Supplemental Data

Methods

Liposome Preparation

The dissolved lipids were mixed with either saline for the preparation of cisplatin liposomes or 10 mM sodium gluconate/150 mM TEA (pH 7) for the preparation of irinotecan liposomes in a glass Applikon bioreactor (Foster City, CA, USA). The solution was mixed and heated to 60ºC with the aid of a water jacket while under continuous nitrogen sparging for 90 minutes. The liposome suspension was passed 10 times through an extrusion apparatus (Northern Lipids Inc., Burnaby, BC, Canada) containing two stacked 100 nm polycarbonate filters. The mean diameter and size distribution of each liposome preparation, analyzed by quasi-elastic light scattering NICOMP Model 270 Submicron particle sizer (Pacific Scientific, Santa Barbara, CA, USA) was typically 110 ± 20 nm. Following extrusion, cisplatin and irinotecan liposomes were buffer exchanged into 150 mM NaCl and 300 mM sucrose/20 mM phosphate, pH 7, respectively, by tangential flow chromatography.

Pharmacokinetic and Tumor Biodistribution Analysis

At various time points, up to 48 hours post-drug administration, blood was collected by cardiac puncture from euthanized mice. Cisplatin concentration was analyzed by atomic absorption spectroscopy (Varian Spectra AA-240 Zeeman Graphite Furnace atomic absorption spectrometer; Varian Canada Inc., Mississauga, Ontario, Canada) and lipid concentration was determined by quantifying tracer quantities of ^{3}H-CHE by liquid scintillation counting (Beckman Coulter, Fullerton, CA, USA).

Irinotecan was quantified as previously described (1). Briefly, separation was performed using a Waters Symmetry C18 column (Waters Corporation, Milford, MA, USA) with a Symmetry Sentry C_{18} guard column and a run time of 20 minutes at a flow rate of 1.5 mL/minute with a multi λ fluorescence detector (Waters Model 2475) set at excitation/emission wavelengths of 362/425 nm. The mobile phase was 1.5 mL/min acetonitrile - 75 mM ammonium acetate containing 7.5 mM tetrabutylammonium bromide (24:76, v/v, pH 6.4).

For tumor biodistribution studies in CD-1 nude mice, H460 human NSCLC xenografts with mean tumor volumes between 150 – 180 mg, were injected with ^{3}H-radiolabeled irinotecan/^{14}C-radiolabeled DPPC CPX-571. Following i.v. injection of radiolabeled CPX-571 in the lateral tail vein, mice were terminated and blood plasma was collected by cardiac puncture. The tumors were excised at time points between 2 and 48 hours post injection and each data point represents the average of five mice per time point. A blood volume correction factor of 6 µl/gram tumor was applied to each data point to account for plasma lipid and drug levels.

Results

Development of Irinotecan and Cisplatin Liposome Formulations that Maintain Drug Ratios *In Vivo*

Cisplatin was encapsulated into preformed liposomes using elevated temperatures in the presence of low amounts of ethanol and an efficient encapsulation method was utilized to entrap irinotecan based on the observation that triethanolamine (TEA)

contained inside the liposomes can actively encapsulate irinotecan through a charge neutral exchange process (2).

Following the preparation of the individual liposomal drugs, the formulations were mixed at an irinotecan:cisplatin molar ratio of 7:1 and injected intravenously into mice. Plasma levels of irinotecan and cisplatin revealed similar plasma clearance rates (Figure 1) with an elimination half-life of 5.7 hours for irinotecan and 6.9 hours for cisplatin. In studies using ^{3}H CHE, a non-exchangeable lipid marker, the plasma elimination half-life for the liposomes was approximately 16 hours (data not shown). This formulation maintained the irinotecan:cisplatin molar ratio in the plasma near the injected 7:1 ratio throughout the 24 hour time course (Figure 1 inset). In addition, an irinotecan:cisplatin liposome formulation containing the drugs at a 1:1 molar ratio also maintained the formulated ratio in the plasma compartment. The similarity in the plasma half-life for irinotecan and cisplatin and the increased plasma half-life for the liposomes relative to the drugs indicates that the two drugs were released from the liposomes and made bioavailable at comparable rates after i.v. injection independent of the starting irinotecan:cisplatin ratio. Plasma drug concentrations following free drug cocktail treatment fell to below detectable levels within 2h. During this time irinotecan:cisplatin molar ratios ranged from 133:1 to 58:1 (data not shown).

Irinotecan:Cisplatin Accumulation in Tumors Following Injection of CPX-571

We have shown enhanced therapeutic efficacy that is far superior to free drug cocktail when CPX-571 is delivered at irinotecan:cisplatin molar ratios greater than >4:1. To determine whether differences in efficacy may be attributed to increased accumulation

within tumor sites, we performed a biodistribution study. Mice bearing NSCLC H460 tumor xenografts were administered with CPX-571 or free drug cocktail at 34 mg/kg irinotecan and 2.1 mg/kg cisplatin. Irinotecan and cisplatin tumor levels were determined via liquid scintillation counting and AA spectroscopy, respectively. Two hours after injection of CPX-571, irinotecan and cisplatin drug concentrations in tumor tissue were 20.3 and 3.1 nmoles/gram tumor respectively, corresponding to a 6.6:1 irinotecan:cisplatin molar ratio (inset of Figure 3). By 24 hours, drug levels within the tumor had decreased to 17.2 nmoles irinotecan/gram tumor and 1.8 nmoles cisplatin/gram tumor resulting in a 9.5:1 irinotecan:cisplatin ratio. The irinotecan:cisplatin molar ratio ranged between 7:1 to 10:1 over the first 24 hours after injection and subsequently slowly decreased to 4:1 by 48 hours. Lipid accumulation in the tumor gradually increased from 116.8 nmoles lipid/gram tumor at 2 hours to 186.2 nmoles/gram tumor at 48 hours (data not shown). For free drug cocktail administered at the same dose, the irinotecan concentration at 2 hours was 6.3 nmoles/gram tumor, approximately 3.2-fold lower than irinotecan concentrations observed for mice administered CPX-571. For the same time point, tumor levels of cisplatin averaged to 1.8 nmoles/gram tumor and were below the limit of quantitation at every subsequent time point. As a result, the free cocktail delivered an irinotecan:cisplatin molar ratio of 3.5:1 to the tumor, a ratio shown to be antagonistic *in vitro*.

References

1. Messerer CL, Ramsay EC, Waterhouse D, et al. Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer. Clinical Cancer Research 2004; 10:6638-6649.

2. Dicko A, Tardi P, Xie X, Mayer L. Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes. Int J Pharma 2007; 337: 219-228.

Figure 1. Plasma drug concentrations for irinotecan and cisplatin determined following i.v. administration of CPX-571 in CD-1 nude mice. Following injection of CPX-571 at an irinotecan dose of 60 μmoles/kg (41 mg/kg) and a cisplatin dose of 8.6 μmoles/kg (2.6 mg/kg), mouse plasma was collected at 1, 2, 4, 8 and 24 hours post drug administration and assayed for (●) irinotecan and (○) cisplatin by HPLC. **Inset:** Based on the plasma drug levels, the molar ratio of irinotecan:cisplatin was calculated for two different molar ratio formulations; (■) 1:1, and (▲) 7:1 at the indicated time intervals for 24 hours. All data points represent the mean values obtained from three mice per time point, and the error bars represent the SE. For some data points, the error bars are smaller than the symbols.

Figure 2. Tumor growth inhibition studies comparing 1:5 and 2:1 irinotecan:cisplatin ratios while maintaining the cisplatin dose. Log cell kill (LCK) values were determined from tumor growth delay studies in mice implanted s.c. with H1299 human NSCLC cells and were administered irinotecan:cisplatin on a Q7Dx3 dosing schedule. The treatment groups consisted of liposomal cisplatin at 3.3 mg/kg, liposomal irinotecan:cisplatin (2:1 molar ratio) at 15/3.3 mg/kg, and liposomal irinotecan:cisplatin (1:5 molar ratio) at 1.5:3.3 mg/kg resulting in LCK values of 0.44, 0.68 and 1.11 respectively.

Figure 3. Accumulation of irinotecan and cisplatin in solid tumors following intravenous injection of CPX-571. Mice bearing H460 tumor xenografts were administered with CPX-571 at a dose of 34:2.1 mg/kg (7:1 molar ratio) irinotecan:cisplatin. Irinotecan (●)

and cisplatin (○) drug levels were measured from excised tumors by liquid scintillation counting and atomic absorption spectrometry, respectively, for up to 48 hours post injection. Inset: Irinotecan:cisplatin molar ratios were determined at each time point. All data points represent the mean values obtained from five mice per time point, and the error bars represent the SD.

Figure 1

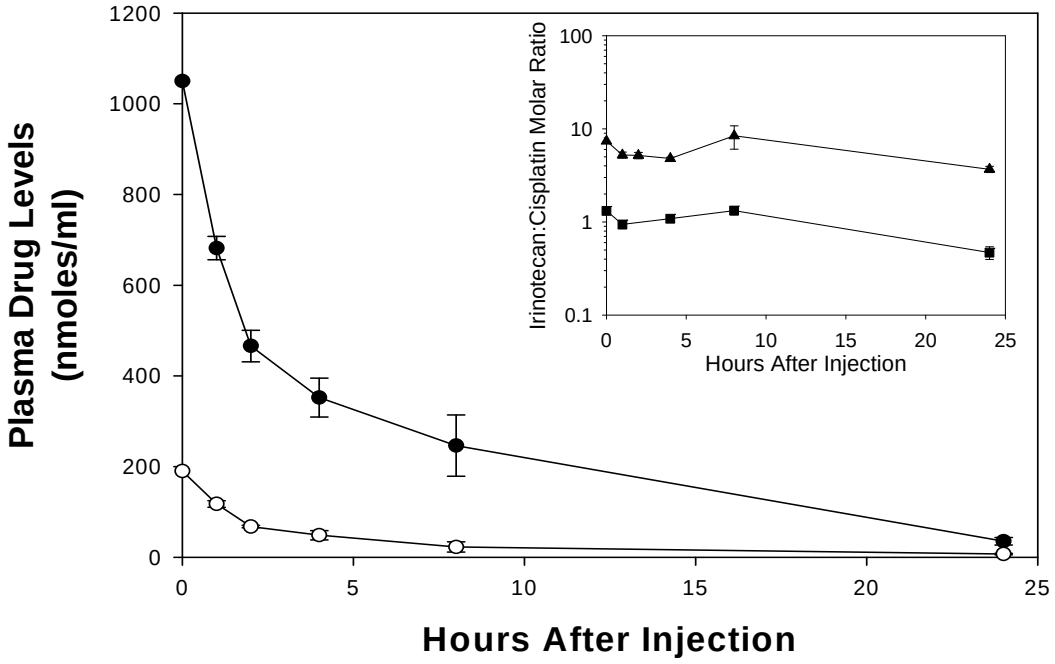

Plasma Drug Levels
(nmoles/ml)
1200
1000
800
600
400
200
0
0
5
10
15
20
25
Hours After Injection
Irinotecan:Cisplatin Molar Ratio
100
10
1
0.1
0
5
10
15
20
25
Hours After Injection

Figure 2

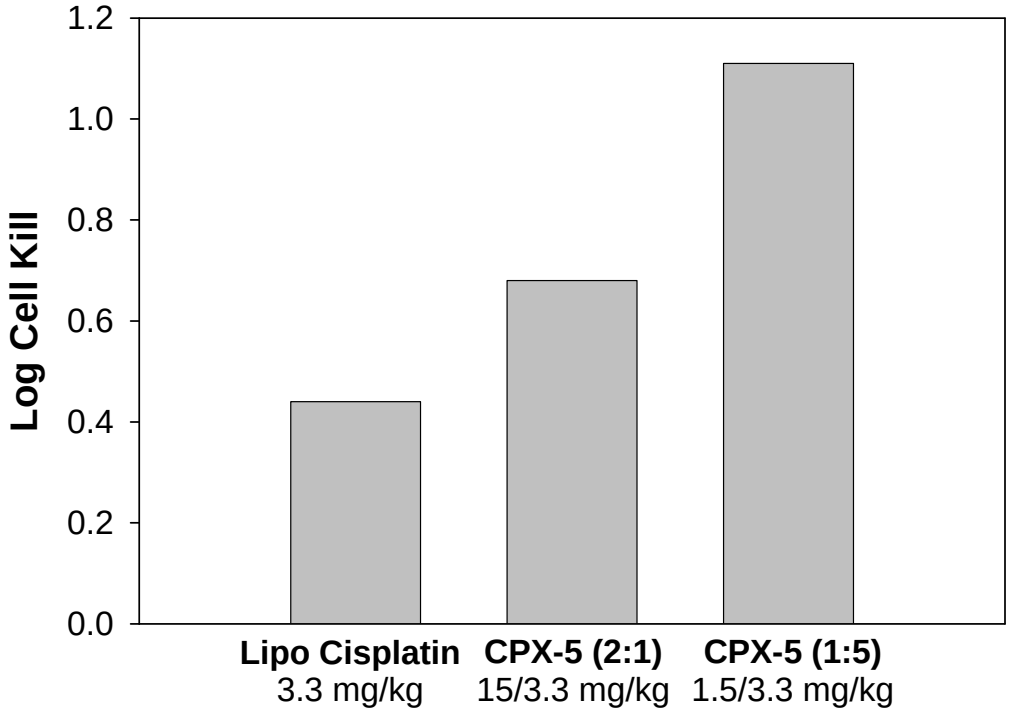
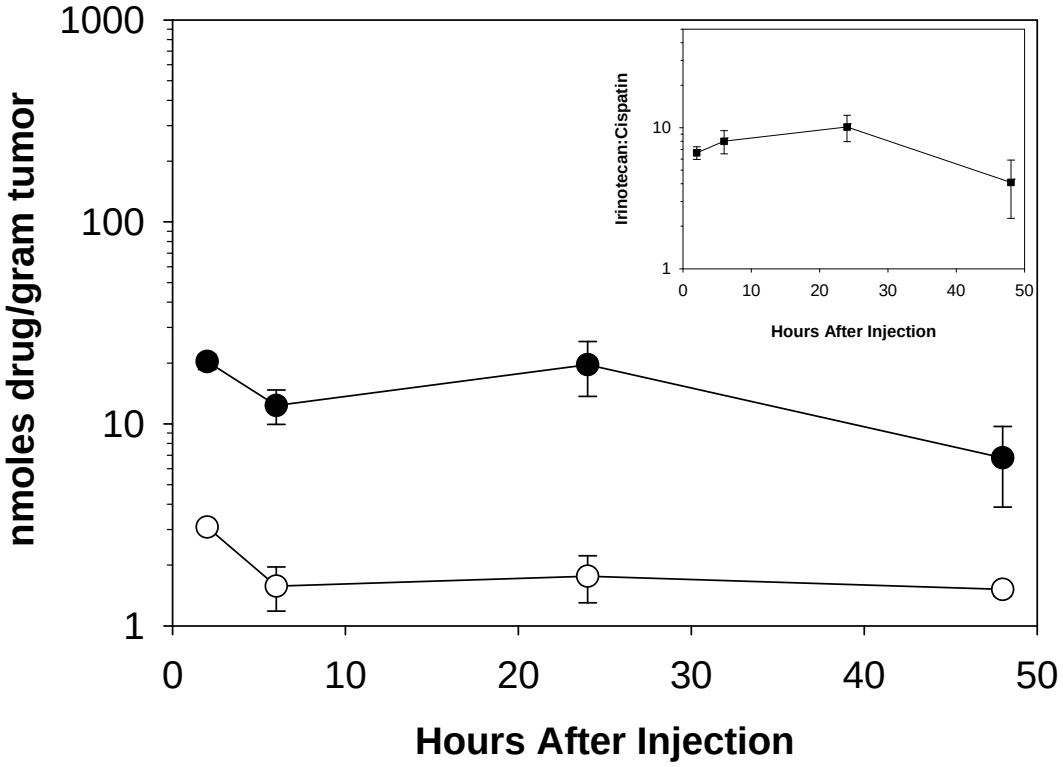

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Log Cell Kill
Lipo Cisplatin
3.3 mg/kg
CPX-5 (2:1)
15/3.3 mg/kg
CPX-5 (1:5)
1.5/3.3 mg/kg

Figure 3


1000
100
10
1
nmoles drug/gram tumor
0
10
20
30
40
50
Hours After Injection
Irinotecan:Cispatin
10
1
0
10
20
30
40
50
Hours After Injection