Supplementary Information

Supplementary Materials and Methods:

Synthesis of CRLX101 for rat PK study

CRLX101 was synthesized with different drug loading and polymer molecular weight for the rat PK experiment (Table 1) using methods described elsewhere (18).

Cryo-TEM Imaging

Samples of CRLX-101 (3 ul of a 2.5 mg/ml solution) were vitrified on R2-2 Quantifoil™ grids in liquid ethane using a Vitrobot™ (FEI Company, Hillsboro) under the conditions: 2 second blot, 1 second drain time, offset -4.5mm, 100% chamber humidity, 22.5 ºC chamber temperature. Images were collected on a Tecnai (FEI Company, Hillsboro) 120keV transmission electron microscope equipped with a Gatan 2k×2k UltraScan CCD camera (Gatan, Pleasanton, CA). At least 500 nanoparticles were sized using the "measure" function on ImageJ.

CRLX101 *In Vitro* Release Kinetics

Release of CPT from CRLX101 was conducted at 0.32 mg CPT/mL in BALB/c mice plasma, human plasma, and PBS at pH 7.4 in the presence and absence of 100 units/mL of butyrylcholinesterase and or 3 mg/mL LDL. CRLX101 was mixed into the relevant pre-warmed media and placed back into humidified incubator at 37 ^{o}C with 5% CO_2. Samples were taken out at predetermined time points and immediately frozen at -80 ^{o}C until time for analysis. Fraction of free and bound CPT was determined as previously described (14).

Plasma and Urine Pharmacokinetics (rat, dog, human)

Rats were dosed at 2.59 mg/kg (Fig. 2) or 3 mg/kg CRLX101 (CPT equivalent) and beagle dogs were dosed at 0.58 m/kg CRLX101 (CPT equivalent) by intravenous injection. Patients from the Phase 1 and 2a clinical trials were dosed as described previously (7). Blood was collected at

various time points and plasma was separated by centrifugation and frozen until sample analysis. Plasma was thawed and analyzed by HPLC for free, conjugated and total CPT, as described previously (14). PK curves were analyzed using WinNonLin (Pharsight Inc.). Patient urine from the Phase 1 and 2a clinical trials was analyzed for conjugated and released CPT using methods described elsewhere (7). Briefly, polymer was collected from the urine of patients and the CPT removed by treatment with NaOH (procedure similar to that used for blood and tissue PK).

Mouse Tumor PK

Tumor samples were homogenized in 1:1, DMSO:water, 0.1% formic acid. The homogenates were centrifuged and the supernatant collected for processing. Samples were treated with 2N NaOH to release all CPT from the polymer. Proteins were precipitated by the addition of acetonitrile. Samples were diluted with aqueous ammonium formate and assayed for CPT by HPLC/MS.

Measurement of Mouse Tumor Topoisomerase-1

$8x10^6$ NCI-H1299 tumor cells in 100 μL RPMI-1640 were implanted in the mammary fat pad of 6-7 week old female homozygous NCR nude mice (Taconic Farms, Germantown, NY) and were dosed intravenously once with 6 mg/kg CRLX101 when the average tumor volume reached 471 ±182 mm^3. At the time points indicated, mice were sacrificed, tumors were harvested and homogenized for immunoblotting. Immunoblotting was performed using anti-topoisomerase 1 (1:1000, Abcam, Cambridge, MA) and anti-actin (1:2500, Santa Cruz Biologicals, Santa Cruz, MA) antibodies. IRDye conjugated anti-mouse and anti-rabbit secondary antibodies (1:10000, LI-COR) were visualized on the Odyssey CLx infrared imaging system, and Western blots were quantified using Image Studio software (LI-COR Biosciences) and presented as the mean of three independent mice.

Tumor Penetration

$5x10^6$ NCI-H2122 tumor cells in 100 μL RPMI-1640 were implanted in the mammary fat pad of 6-7 week old female homozygous NCR nude mice (Taconic Farms, Germantown, NY) and were dosed intravenously once with 10 mg/kg CRLX101 when the average tumor volume reached 677 ± 85 mm^3. At the time points indicated, mice were sacrificed and the tumors were embedded in OCT and frozen. In order to positively identify CPT in these tissues, the entire tissue was scanned under epifluorescence that facilitated rapid screening of large areas of tissue for signal with CPT specific features, e.g., correct color and punctate signal as seen in the mouse xenograft images). Human gastric adenocarcinoma sample was obtained from a patient enrolled on a CRLX101 pilot trial and consented in accordance with City of Hope IRB guidelines (City of Hope IRB protocol #11276). The patient underwent endoscopic biopsies 48 hours post cycle 1, day 1 infusion of CRLX101. The biopsies were immediately frozen in OCT media on dry ice, and transferred to the City of Hope Translational Research Laboratory prior to processing. Sections of mouse xenografts and the human tumor biopsies were imaged on a Zeiss LSM 510 inverted confocal scanning microscope (Zeiss PlanApochormat×63/1.4 oil objective). The excitation wavelength for camptothecin was 710 nm (two-photon laser) and 488 nm for Sytox Green and Alexafluor488 labeled anti-rabbit secondary antibody for CD31 detection (CD31 antibody, BD biosciences). Their corresponding emission filters were 390-465 nm, 565-615 nm, and LP 510 respectively.

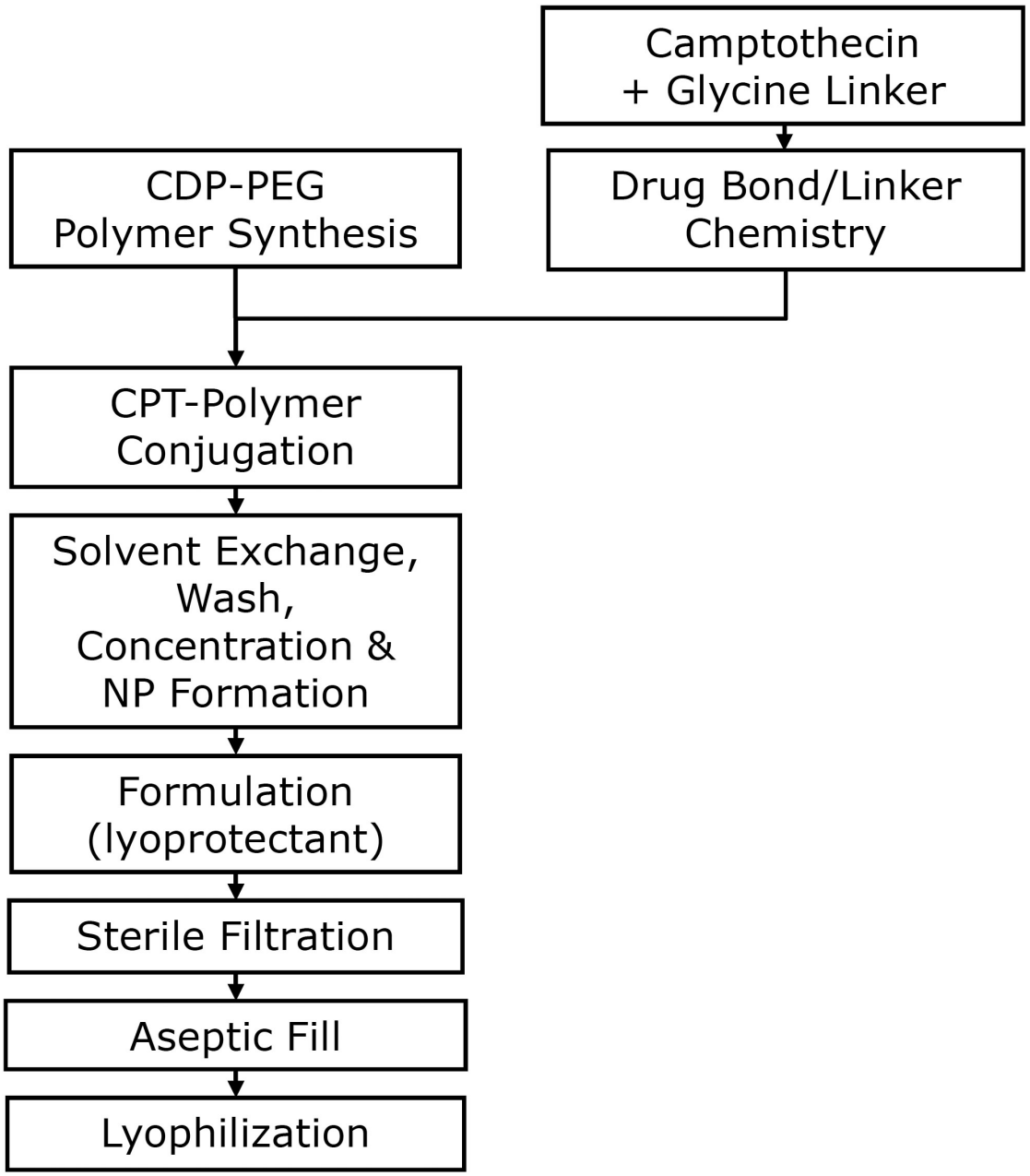


Figure S1. Schematic diagram of how CRLX101 is synthesized and formulated into vials.

Lot	Parent Polymer MW (kDa)	CPT Loading (wt%)	Particle Diameter (nm)
A	64	11.4	20 (22)[a]
B	65	10.0	20 (25)[a]
C	65	11.3	18 (18)[a]
D	63	10.7	21
E	66	8.6	23

[a]Value in parenthesis is from the size measured from cryo-TEM images.
Zeta potential measured in 10 mM phosphate buffer is ca. -5 mV.

Table S1. **Properties of clinical lots of CRLX101.** Nanoparticle sizes were measured using DLS (Brookhaven Instrument) or cryo-TEM (in parenthesis). Zeta potentials were measured on a Zeta Pals Instrument.

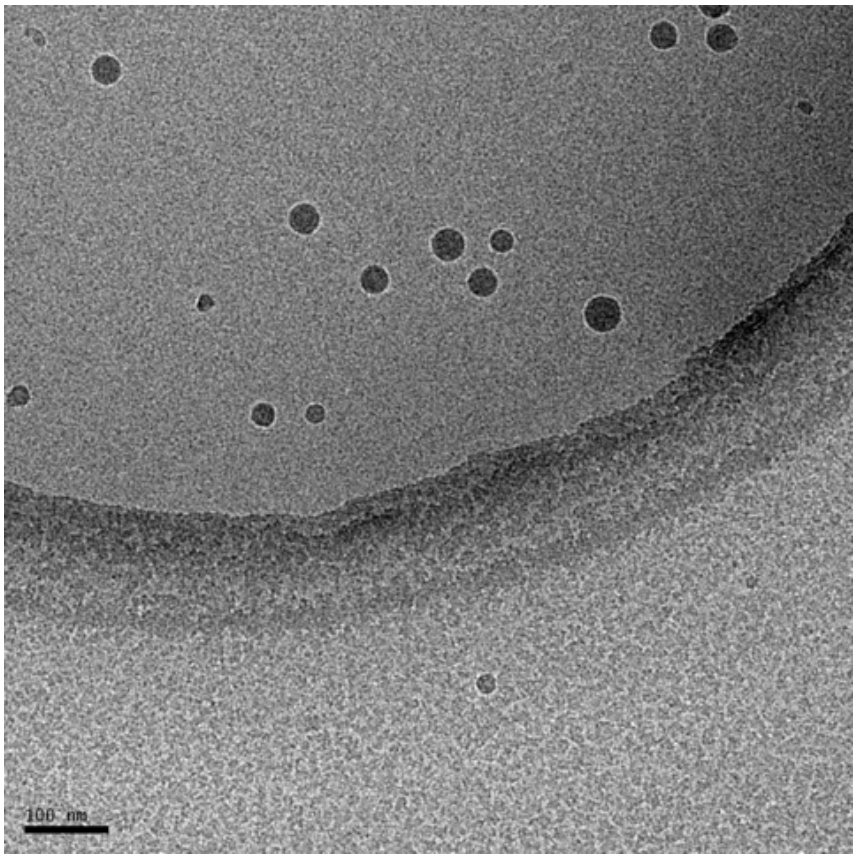


Figure S2. Representative cryo-TEM of CRLX101 (lot A).

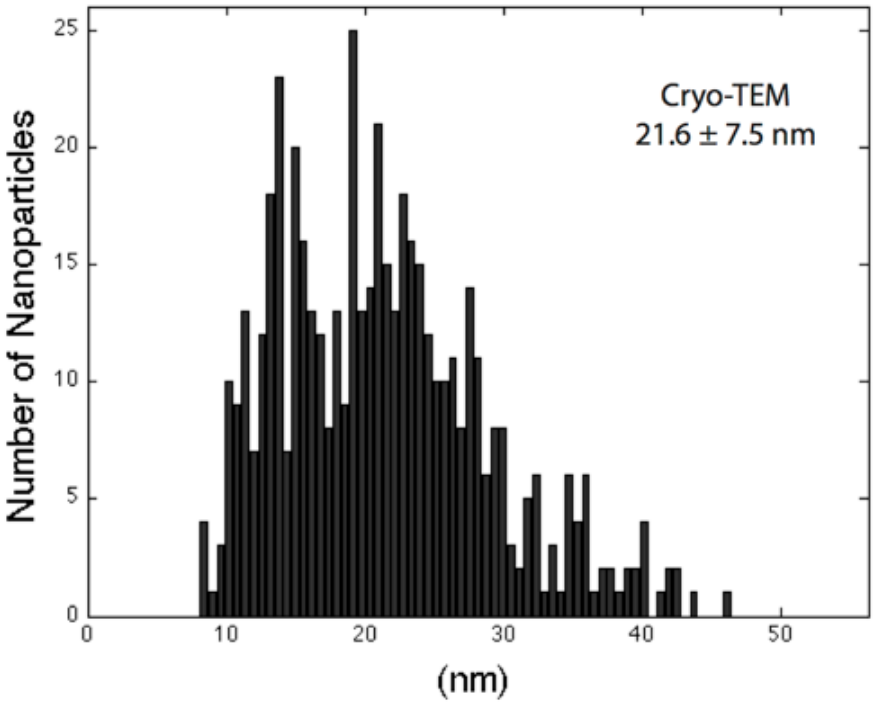


Figure S3. Cryo-TEM nanoparticle size distribution for lot A.

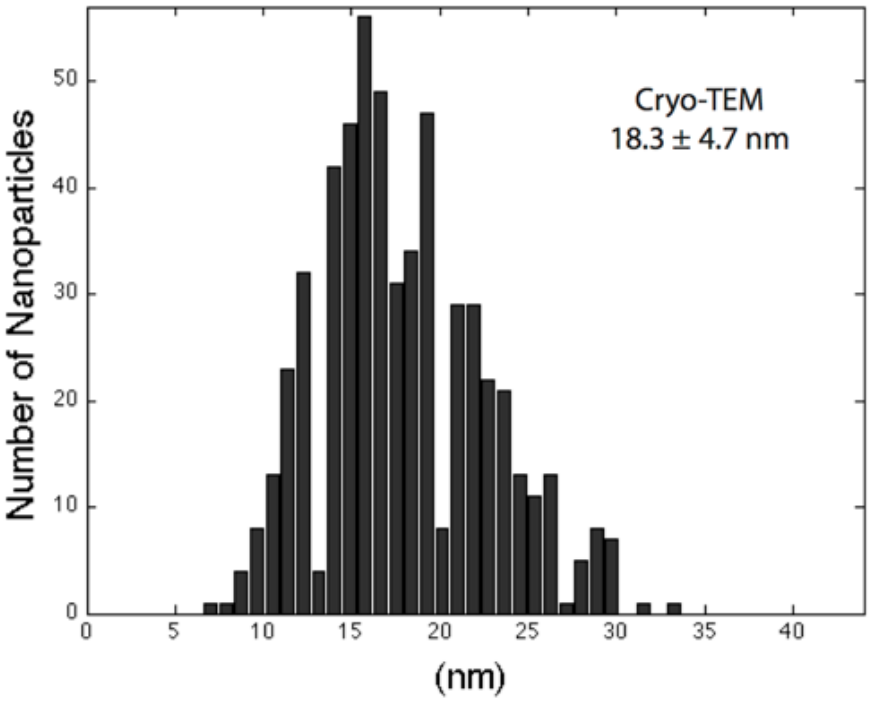


Figure S4. Cryo-TEM nanoparticle size distribution for lot C.

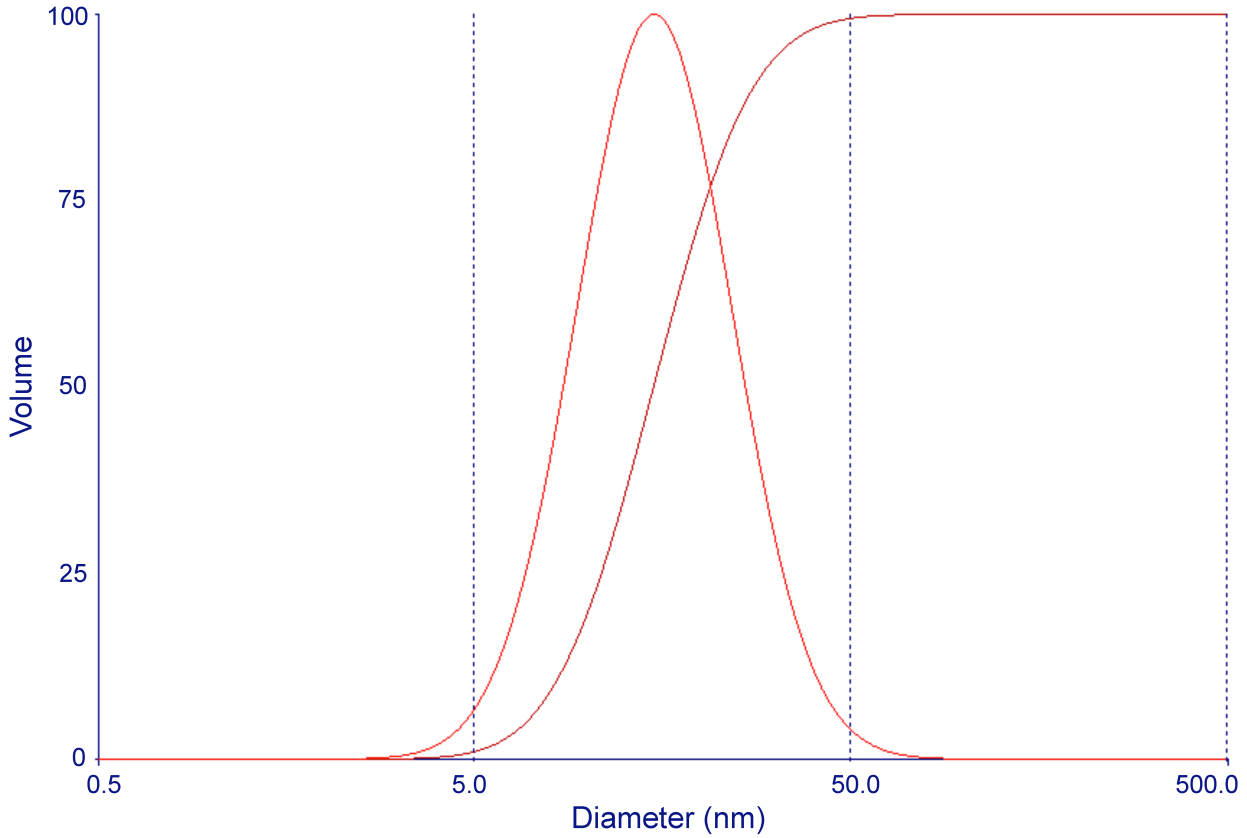


Figure S5. DLS nanoparticle size distribution for lot C.

Dose Level (mg/m^2)	Conjugated CPT					Unconjugated CPT			
	C_{max} (µg/L)	$T_{1/2}$ (h)	AUC_{inf} (h*mg/L)	CL (mL/h)	V_{ss} (L)	C_{max} (µg/L)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{inf} (h*mg/L)
6	3580 (457)	28.9 (5.0)	116 (22)	99 (22)	3.3 (0.6)	116 (61)	17.7 (10.3)	48.4 (16.1)	13.2 (7.0)
12	5620 (872)	30.2 (5.2)	188 (38)	132 (24)	4.6 (1.1)	203 (63)	19.3 (7.5)	50.1 (30.6)	16.6 (3.1)
15	7190 (1800)	31.5 (1.5)	335 (122)	94 (41)	2.3 (1.3)	268 (93)	24.5 (1.1)	43.3 (7.9)	25.4 (8.5)
18	8770 (1830)	33.5 (6.9)	291 (71)	103 (45)	3.5 (1.6)	351 (146)	22.8 (6.5)	41.9 (10.6)	31.5 (10.9)

Table S2. Human PK parameters as a function of dose level in the Phase 1a clinical trial. Numbers are represented as mean and standard deviation (in parentheses). Six patients were evaluated at each dose level. CRLX101 lots A and E were used in this study.

Dose #	N	Conjugated CPT					Unconjugated CPT	
		C_{max} (μg/L)	$t_{1/2}$ (h)	AUC_{inf} (h*mg/L)	CL (mL/h)	V_{ss} (L)	C_{max} (μg/L)	t_{max} (h)
Cycle 1 Day 1	6	7333 (1777)	27.6 (2.)	270 (53)	105.7 (19.1)	2.8 (0.5)	363 (292)	24.9 (.2)
Cycle 6 Day 1	6	7188 (1795)	27.5 (3.8)	335 (122)	94 (41)	2.3 (1.3)	302 (90)	22.1 (7.3)

Table S3. Human PK parameters as a function of dose from the Phase 2a clinical trial. All patients were dosed at 15 mg/m^2 every 14 days. Numbers are represented as mean and standard deviation (in parentheses). There were no statistically significant differences between doses, as determined using unpaired, two-tailed t-test (non-equal variances). CRLX101 lots A, B and C were used in this study.

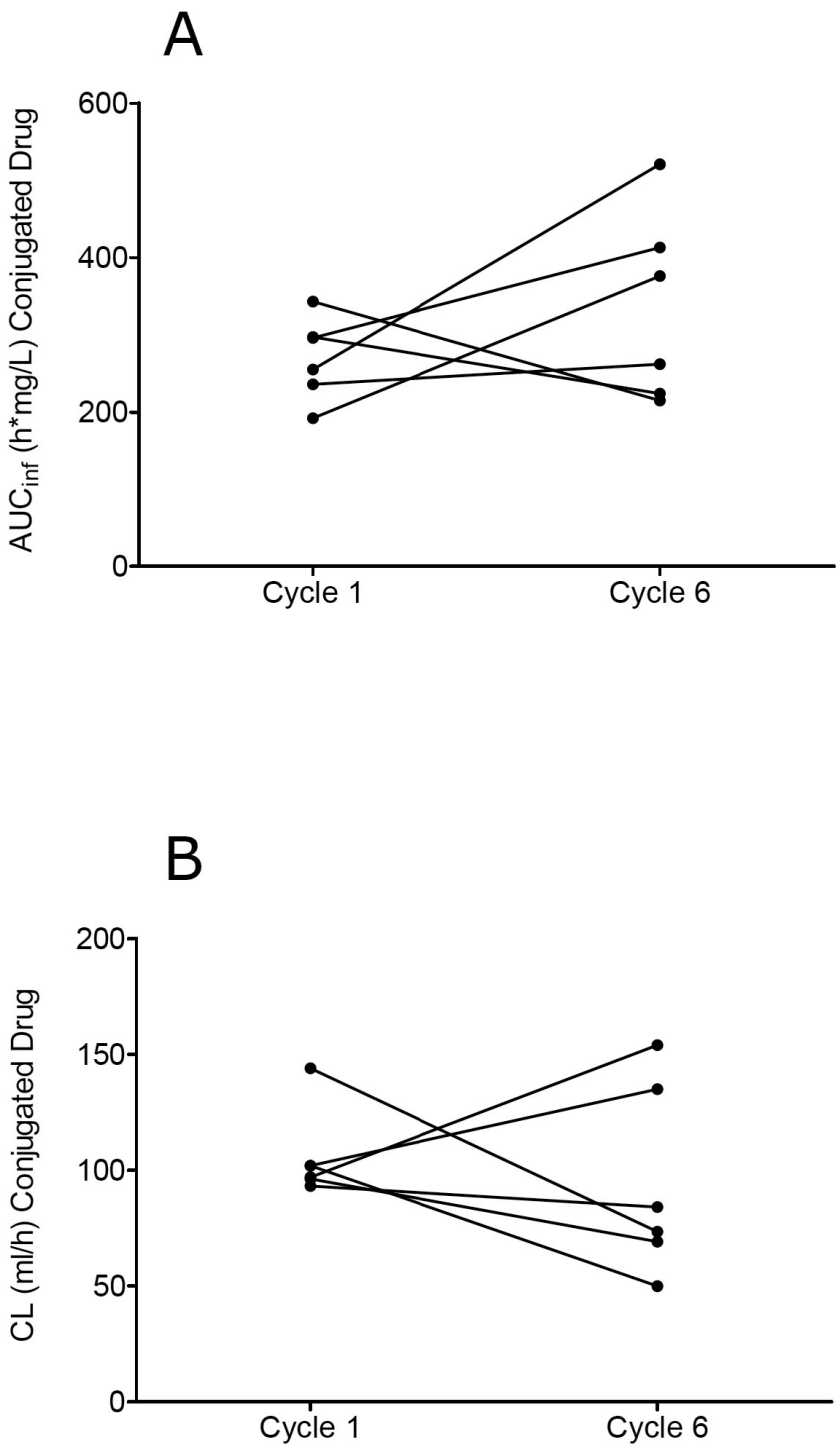
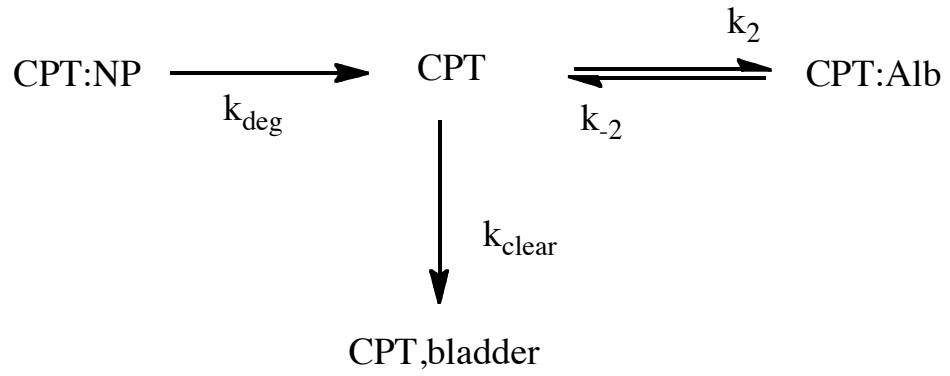


Figure S6. Human PK did not change between the first dose (Cycle 1, Day 1) and the eleventh dose (Cycle 6, Day 1). Shown are (A) exposure (AUC) and (B) clearance. Lines connect individual patients. CRLX101 lots A, B and C were used in this study.

Text

Modeling of the PK Dynamics

In order to model the dynamics of the various forms of CPT in the blood, we developed a mathematical model from the following scheme that is used to represent the dynamics in the blood of animals and humans:



CPT = Camptothecin that is not in the nanoparticle or bound to albumin (moles)
CPT:NP = Camptothecin that is in the nanoparticles (moles)
CPT:Alb = Albumin bound camptothecin (moles)
CPT,bladder = Camptothecin excreted by kidneys into the urine (moles)

The rate equations for the various forms of CPT can be written as a series of ordinary differential equations:

$$\frac{dN_{CPT:NP}}{dt} = -k_{deg}N_{CPT:NP}$$

$$\frac{dN_{CPT,bladder}}{dt} = k_{clear}N_{CPT}$$

$$\frac{dN_{CPT:Alb}}{dt} = k_2 N_{Alb} N_{CPT} - k_{-2}N_{CPT:Alb}$$

$$\frac{dN_{CPT:Alb}}{dt} = k_{deg}N_{CPT:NP} - k_{clear}N_{CPT} - k_2 N_{Alb} N_{CPT} + k_{-2}N_{CPT:Alb}$$

with initial conditions of (the initial amount of CPT in the nanoparticles is representative of the values used in our animal and human studies):

$N_{CPT:NP} = 1.45 \times 10^{-4}\ mol$
$N_{CPT} = 0\ mol$
$N_{CPT,bladder} = 0\ mol$
$N_{CPT:Alb} = 0\ mol$

These equations describe the nanoparticle release of CPT, the urinary clearance of non-bound CPT and the binding of CPT to albumin. In order to have estimates of the rates of CPT release and non-bound CPT clearance, we calculated rate constants from the experimental half-lives of the nanoparticles and CPT, respectively:

$$t_{\frac{1}{2}}(CPT:NP) = 24\ hr \quad \rightarrow \quad k_{deg} = {-\log(0.5)}/{24\ hr} = 0.0125\ hr^{-1}$$

$$t_{\frac{1}{2}}(CPT) = 0.26\ hr \quad \rightarrow \quad k_{clear} = {-\log(0.5)}/{0.26\ hr} = 1.16\ hr^{-1}$$

CPT is assumed to quickly reach equilibrium with CPT:Alb and so constants k_2 and k_{-2} can be constrained by:

$$K_{eq} = \frac{N_{CPT:Alb}}{N_{CPT}N_{Alb}} = \frac{k_2}{k_{-2}}$$

The amount of albumin in humans is 3.3 X 10^{-3} moles. Since this value is well above the amount of CPT administered, the fraction of albumin that is bound with CPT at any given time is small. Thus, for the simulations, we let the value for unbound albumin be constant. We solved the set of governing differential equations using Matlab with ode solver ode15s. The values for k_2 and K_{eq} are not known and were chosen in order to mimic the human data. The results give $k_2 = 6 \times 10^3$ hr^{-1} $mole^{-1}$ and $K_{eq} = 3 \times 10^3$ $mole^{-1}$ and the dynamics are shown in Fig. S7.

The same governing differential equations, initial conditions, and rate constants apply to SN-38, except for its binding strength to human albumin, described by K_{eq}, that is reported to be 50 times lower than CPT (1). Decreasing K_{eq} to 60 $mole^{-1}$ the blood profiles for SN-38 are illustrated in Fig. S8.

References:

1. Y. Kurono, M. Miyajima, K. Ikeda, Interaction of Camptothecin Derivatives with Human Plasma Proteins *in Vitro*. *Yagaku Zasshi* **113**, 167-175 (1993).
2. Z. Mi, T. G Burke, Marked Interspecies Variations Concerning the Interactions of Camptothecin with Serum Albumins: A Frequency-Domain Fluorescence Spectroscopic Study. *Biochemistry* **33**, 12540-12545 (1994).

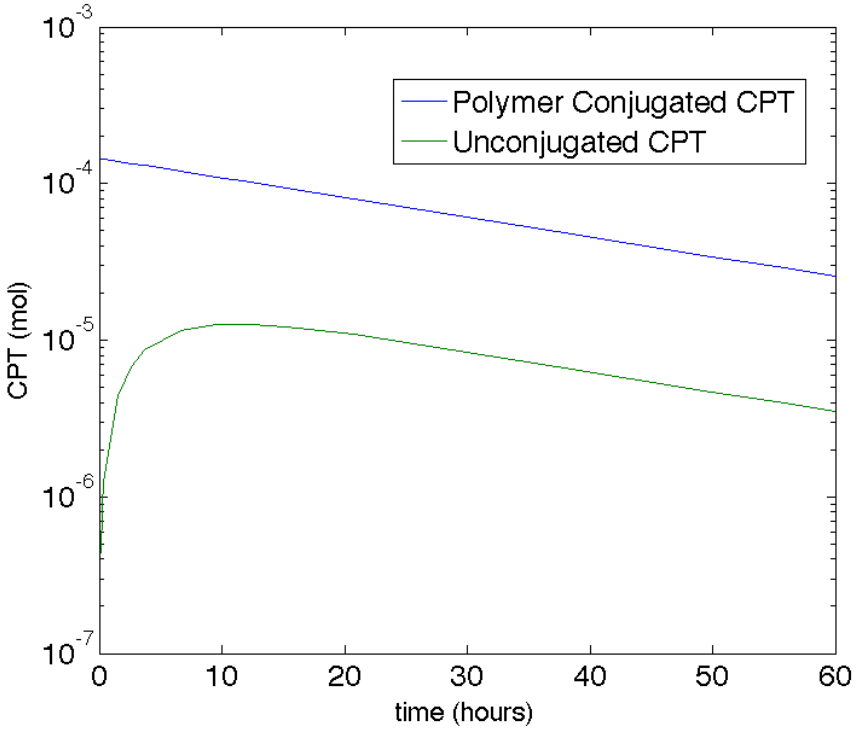


Figure S7. Simulated CPT profiles.

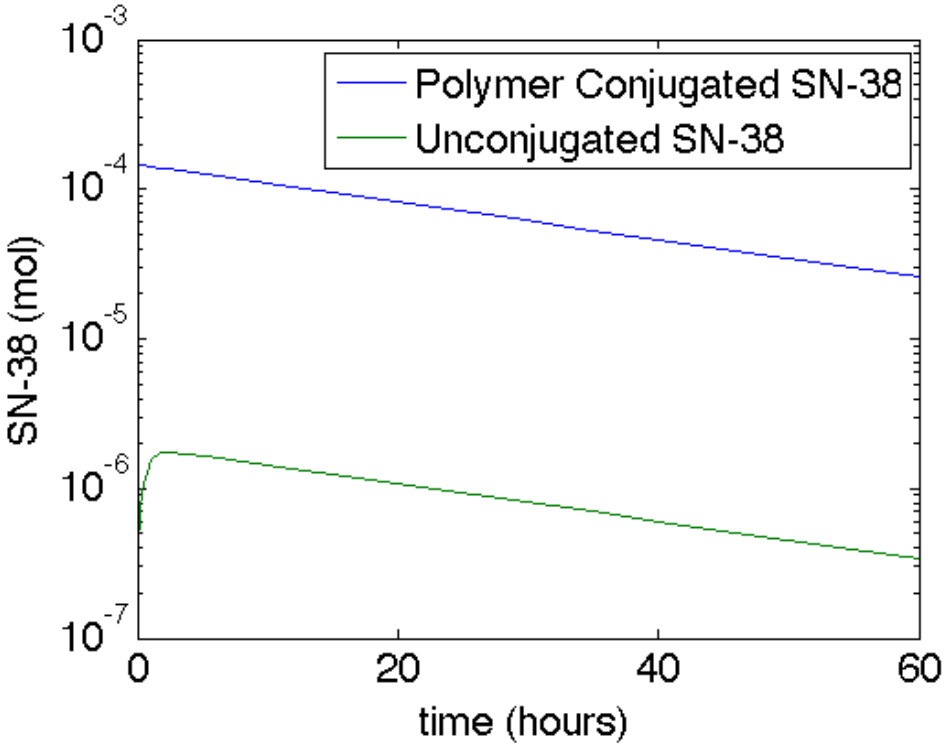


Figure S8. Simulated SN-38 profiles.

Formulation	Physical Properties			Conjugated CPT			Unconjugated CPT	
	Parent Polymer MW (kDa)	Loading (weight %)	Particle Size (nm)	Cmax (ug/mL)	$T_{1/2}$ (h)	AUC (hr*ug/mL)	Cmax (ug/mL)	AUC (hr*ug/mL)
CRLX101	64	11.4	20	74.5 (13.9)	18.9 (2.4)	874 (29)	1.27 (0.78)	5.69 (1.38)
LMW-LL	47.5	5.4	23	84.1 (10.8)	18.1 (3.7)	768 (36)	1.53 (0.39)	6.98 (1.53)
LMW-HL	47.5	10.8	17	85.2 (2.8)	23.9 (1.6)	931 (25)	1.05 (0.21)	4.92[3] (0.70)
HMW-LL	86.6	6.4	31	93.2 (4.8)	18.0 (3.4)	1200 (78)	1.57 (0.16)	8.95 (0.61)
HMW-HL	86.6	9.9	31	78.4 (5.2)	21.2 (3.3)	1069 (61)	0.88 (0.18)	5.00 (0.79)

Table S4. Rat PK parameters as a function of formulation, where polymer molecular weight and drug loading were varied.

Lot	N	Conjugated CPT					Unconjugated CPT			
		C_{max} (µg/L)	$T_{1/2}$ (h)	AUC_{inf} (h*mg/L)	CL (mL/h)	V_{ss} (L)	C_{max} (µg/L)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{inf} (h*mg/L)
A	24	8818 (1300)	28.3 (3.7)	298 (67)	97 (30)	2.8 (1.1)	297 (80)	25.6 (8.6)	46.2 (11.5)	31.9 (11.5)
B	18	7694 (1238)	28.5 (1.9)	312 (48)	88 (22)	2.3 (0.6)	305 (217)	21.3 (7.9)	42.5 (11.)	23.4 (4.3)

Table S5. Human PK parameters from patients receiving doses from two different lots in the Phase 2a clinical trial. Numbers are represented as mean and standard deviation (in parentheses). N= the number of patients.

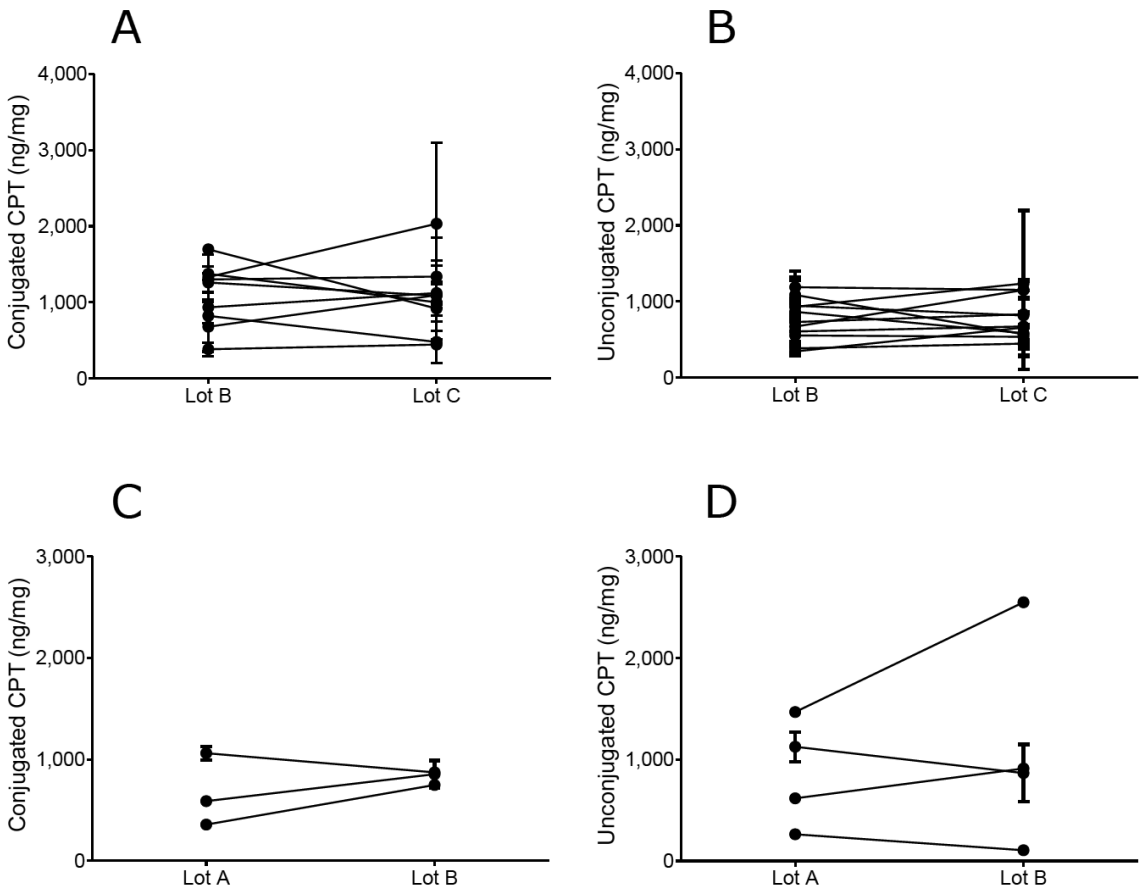


Figure S9. No consistent changes in patient urine excretion were observed when individual patients were dosed with two different lots of CRLX101. Data points represent total conjugated (A,C) and unconjugated (B,D) CPT measured in urine, normalized to the dose delivered to patients that were dosed with two different lots of CRLX101 at different times during their treatment course. Data points represent mean and error bars represent standard deviation. Lines connect individual patients.

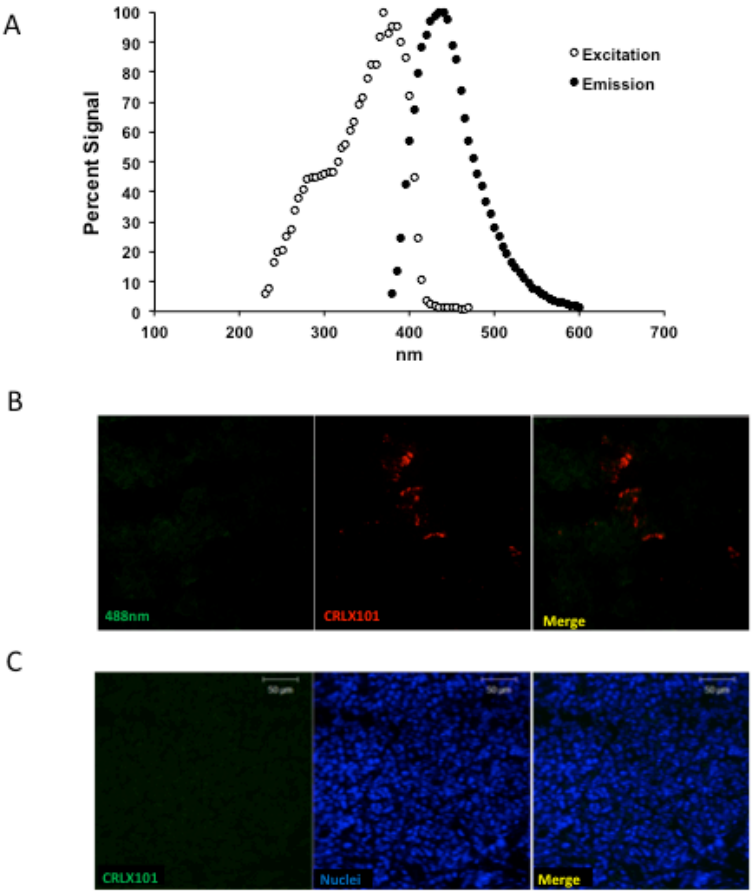


Figure S10. Fluorescence from CPT component of CRLX101. (A) Fluorescence spectrum of camptothecin. Ex_{max} = 370 nm and Em_{max} = 435nm. (B) Confocal micrograph of a section of NCI-H2122 Kras mutant NSCLC xenografts 24hrs after dosing with CRLX101. Sections with anti-rabbit secondary only, demonstrating no non-specific signal in the 488nm channel. (C) Confocal micrograph of a section of NCI-H2122 Kras mutant NSCLC xenografts 24hrs after dosing with vehicle control only (no CPT). Autofluorescence was observed in 370nm channel used to image CRLX101.

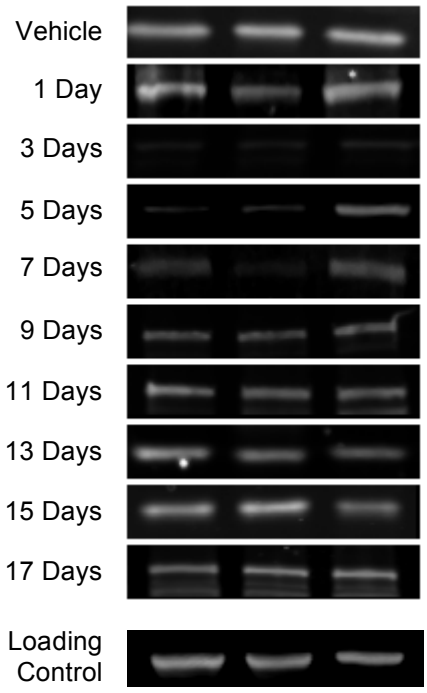


Figure S11. Inhibition of Topoisomerase-1 by CRLX101. NCI-H1299 NSCLC tumor-bearing mice were injected IV with CRLX101 at 6 mg/kg and topoisomerase-1 expression was measured in tumors at the time points indicated after treatment via Western blots. These data were used to generate the plot shown in Fig. 5D.

Figure S12. Inhibition of HIF-1alpha by CRLX101. NCI-H1299 NSCLC tumor-bearing mice were injected IV with CRLX101 at 6 mg/kg and HIF-1alpha expression was measured in tumors 72 hours after treatment via Western blots.