Supporting Information

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SI Materials and Methods

General Method for Synthesis of Ionizable β-Cyclodextrins. β-Cyclodextrin (Sigma) was monotosylated with a 0.9 molar equivalent of tosyl chloride in pyridine at the primary 6′-hydroxyl group to afford the corresponding tosylate, which was converted to the iodo derivative by treatment with a large excess of sodium iodide in acetone. The iodo derivative was converted to the desired aminated cyclodextrin by heating at 80 °C for 8–12 h with a fivefold molar excess of the appropriate amine (1). 6′-Mono-succinyl-β-cyclodextrin was synthesized by treatment of parent β-cyclodextrin with a 0.9 molar equivalent of succinic anhydride in dimethylformamide (DMF) (2). The product was precipitated in acetone and purified by HPLC before use. 6′,6′,6′,6′,6′,6′,6-Heptakis-succinyl-β-cyclodextrin was synthesized from β-cyclodextrin by treatment with excess succinic anhydride in DMF and precipitated with acetone. Fractional crystallization afforded the desired compound at ∼85% purity.

Dansylated cyclodextrins I, IV, and V were synthesized from commercially available β-cyclodextrin and compounds II and III, respectively, by treatment with a 0.9 molar equivalent of dansyl chloride in pyridine.

Each intermediate and the final product were purified by HPLC using a preparative C18 column and linear gradients of 0–95% solvent B (acetonitrile) in solvent A (water). All cyclodextrins were characterized by ^{1}H NMR and electrospray ionization MS and matched with previously published literature references.

6′,6′,6′,6′,6′,6′,6′-Heptakis-amino-β-cyclodextrin was purchased from CTD Holdings and used without further purification.

BI-2536 (3) and PD-0325901 (4) were synthesized and formulated for in vivo experiments as previously described.

General Procedure for the Preparation of Liposomes. Hydrogenated egg phosphatidylcholine (Avanti Polar Lipids), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG 2000) (Avanti Polar Lipids) (molar ratios 50:45:5) were dissolved in chloroform (20 mL). The solvent was removed in vacuo to give a thin lipid film, which was hydrated by shaking in the appropriate buffer (PBS, pH 7.4; 200 mM citrate, pH 4.0; or 80 mM Arg·Hepes, pH 9.0) at 50 °C for 2 h. The vesicle suspension was sonicated for 30 min and then extruded successively through 0.4-, 0.2-, and 0.1-μm polycarbonate membranes (Whatman; Nuclepore Track-Etched Membrane) at 50 °C to obtain the final liposomes with low polydispersity at the desired size. The transmembrane gradient was then created by equilibrium dialysis of the liposomes against 300 mM sucrose or PBS overnight. The average size and polydispersity index were then measured by dynamic light-scattering experiments on a Zetasizer Nano ZS90 (Malvern Instruments) at a wavelength of 633 nm and a 90° detection angle.

Protocol for Passive Loading of Liposomes. ***Method 1: Encapsulation of BI-2536 in the lipid layer.*** Hydrogenated egg phosphatidylcholine (Avanti Polar Lipids), cholesterol, and DSPE-PEG2000 (Avanti Polar Lipids) (molar ratios 50:45:5) were dissolved in chloroform (20 mL). Ten milligrams of BI-2536 (in 1 mL chloroform) was added and the solvent was evaporated to generate a thin film. One milliliter of PBS (pH 7.4) was added to hydrate the lipid layer, and the mixture was shaken at 50 °C for 2 h as previously described. The vesicle suspension was sonicated for 30 min and then extruded successively through 0.4-, 0.2-, and 0.1-μm polycarbonate membranes (Whatman; Nuclepore Track-Etched Membrane) at 50 °C to obtain the final liposomes with low polydispersity at the desired size. The liposomes were then dialyzed in PBS overnight to remove unentrapped drug. The average size and polydispersity index were then measured by dynamic light-scattering experiments on a Malvern ZS90. Drug content was calculated by rupturing the liposomes with an equal volume of methanol and measuring the UV-vis absorbance on a NanoDrop 1000.

Method 2: Hydration of the lipid layer with an aqueous formulation of BI-2536. Hydrogenated egg phosphatidylcholine (Avanti Polar Lipids), cholesterol, and DSPE-PEG2000 (Avanti Polar Lipids) (molar ratios 50:45:5) were dissolved in chloroform (20 mL), and the solvent was evaporated in vacuo to generate a thin film. One milliliter of aqueous BI-2536 (4 mg; pH 5.5) was added to hydrate the lipid layer, and the mixture was shaken at 50 °C for 2 h as previously described. The vesicle suspension was sonicated for 30 min and then extruded successively through 0.4-, 0.2-, and 0.1-μm polycarbonate membranes (Whatman; Nuclepore Track-Etched Membrane) at 50 °C to obtain the final liposomes with low polydispersity at the desired size. The liposomes were then dialyzed against PBS overnight to remove unentrapped drug. The average size and polydispersity index were then measured by dynamic light-scattering experiments on a Malvern ZS90. Drug content was calculated by rupturing the liposomes with an equal volume of methanol and measuring the UV-vis absorbance on a NanoDrop 1000.

General Procedure of Preparation of Encapsulated Complexes. Equimolar quantities of the drug (0.1 mmol; 30–50 mg) and appropriate cyclodextrin (0.11 mmol; 110–185 mg) were dissolved separately in methanol (nearly saturated ∼1–2 mL) and deionized water (∼10–20 mg/mL), respectively. The methanolic solution of the drug was then added dropwise into the cyclodextrin solution with agitation, ensuring a uniform suspension. This suspension was then shaken at 55 °C for 36–48 h using an Eppendorf Thermomixer R. The solution was filtered to remove particulate matter and flash-frozen in a dry ice/acetone bath followed by lyophilization. The lyophilized complex was stored at −20 °C until further use.

General Protocol for Remote Loading of Liposomes. The lyophilized powder complex previously described was pulverized and incubated with appropriate liposomal solutions (30–40 mg drug equivalent in 6 mL liposomal solution, to achieve loading ratios of 5–8 mg/mL concentrations) for 1 h at 65 °C. They were centrifuged at 1,000 × *g* for 3 min to remove particulate matter and then dialyzed against 300 mM sucrose or commercial PBS solution (pH 7.4) overnight to remove material that had not been loaded into the liposomes. The size distributions of the liposomal formulations were characterized using a Malvern ZS90. Concentrations of BI-2536 and PD-0325901 in liposomes were measured in triplicate using a NanoDrop 1000 after disruption of the liposomal solutions with equal volumes of methanol at 367 nm for BI-2536 and 277 nm for PD-0325901.

Tissue Biodistribution Study. Coumarin 334 was used as a drug surrogate to assess biodistribution and pharmacokinetics of cyclodextrin-encapsulated liposomes. Coumarin 334 (3 mg) was dissolved in methanol (6 mL) and added dropwise to an aqueous solution of cyclodextrin VI (14 mg in 20 mL water). The solution was shaken at 55 °C for 48 h and lyophilized. The lyophilized powder was incubated with citrate liposomes (internal pH 4.0) at 65 °C for 1 h. The liposomal solution was dialyzed against PBS

overnight. To assess loading efficiency, 100 μL liposomes was broken with 100 μL methanol and analyzed for fluorescence. The loading efficiency was found to be 90%. Female athymic BALB/c nude mice bearing HCT116 subcutaneous xenografts were used in the study following a modified protocol (5). When the tumor volumes reached 400–600 mm^3, 12 mice were treated i.v. with 200 μL cyclodextrin-encapsulated, liposomal (CYCL-)coumarin 334 (0.5 mg/mL). Posttreatment, four mice were euthanized at time points 2, 24, and 48 h and tumor, spleen, liver, kidneys, heart, and lungs were excised and weighed. Blood was also collected, and plasma was separated and stored at 4 °C. Except for plasma, each frozen tissue was homogenized and sonicated in 0.9% saline [3× volume (μL) of tissue mass (mg)]. Methanol was added to a final volume of 33% (vol/vol) with vortexing. The samples were centrifuged (6,000 × *g*, 10 min) and the fluorescence in the supernatants was measured by a CytoFluor II fluorescence multiwall plate reader (Applied Biosystems) using excitation 485 nm/emission 530 nm. As a control for tissue autofluorescence, tumor-bearing animals treated with equivalent volumes of empty liposomes were euthanized and their tissue and plasma were harvested.

Studies in Mice. All animal experiments were designed in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* (6) and were approved by The Johns Hopkins University's Institutional Animal Care and Use Committee.

Five million HCT116 ($p53^{-/-}$), HCT116 ($p53^{+/+}$), or RKO cells were injected s.c. into the flanks of female athymic BALB/c nude mice and allowed to grow for 3 wk, reaching 300–400 mm^3 in volume. In the case of CYCL-BI-2536, the animals were then randomly segregated into four arms. In all cases, liposomal formulations (CYCL-drug) have been reported as equivalents of free drug. Over the course of 2 wk, the first arm received empty liposomes; the second arm received a single dose of a 100 mg/kg formulation of the free drug twice using a formulation reported in literature (at days 0 and 7); the third and fourth arms received 100 and 400 mg/kg, respectively, of the CYCL-BI-2536 liposomal formulation at the same time points. Tumor volume was recorded every 48 h. The average tumor size for each respective group was normalized to the tumor volume on the first day of treatment. In the case of CYCL-PD-0325901, the first arm was treated twice with empty liposomes at days 0 and 8, whereas the other two arms received two doses of free PD-0325901 and CYCL-PD-0325901, respectively, at the same time points. For clarity of the experimental outcome, the data are presented as the average tumor size of each group normalized to the tumor volume on day 0. The tumor regression experiments in each case were terminated and the animals were euthanized when the tumors on the control animals reached 2,000 mm^3.

1. Tang W, Ng SC (2008) Facile synthesis of mono-6-amino-6-deoxy-alpha-, beta-, gamma-cyclodextrin hydrochlorides for molecular recognition, chiral separation and drug delivery. *Nat Protoc* 3(4):691–697.
2. Cucinotta V, et al. (2005) The 6-derivative of beta-cyclodextrin with succinic acid: A new chiral selector for CD-EKC. *J Pharm Biomed Anal* 37(5):1009–1014.
3. Hoffmann M, et al. (2004) Dihydropteridinones, method for the production and use thereof in the form of drugs (WO Patent 2,004,076,454).
4. Warmus JS, et al. (2008) 2-Alkylamino- and alkoxy-substituted 2-amino-1,3,4-oxadiazoles-O-alkyl benzohydroxamate esters replacements retain the desired inhibition and selectivity against MEK (MAP ERK kinase). *Bioorg Med Chem Lett* 18(23):6171–6174.
5. MacDiarmid JA, et al. (2007) Bacterially derived 400 nm particles for encapsulation and cancer cell targeting of chemotherapeutics. *Cancer Cell* 11(5):431–445.
6. Committee on Care and Use of Laboratory Animals (1985) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85–23.

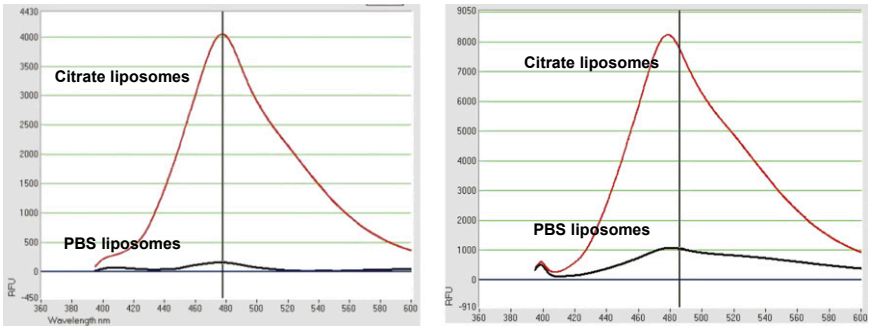


Fig. S1. Incorporation of dansylated cyclodextrins into citrate liposomes. Fluorescence analyses in relative fluorescence units (RFU) of dansylated I and compound IV in citrate liposomes and control (PBS) liposomes are presented.

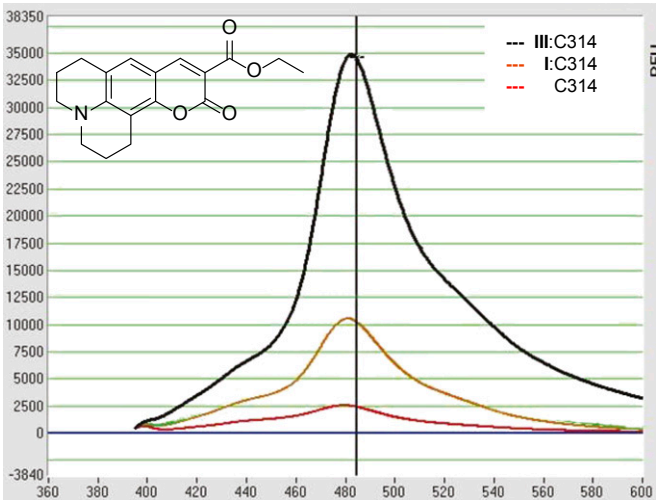
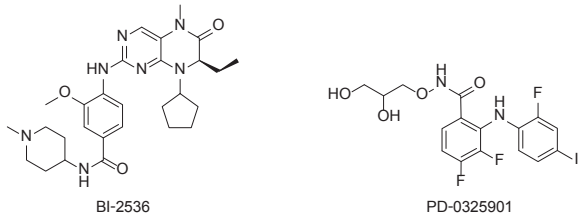


Fig. S2. Ability of various cyclodextrins to transfer coumarin 314 into citrate liposomes. Fluorescence of uncomplexed coumarin 314 and coumarin 314 complexed with III (ionizable mono-6′-ethylenediamino-6′-deoxy-cyclodextrin) and I (unionizable β-cyclodextrin) followed by remote loading into citrate liposomes.



Compound	BI-2536	PD-0325901
Solubility in water at 25 ºC at pH 7.4	0.5 mg/ml	0.01mg/ml
IC_{50} against HCT 116	0.01nM	0.3nM
Dose limiting toxicity	Neutropenia	Retinal vein occlusion

Fig. S3. Structure and physical properties of BI-2536 and PD-0325901.

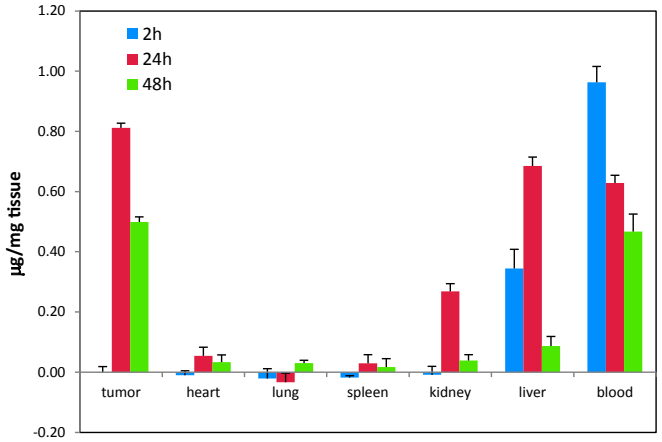


Fig. S4. Tissue biodistribution of CYCL-coumarin 334 at 2, 24, and 48 h time points (data is presented as mean and SD).

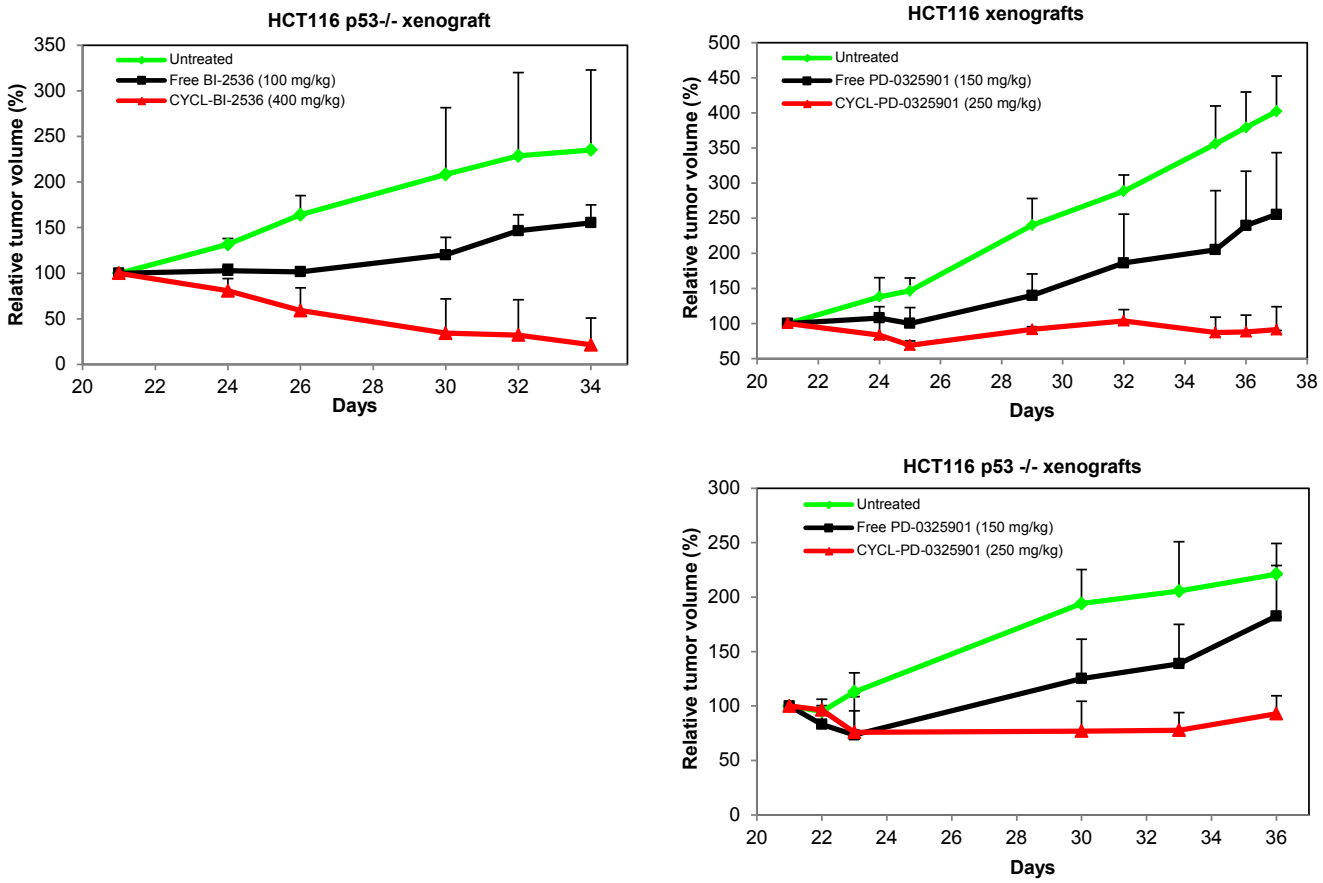
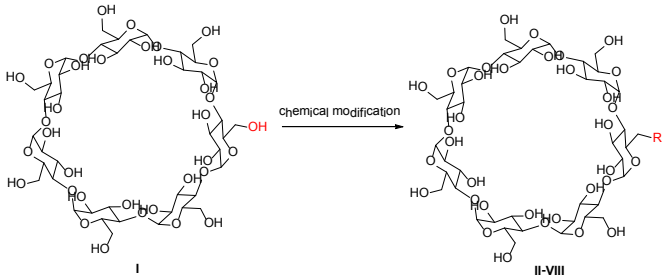


Fig. S5. Antitumor activity of CYCL-BI-2536 and CYCL-PD-0325901 in a second xenograft model. Liposomal formulations have been reported as equivalents of free drug; relative tumor volume and SD of each experimental arm are shown.



No	R
II	mono 6'-amino
III	mono 6'-ethylene diamino
IV	mono 6'-dansylamido
V	mono 6'-dansylamido ethylene amino
VI	6'-per amino
VII	6'-per ethylene diamino
VIII	6'-succinyl
IX	6'-per succinyl

Scheme S1. Table of synthesized ionizable cyclodextrins.