0.93	0.94	0.81	0.75	0.80	0.81	0.87	0.70	0.81	0.81	1.20	1.03	1.25
Capan-1	Pancreatic	1.46	1.22	1.11	1.25	0.86	0.89	1.12	1.17	0.69	0.89	0.83	0.71	0.99
BXPC-3	Pancreatic	1.00	1.02	0.91	1.10	0.81	0.99	1.04	0.88	0.70	0.60	0.49	0.64	0.61
N87	Gastric	1.76	1.87	1.35	1.55	1.65	1.09	1.05	0.68	0.51	0.18	0.11	0.06	0.04
A253	H&N	0.85	0.84	0.81	0.92	0.76	0.63	0.79	0.78	0.86	0.83	0.90	0.82	0.84

NOTE: Irinotecan and cisplatin were simultaneously exposed to cells for 72 h at indicated molar ratios, and the dose-response curve for each cell line was determined by an MTT cytotoxicity assay. The dose-response curves were subsequently evaluated by the Chou and Talalay median-effect method to calculate the CI at ED$_{80}$ (Fa = 0.8) and summarized in a heat map. Two areas of synergy are observed at <1:2 and >4:1 irinotecan/cisplatin ratios. Green indicates synergy (CI < 1); yellow indicates additivity (CI ~ 1); and red indicates antagonism (CI > 1). Results reflect compiled results from multiple experiments.
Abbreviation: H&N, head and neck.

Table 2. Tumor growth delay and LCK values for mice given synergistic (7:1) and antagonistic (3:1) irinotecan/cisplatin ratios in an H460 NSCLC xenograft model

Treatment	Irino/Cis ratio	Dose (mg/kg; Irino/Cis)	TGD	%TGD	LCK*
CPX-5	7:1	40/2.5	23.3	131	2.06
Lipo-irinotecan		40	13.8	78	1.22
Lipo-cisplatin		2.5	2.8	16	0.24
Predicted additive value[†]	7:1		16.6		1.46
CPX-5	3:1	20/3	13.5	76	1.20
Lipo-irinotecan		20	6	34	0.53
Lipo-cisplatin		3	7.3	41	0.64
Predicted additive value[†]	3:1		13.3		1.17

Abbreviations: TGD, tumor growth delay; Cis, cisplatin; Irino, irinotecan.
*LCK = $[T − C] / (3.32 \times T_d)$, where $T − C$ is the treatment-induced delay for tumors to reach 400 mg and T_d is the tumor doubling time of the control group.
[†]Value determined from adding the sum of TGD or LCK values for individual liposomal drugs.

examining the effect of bilayer composition on the *in vivo* activity of liposomal cisplatin, we identified the formulation of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine/ 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine/1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol/cholesterol (35:35:20:10, mole ratio) to be the most efficacious (20). As a result, formulation efforts focused on this membrane composition to coordinate the release rates of irinotecan and cisplatin while maintaining the therapeutic activity of the cisplatin. Coordinated pharmacokinetics were achieved for a range of different irinotecan/cisplatin ratios *in vivo* (Supplementary Fig. S1).

Drug Ratio–Dependent Activity of Liposomal Irinotecan:Cisplatin Formulations *In vivo*

In vitro screening studies of irinotecan/cisplatin drug ratios in a panel of 20 tumor cell lines discerned two areas of synergy: <1:2 and >4:1 irinotecan/cisplatin molar ratios. Identifying which of these irinotecan/cisplatin molar ratios would yield the most efficacious combination for preclinical studies was pertinent. In addition, we wanted to examine drug ratio–dependent efficacy *in vivo* by comparing synergistic and antagonistic irinotecan/cisplatin ratios encapsulated in liposomes. One of the challenges in comparing the molar ratios of liposome-encapsulated irinotecan/cisplatin *in vivo* was the low MTD of liposomal cisplatin (~3 mg/kg). Because of this, high irinotecan/cisplatin molar ratio combinations could bias efficacy results to favor the synergistic ratio based on dose intensity rather than drug ratio–dependent synergy. Therefore, we selected irinotecan/ cisplatin molar ratios of 1:5 and 2:1 for drug ratio–dependent efficacy comparisons, which represented ratios found *in vitro* to be synergistic and antagonistic, respectively. For the efficacy comparison of these two formulations, the cisplatin dose could be maintained at 3.3 mg/kg while the irinotecan dose differed 10-fold between the 1:5 (1.5 mg/kg) and 2:1 (15 mg/kg) irinotecan/cisplatin molar ratios. Consequently, an increase in efficacy for the synergistic 1:5 ratio formulation over the 2:1 ratio would clearly reflect drug ratio–dependent efficacy given that increased antitumor activity would be associated with a reduced drug dose.

We examined the therapeutic activity of fixed molar ratios of 1:5 and 2:1 in the H1299 NSCLC model (Supplementary Fig. S2). This model was chosen based on the strong antagonism obtained at the 2:1 irinotecan/cisplatin ratio for this cell line *in vitro* (CI, 2.57) as well as the large absolute difference in CI value observed for these two drug ratios (see Table 1). We compared the degree of antitumor activity for the different treatment groups based on the treatment-induced delay for tumors to reach a size of 400 mg. We correlated the degree of tumor growth delay to a more quantifiable measure of antitumor activity using the established relationship (21) of:

$$\log_{10} \text{cell kill} = (T−C)/(3.32 \times T_d)$$

$$\text{where: } (T−C) = \text{tumor growth delay}$$

$$T_d = \text{tumor doubling time}$$

The liposome-encapsulated irinotecan:cisplatin formulation containing the drugs at a 1:5 molar ratio, identified as synergistic *in vitro*, exhibited a tumor growth delay of 26 days and a corresponding log cell kill (LCK) of 1.11 (Supplementary Fig. S2). Increasing the irinotecan dose 10-fold to 15 mg/kg while maintaining the cisplatin dose at 3.3 mg/kg (reflecting a 2:1 irinotecan/cisplatin molar ratio, identified as antagonistic *in vitro*) resulted in a reduced tumor growth delay of 16 days and a LCK of only 0.68. Liposomal cisplatin administered alone provided a tumor growth delay of 10.5 days and LCK of 0.44. Consequently, the addition of 1.5 mg/kg liposomal irinotecan at the synergistic 1:5 ratio increased the LCK by 0.67, whereas the addition of 15 mg/kg liposomal irinotecan at the antagonistic 2:1 ratio increased the LCK by only 0.24. Strikingly, the 1:5 molar ratio formulation of irinotecan/cisplatin provided comparable therapeutic activity to liposomal irinotecan administered at a 27-fold higher dose of 40 mg/kg, for which a LCK of 1.2 was observed (data not shown). These results highlight the *in vivo* importance of drug ratios and indicate that higher irinotecan doses may not necessarily result in higher therapeutic activity if antagonistic drug ratios ensue.
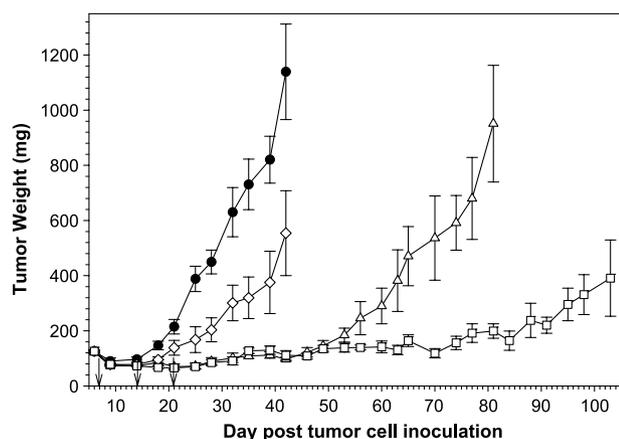
Figure 1. Antitumor efficacy of individual and combined irinotecan/cisplatin liposomal drugs. Tumor growth delay was assessed in the H69 SCLC human tumor xenograft model using a q7d×3 dosing schedule. Mice received injections of saline (●), liposomal cisplatin at 2.5 mg/kg (◊), liposomal irinotecan at 39 mg/kg (△), or liposomal irinotecan/cisplatin (7:1 molar ratio) at 39:2.5 mg/kg (□). *Downward arrows,* injection days.

To assess the *in vivo* drug ratio dependency at high irinotecan/cisplatin molar ratios, we conducted two efficacy comparisons using the H460 human NSCLC xenograft tumor model. *In vitro,* this tumor cell line displayed a very sharp transition from antagonism at an irinotecan/cisplatin molar ratio of 4:1 (CI, 2.03) to synergy at a molar drug ratio of 8:1 (CI, 0.53) and higher (Table 1). Assessing the degree of synergy or antagonism for these two formulations required the comparison of the liposomal combination with matched doses of the individual liposome-encapsulated agents rather than comparisons between the two fixed-ratio formulations. This is due to the fact that increasing irinotecan/cisplatin ratios in this range also increased total drug doses because the cisplatin component predominated toxicity effects, and thus increased efficacy at higher irinotecan/cisplatin ratios could not be attributed solely to drug ratio–dependent synergy.

We evaluated the efficacy of fixed-ratio liposome formulations of irinotecan/cisplatin encapsulated below (3:1 molar ratio) and above (7:1 molar ratio) the *in vitro* transition point between antagonism and synergy. As shown in Table 2, administering liposome-encapsulated irinotecan/cisplatin with a 7:1 molar ratio at its MTD of 40:2.5 mg/kg yielded a LCK of 2.06. The LCK values of the individual liposomal drugs at matched doses were 1.22 for irinotecan and 0.24 for cisplatin, which would predict a LCK of 1.46 for the liposome-encapsulated combination if the two drugs combined in an additive fashion. The observed LCK of 2.06 for the 7:1 molar ratio formulation is ~4-fold greater antitumor activity than predicted for additivity based on the
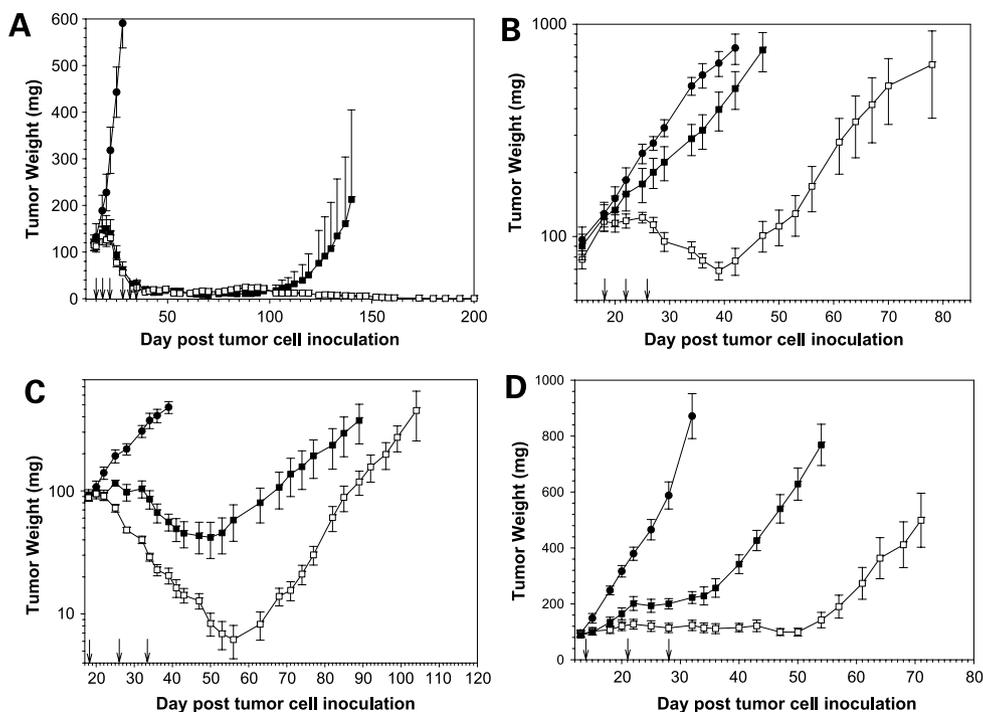

Figure 2. Antitumor efficacy of CPX-571 compared with free-drug cocktail in SCLC, colon, and pancreatic human xenograft models. **A,** H69 human SCLC xenograft growth curves using a (q4d×3)×2 dosing schedule. Mice received injections of saline (●), free-drug cocktail (7:1 molar ratio) at 34:2.1 mg/kg (■), or liposomal irinotecan/cisplatin (7:1 molar ratio) at 34:2.1 mg/kg (□). **B,** HT29 human colon xenograft growth curves using a q4d×3 dosing schedule. Mice received injections of saline (●), free-drug cocktail of irinotecan/cisplatin (7:1 molar ratio) at 47:3 mg/kg (■), or liposomal irinotecan/cisplatin (7:1 molar ratio) at 47:3 mg/kg (□). **C** and **D,** Capan-1 human pancreatic xenograft growth curves (**C**) and HCT-116 human colon xenograft growth curves (**D**) both used a q7d×3 dosing schedule. For both, mice received injections of saline (●), free-drug cocktail of irinotecan/cisplatin at 47:3 mg/kg (■), or liposomal irinotecan/cisplatin at 47:3 mg/kg (□). *Downward arrows,* injection days.

individual liposomal drugs, consistent with *in vivo* synergy. In contrast, when irinotecan and cisplatin were formulated in liposomes at a 3:1 molar ratio and administered at MTD to tumor-bearing mice, the degree of antitumor activity was consistent with additivity, where the observed LCK value for the combination of 1.20 was comparable to the predicted LCK value of 1.17 based on the additive activities of the dose-matched individual liposomal agents (Table 2).

Further studies investigated the therapeutic activity of synergistic irinotecan/cisplatin molar ratios of 5:1, 7:1, and 10:1 in the H460 NSCLC tumor xenograft model to determine which ratio may provide optimal therapeutic activity when administered at MTD. Although synergistic activity may be obtained for all three irinotecan/cisplatin
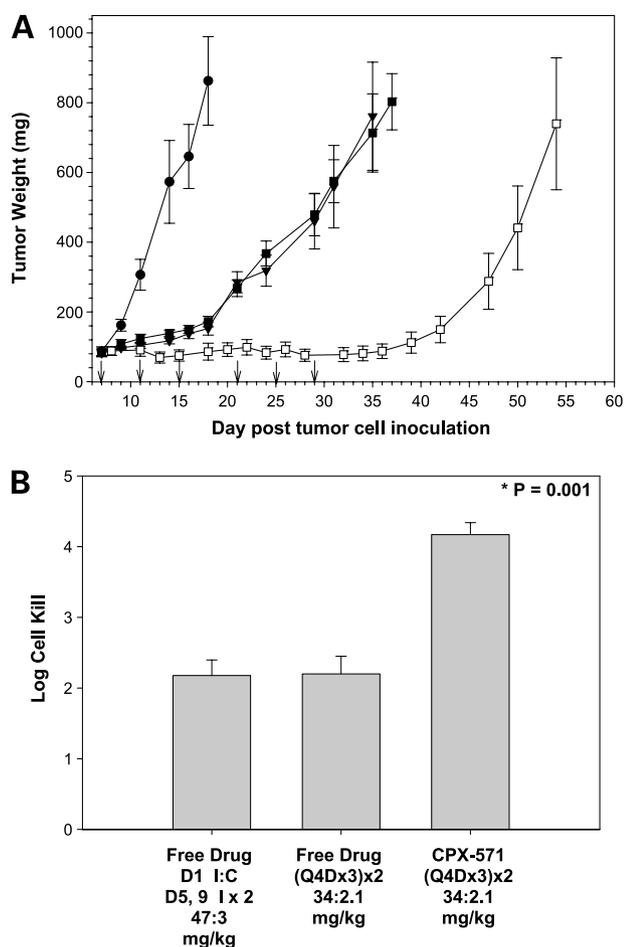
Figure 3. Antitumor efficacy of CPX-571 compared with optimized free-drug cocktail schedules. Two free-drug dosing schedules were compared directly with CPX-571 on a (q4d×3)×2 schedule in the human H460 NSCLC xenograft model. **A**, mice received injections of saline (●); two courses of free-drug cocktail containing irinotecan and cisplatin at a 7:1 molar ratio (47:3 mg/kg dose; *I:C*) on day 1 and 47 mg/kg irinotecan only (*I*) on days 5 and 9 (▼); free-drug cocktail of irinotecan and cisplatin at a 7:1 molar ratio (34:2.1 mg/kg) dosed on a (q4d×3)×2 schedule (■); or CPX-571 dosed at 34:2.1 mg/kg on a (q4d×3)×2 schedule (□). *Downward arrows,* injection days. **B**, LCK achieved by the different free-drug and liposomal irinotecan/cisplatin combinations. *, *P* value of CPX-571 compared with both of the free-drug cocktail dosing schedules.

ratios, absolute drug doses of the two agents vary when the fixed-ratio liposomal formulations are administered at their MTDs (47:2.1, 40:2.5, and 34:3.0 mg/kg for liposome formulations containing irinotecan/cisplatin molar ratios of 10:1, 7:1, and 5:1, respectively). Over this range of irinotecan/cisplatin ratios delivered in liposomes, equivalent LCK values of 2.00, 1.93, and 2.05, respectively, were obtained (data not shown).

Expanded Efficacy Evaluations of Irinotecan/Cisplatin Formulated at a 7:1 Molar Ratio

The liposomal formulation of irinotecan/cisplatin at a 7:1 molar ratio (hereafter referred to as CPX-571) was selected for further efficacy testing based on the fact that it delivered intermediate doses of both agents and may provide more consistent activity against tumor types that exhibit a range of sensitivities to irinotecan and/or cisplatin. Based on the established clinical activity of irinotecan/cisplatin combination therapy in SCLC, we evaluated the efficacy of CPX-571 in the H69 SCLC human solid tumor xenograft model (Fig. 1). We compared the efficacy of CPX-571 to that of the individual liposome-encapsulated drugs to elucidate the contribution of each agent to the overall therapeutic effect. Treatment with liposomal cisplatin at a dose of 2.5 mg/kg resulted in a modest tumor growth delay of 15 days (LCK, 0.72) relative to the saline control group, whereas liposomal irinotecan at a dose of 39 mg/kg resulted in a tumor growth delay value of 39 days (LCK, 1.95). Treatment with a dose-matched CPX-571 resulted in a significantly higher tumor growth delay of 76 days (LCK, 4.58). Not only did both liposome-encapsulated agents contribute significantly to the overall therapeutic effect but also the degree of antitumor activity observed reflected nearly 100-fold greater tumor growth inhibition than predicted for additivity based on the LCK values of the individual liposomal drugs (e.g., LCK values of 0.72 + 1.95 predict LCK value of 2.67 for additivity), consistent with strong *in vivo* synergy.

We expanded the efficacy evaluation of CPX-571 to compare its activity to that of the free-drug cocktail in several additional tumor xenograft models. In the case of the free-drug cocktail, the molar ratio of irinotecan/cisplatin reflecting both agents pushed to their respective MTDs was 7:1, equivalent to the optimal ratio when delivered in liposomes. In addition, the MTD of the free-drug cocktail was equivalent to that exhibited by CPX-571 when examined across a panel of tumor models (irinotecan/cisplatin doses ranging from 34:2.1 to 47:3 mg/kg, depending on the tumor model; see Fig. 2). These two features simplified direct efficacy comparisons between CPX-571 and free irinotecan/cisplatin cocktail groups because multiple comparisons based on matched ratios versus MTDs or relative potencies were not necessary.

In H69 human SCLC tumor xenograft studies, administration of CPX-571 on a (q4d×3)×2 schedule significantly enhanced survival of treated mice, resulting in 100% long-term tumor elimination and survival (>300 days after treatment) shown in Fig. 2A. Preclinical studies in the HT29 colon xenograft model showed that CPX-571 had substantially

greater tumor growth inhibition including tumor regression (Fig. 2B). The mean tumor volume regressed from 150 to 60 mg following completion of treatment with CPX-571. The free-drug combination counterpart did not cause any regression and tumor growth delay was minimal. Similarly, CPX-571 caused greater tumor regression than its free-drug counterpart against the Capan-1 pancreatic xenograft model (Fig. 2C). In this model, treatment with the CPX-571 resulted in a 60-day regression of all tumors, with the mean tumor size reduced to a low of 6 mg. During the same 60-day period in this experiment, the unencapsulated drugs caused minimal tumor regression followed by tumor progression. The effects of CPX-571 on tumor growth inhibition were also tested in the HCT-116 human colon xenograft model, and although no tumor regression was observed, CPX-571 treatment resulted in tumor growth delay of 44.5 days, compared with 19.5 days for free-drug cocktail (Fig. 2D).

CPX-571 was also compared with free-drug cocktail administered at varying dosing schedules, including a dosing schedule that mimics the clinical regimen where both cisplatin and irinotecan are administered on day 1 followed by irinotecan administered independently for the following two doses per cycle (22). This study was undertaken to ensure that the efficacy improvements observed above for CPX-571 were not biased due to suboptimal dosing of the free-drug cocktail. We observed that the absolute therapeutic activity of the free-drug cocktail could be increased by administering two courses of treatment with each course, consisting of three injections on a q4d schedule (Fig. 3). Alterations to the dosing schedule to mimic the clinical regimen provided equivalent efficacy. Administering CPX-571 on the (q4d×3)×2 schedule also increased the degree of tumor growth inhibition over single-course CPX-571 treatment with a LCK value of 4.2 (and 1 complete regression), a value markedly higher than that obtained with free-drug cocktail and one considered highly active for antitumor agents (23).

Efficacy of CPX-571 in an Irinotecan-Resistant Tumor Xenograft Model

One of the primary hurdles to successful treatments for SCLC is the emergence of resistant disease. Consequently, we examined the efficacy of free-drug cocktail and CPX-571 in an irinotecan-resistant variant of the HCT-116 solid tumor model (HCT-116IR, Fig. 4) to determine if controlling drug ratios *in vivo* may enhance the activity of this combination in resistant tumors. Mice were given saline, individual free drugs or free cocktail (Fig. 4A), or individual liposomal drugs or CPX-571 (Fig. 4B). In Fig. 4A, the tumor growth delay, based on the time for tumors to reach 400 mg, was determined for free-drug comparisons. The tumor growth delay was 7 days for cisplatin (LCK, 0.35), 14.5 days for irinotecan (LCK, 0.73), and 19 days for the combination of irinotecan and cisplatin (LCK, 0.95). The free-drug cocktail LCK value of 0.95 was less than the predicted additive value of 1.08 for the individual free cisplatin (LCK, 0.35) and free irinotecan (LCK, 0.73). Tumor growth delays for liposomal cisplatin, liposomal irinotecan, and CPX-571 were 10
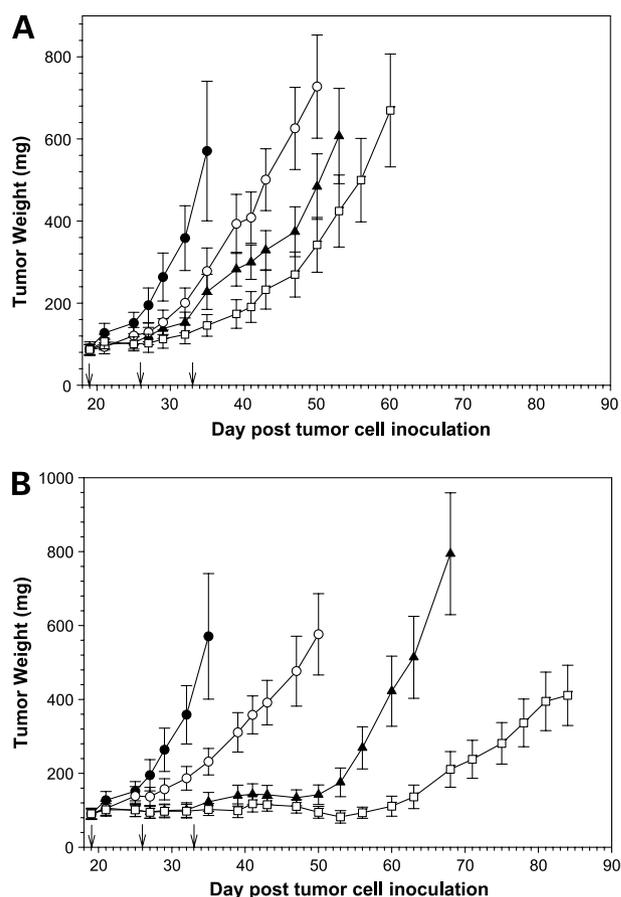
Figure 4. Antitumor efficacy of free-drug cocktail and CPX-571 in the irinotecan-resistant HCT-116IR colon tumor xenograft model. Tumor growth delay was assessed in this model using a q7d×3 schedule. **A,** mice received injections of saline (●), free cisplatin at 3 mg/kg (○), free irinotecan at 47 mg/kg (▲), or free-drug cocktail of irinotecan/cisplatin (7:1 molar ratio) at 47:3 mg/kg (□). **B,** mice received injections of saline (●), liposomal cisplatin at 3 mg/kg (○), liposomal irinotecan at 47 mg/kg (▲), or liposomal irinotecan/cisplatin (7:1 molar ratio) at 47:3 mg/kg (□). *Downward arrows,* injection days.

days (LCK, 0.5), 26 days (LCK, 1.31), and 48 days (LCK, 2.41), respectively. Not only was the absolute extent of antitumor activity for CPX-571 increased compared with free-drug cocktail but also the LCK value of 2.41 for CPX-571 was greater than the predicted additive value of 1.81 based on liposomal cisplatin (LCK, 0.50) and liposomal irinotecan (LCK, 1.31).

Discussion

Human clinical studies have shown that combination treatment with irinotecan and cisplatin is efficacious in a wide range of cancer types including SCLC (9–11, 24–26), NSCLC (27–29), ovarian cancer (30), upper gastrointestinal cancers (31–35), and other less prevalent tumor types (36–38). Based on phase III randomized trials, this combination remains a prospective treatment for extensive-stage SCLC, with response and survival end points comparable to that

of etoposide/cisplatin in the presence of reduced myelosuppression. However, these results have been somewhat disappointing in view of the promising synergy shown preclinically (5, 6), the broad-spectrum activity, and the historical clinical superiority of irinotecan over etoposide in a monotherapy setting (3), which together would suggest that irinotecan/cisplatin therapy should be therapeutically superior. In view of these apparent discrepancies, we investigated whether this drug combination exhibited drug ratio–dependent synergy and if ratiometric dosing using liposome delivery vehicles could significantly augment therapeutic activity.

Ratiometric dosing is an approach to develop drug combinations based on drug ratio–dependent synergy using nanoscale drug carriers *in vivo*. If the degree of synergy (and/or antagonism) depends on the molar ratio of irinotecan/cisplatin, then it may be expected that the differing distribution and elimination kinetics of the two individual agents administered in conventional aqueous formulations could lead to rapid changes in circulating and tissue-associated drug ratios resulting in the exposure of tumor cells to antagonistic drug ratios. This is relevant given that the treatment of SCLC cancer patients typically uses a regimen of irinotecan at 60 mg/m^2 i.v. on days 1, 8, and 15 and cisplatin at 60 mg/m^2 i.v. on day 1 of a 28-day cycle (22), resulting in constantly changing irinotecan/cisplatin ratios during the entire course of treatment. Controlling drug pharmacokinetics through liposome delivery provides an avenue whereby synergistic irinotecan/cisplatin ratios identified *in vitro* can be maintained for extended times after administration *in vivo* and antagonistic drug ratios can be avoided, with the potential benefit of improved efficacy for the combination.

Individual irinotecan and cisplatin liposomes were developed and evaluated for their ability to maintain coordinated pharmacokinetics. A liposome formulation was identified that coordinated the release of irinotecan and cisplatin such that the administered drug ratio was maintained within the plasma for more than 24 hours following injection, independent of the encapsulated drug ratio (Supplementary Fig. S1). The plasma half-life of the liposomes was ~16 hours, with drug half-lives of 6 hours. The decreased drug half life relative to the liposome half life reflected that the drugs were being made bioavailable in a time course commensurate with the accumulation of liposomes in solid tumors via the enhanced penetration and retention effect (39). These features allowed drug ratio dependency to be evaluated *in vivo* by comparing the efficacy of liposome formulations containing synergistic and antagonistic irinotecan/cisplatin ratios.

Irinotecan/cisplatin combinations were systematically screened against a panel of 20 tumor cell lines, which identified trends in synergy and antagonism. Unlike the combination of irinotecan and floxuridine investigated previously (13, 14), a 1:1 molar ratio of irinotecan/cisplatin was strongly antagonistic, consistent with previously published results (40). In the case of irinotecan/cisplatin, either high irinotecan concentrations (>4:1 irinotecan/cisplatin) or high cisplatin concentrations (≤1:4 irinotecan/cisplatin) were

synergistic. With this knowledge, liposome irinotecan:cisplatin formulations were tested against a variety of human tumor xenograft models at synergistic and antagonistic ratios. Liposome-encapsulated irinotecan/cisplatin molar ratios above 4:1 could be administered at high relative dose intensities and were more efficacious than molar ratios of 1:4 and below where low irinotecan doses resulted. Consistent with the *in vitro* studies, drug ratios identified as antagonistic provided inferior therapeutic activity *in vivo* compared with a synergistic drug ratio despite using 10-fold greater doses of irinotecan (with the same cisplatin dose). These results confirm that the enhanced efficacy observed for the synergistic ratio liposome formulations was not simply due to a general liposome effect on drug pharmacokinetic properties but rather showed the ability to translate *in vitro* informatics on drug ratio–dependent synergy to *in vivo* efficacy using drug delivery technology.

We found that fixed irinotecan/cisplatin molar ratios ranging from 5:1 to 10:1 were highly active in tumor xenograft models, resulting in greater antitumor activity than with the free-drug cocktail or either of the individual liposomal drugs at their respective MTDs. Because the 7:1 ratio was highly active, intermediate between the 5:1 and 10:1 ratios and further away from the antagonistic region, the 7:1 ratio formulation (CPX-571) became the lead candidate for further study. CPX-571 was markedly superior to the unencapsulated drugs using all dosing regimens tested. The superior therapeutic activity attained by CPX-571 compared with unencapsulated irinotecan and cisplatin may partially be explained by our results showing that administration of CPX-571 in H460 tumor–bearing mice leads to tumor accumulation of irinotecan/cisplatin molar ratios that were in the synergistic range (Supplementary Fig. S3), which was not observed for unencapsulated drugs. One of the main obstacles in the treatment of SCLC is recurrent disease and the emergence of drug-resistant tumor cells. Therefore, we evaluated CPX-571 compared with the irinotecan/cisplatin free-drug cocktail in the HCT-116 irinotecan-resistant tumor model. In these studies, the magnitude of CPX-571 therapeutic activity was maintained and evidence of synergy preserved as it was in the parental drug-sensitive tumor xenograft. These results indicate that CPX-571 may be a promising treatment for recurrent disease where response rates with currently available agents are 2- to 3-fold lower than for first-line treatment, presumably reflecting an increased resistance to chemotherapy.

The *in vitro* synergy from concomitant administration of irinotecan and cisplatin may arise through reciprocal effects whereby the drugs enhance each other's activity. Specifically, irinotecan/SN-38 inhibition of the topoisomerase I DNA repair activity may augment the persistence of DNA adducts following their initial formation (41) and enhance cisplatin accumulation (42). Conversely, cisplatin has been shown to down-regulate topoisomerase I activity, which correlates with increased irinotecan sensitivity (42, 43). Topoisomerase inhibitors also down-regulate the antiapoptotic protein Bcl-2 thereby sensitizing the cells to apoptosis (44). Thus, it is apparent that both drugs have the potential to

improve each other's relative efficacy by enhancing sensitivity to the other drug.

Based on the above putative interactions, however, one can also envisage conditions under which certain irinotecan/cisplatin ratios may be rendered antagonistic rather than synergistic. For example, exposure of irinotecan and cisplatin to cells results in single- and double-strand breaks and, ultimately, G_2 cell cycle arrest. It is known that irinotecan is most active during the S phase of the cell cycle and more cytotoxic with increasing time of drug exposure. Therefore, drug interactions that result in G_2 arrest but not sufficient for inducing apoptosis could result in antagonistic interactions. We observed a high degree of synergy at higher irinotecan/cisplatin ratios, which is consistent with previous findings where high drug concentrations increase the number of cells in G_1-S phases (45) and also exhibit cytotoxic activity against cells that are not in S phase (46). Such mechanisms could render either synergistic or antagonistic interactions between irinotecan and cisplatin depending on the drug/drug ratios, consistent with our observations here for both *in vitro* and *in vivo* systems.

The application of ratiometric screening data to identify synergistic drug combinations and deliver them within liposomal formulations has led to two ongoing clinical programs, one in colorectal cancer patients (CPX-1, a liposomal combination of floxuridine and irinotecan; see ref. 47) and one in acute myeloid leukemia patients (CPX-351, a liposomal combination of cytarabine and daunorubicin; see ref. 48), with encouraging early signs of antitumor activity in both cases. CPX-571, a fixed-ratio drug combination of irinotecan and cisplatin at a 7:1 molar ratio, optimizes enhanced synergy and improved efficacy with potential for clinical applications in the treatment of a range of solid tumors.

Disclosure of Potential Conflicts of Interest

All authors are employees of Celator Pharmaceuticals Corp. and have no other conflicts of interest with other companies to declare.

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