Novel Delivery of SN38 Markedly Inhibits Tumor Growth in Xenografts, Including a Camptothecin-11 – Refractory Model

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Abstract

Purpose: Clinical development of SN38, the active metabolite of camptothecin-11 (CPT-11), has been hampered due to its poor solubility. We have developed a novel polymer-drug conjugate, EZN-2208, made by linking SN38 with a multiarm polyethylene glycol via a glycine linker.

Experimental Design: The *in vitro* cytotoxicity of EZN-2208 was tested using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The therapeutic efficacy of EZN-2208 was evaluated in various xenografts, including an *in vivo* – selected CPT-11 – refractory model. Tumor and blood concentration of EZN-2208, CPT-11, and SN38 was determined by high-performance liquid chromatography.

Results: *In vitro*, EZN-2208 was 10- to 245-fold more potent than CPT-11 in a panel of human tumor cell lines. In xenograft models of MX-1 breast, MiaPaCa-2 pancreatic, or HT-29 colon carcinoma, treatment with either a single dose or multiple injections of EZN-2208 was more efficacious (and in some cases produced tumor eradication for >16 weeks) compared with CPT-11 at their respective maximum tolerated doses or corresponding dose levels ($P < 0.01$). Most interestingly, EZN-2208 showed marked antitumor activity in animals that developed resistance to an 8-day course of CPT-11 treatment, as well as outperformed CPT-11 as second-round therapy in mice initially sensitive to CPT-11. EZN-2208 had prolonged circulation in the blood compared with CPT-11, resulting in high tumor exposure. This resulted in higher and longer-lasting tumor exposure of free SN38 in mice given EZN-2208 compared with those given CPT-11.

Conclusions: Preclinical data suggest that EZN-2208 may be a promising anticancer agent in a wide variety of clinical settings, including tumors refractory to CPT-11 treatment.

Camptothecin-11 (CPT-11; Camptosar, irinotecan) is approved as a component of first-line combination therapy with 5-fluorouracil and leucovorin for the treatment of patients with metastatic colorectal cancer (1, 2). In addition, CPT-11 has been prescribed for the treatment of small cell lung (3), breast (4), central nervous system (5), cervical (6), esophageal (7), gastric (8), and pancreatic (9) cancers as well as non–Hodgkin's lymphoma (10). The parent compound, camptothecin, is derived from the bark of the Chinese tree *Camptotheca acuminata*. The primary, if not sole, target of camptothecin is topoisomerase I (TOP1), which is involved in controlling DNA replication and transcription (11). CPT-11 is a derivative of camptothecin. Addition of a bis-piperidine group to camptothecin via an ester linkage renders CPT-11 water soluble and allows systemic delivery (Fig. 1A). In the body, carboxylesterase-2 removes the bis-piperidine group and

produces 10-hydroxy-7-ethyl camptothecin or SN38 (Fig. 1B). SN38 is the active ingredient in CPT-11, as it has 100- to 1,000-fold more potent cytotoxicity *in vitro* compared with CPT-11 (12).

Although CPT-11 has clinical utility, several limitations suggest that chemical modifications may further improve the therapeutic index of the compound. First, only 3% to 4% of an injected dose of CPT-11 is converted to SN38 (12, 13), and 55% is excreted as intact CPT-11 in humans (13). Second, the metabolic conversion of CPT-11 to SN38 depends on genetic interindividual variability of carboxylesterase activity (14). Third, both CPT-11 and SN38 in their active forms have a closed lactone ring (E-ring) and can be metabolized to inactive or carboxylate forms by opening of this ring (Fig. 1A; refs. 13, 15, 16). In particular, it is known that, 24 h after CPT-11 infusion in humans, only about 25% to 30% and 50% to 64% of CPT-11 and SN38, respectively, are in the lactone (closed) form (12) compared with the total amount of CPT-11 and SN38. Hence, if the conversion to the carboxylate form of the molecule was inhibited, it may have therapeutic benefit. Finally, resistance to CPT-11 has been observed frequently. Although the basis of resistance to CPT-11 in patients is not clearly understood, in the laboratory, resistance to SN38 may be mediated by exclusion of the active ingredient from the cell via efflux pumps (11, 17), mutations in TOP1 (11), or inactivation of the compound by the addition of a glucuronide moiety to SN38 (i.e., SN38 glucuronide).

Fig. 1. *A,* chemical structure of CPT-11 and its metabolites. The bis-piperidine moiety within CPT-11 is removed by carboxylesterase in blood and liver to create the highly cytotoxic molecule SN38. Both CPT-11 and SN38 can be metabolized to inactive forms by opening of the lactone ring (E-ring). The site of PEGylation of SN38 is indicated as carbon 20 in the E-ring within SN38.
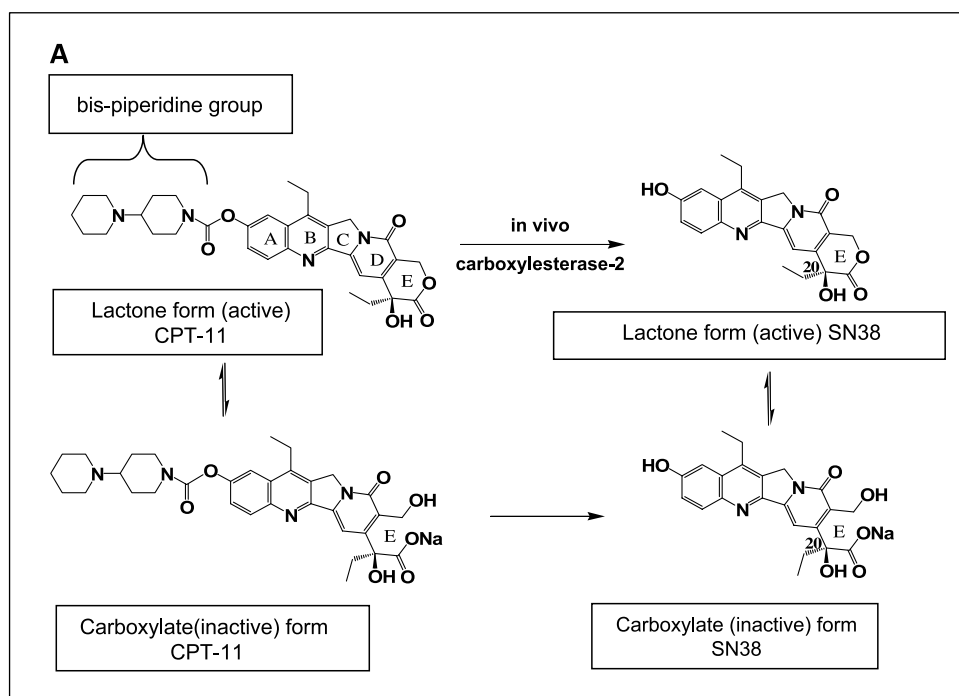
Because CPT-11 has limitations, new CPT-11 analogues are sought that maintain good water solubility, are potent inhibitors of TOP1, shift the equilibrium toward the active (lactone closed) form of the molecule, do not mediate the production of toxic metabolites, and may overcome resistance. Although SN38 itself might seem to be a good candidate to address many of these concerns, the compound has poor solubility in any pharmaceutically acceptable excipient. However, the solubility of SN38 can be greatly improved by linking SN38 to high molecular weight polyethylene glycol (PEG). In addition, PEGylation is known to increase the passive accumulation of polymeric compounds in solid tumors (enhanced permeation retention effect), including camptothecin analogues, and could assist in improved delivery to tumors (18). This is because there exists discontinuous endothelial lining and other capillary anomalies in tumor vasculature. These so-called leaky vessels facilitate the extravasation of supramolecular structures, such as polymers and liposomes, into the interstitial space in solid tumors. Thereby, the polymers accumulate and function as a sustained drug release system (19, 20). Beyond this, it also may be that macromolecular drugs, such as polymer-based drugs, may overwhelm or bypass efflux pumps present on the plasma membrane and hence overcome ATP-binding cassette transporter – mediated multidrug resistance. Finally, our approach was to attach PEG at the C20 position of SN38 (within the lactone E-ring).[1] This strategy serves to stabilize the ring in the closed or active form, which may result in a higher ratio of the active conformation for SN38 compared with CPT-11.

We have used our Customized Linker Technology to make novel PEGylated conjugates of SN38,[1] of which one conjugate, EZN-2208 (compound 6 in Fig. 1B), was selected for further development. EZN-2208 is composed of a four-arm 40-kDa PEG linked via a glycine residue to SN38 (Fig. 1B). Because the linkage between the amino acid spacer and SN38 is an ester bond, it will hydrolyze and release the intact SN38 under basic conditions. In addition, under physiologic conditions, esterase will hydrolyze the ester bond. Approximately 3.5 to 4.0 SN38 molecules are attached to the PEG backbone. EZN-2208 has good aqueous solubility of 180 mg/mL (equivalent to 6.7 mg/mL of SN38) that allows convenient i.v. delivery.[1] In this article, we show that EZN-2208 has significantly greater antitumor activity than CPT-11 in several human tumor xenograft models. The high antitumor activity is attributed to higher exposure of tumors to PEG-SN38 via enhanced permeation retention effect compared with CPT-11. We also show that EZN-2208 has excellent efficacy in mice that failed to respond to an initial course of multiple-dose therapy with CPT-11. Further, we show that treatment with EZN-2208 is significantly more effective than treatment with CPT-11 as a second-round therapy in animals that initially responded to CPT-11.

Materials and Methods

Materials, cell lines, and animals. Irinotecan (CPT-11, Camptosar) was obtained from Bell Medical Services. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium CellTiter 96 Aqueous reagent was purchased from Promega. All other chemicals were of analytic grade purity. The following cells were obtained from the American Type Culture Collection and grown in the listed medium: Colo 205, AsPC-1, BxPC-3, and OVCAR-3 [RPMI 1640 with 4.5 g/L glucose, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 10% fetal bovine serum (FBS)]; HT-29 and SK-OV-3 (McCoy's 5a with 1.5 mmol/L L-glutamine, 10% FBS); MiaPaCa-2 and PANC-1 (DMEM with 4 mmol/L L-glutamine, 4 mmol/L glucose, 10% FBS);

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[1] H. Zhao et al. Novel prodrugs of SN38 using multi-arm polyethylene glycol (PEG) linkers. Bioconjugate Chemistry, submitted for publication.
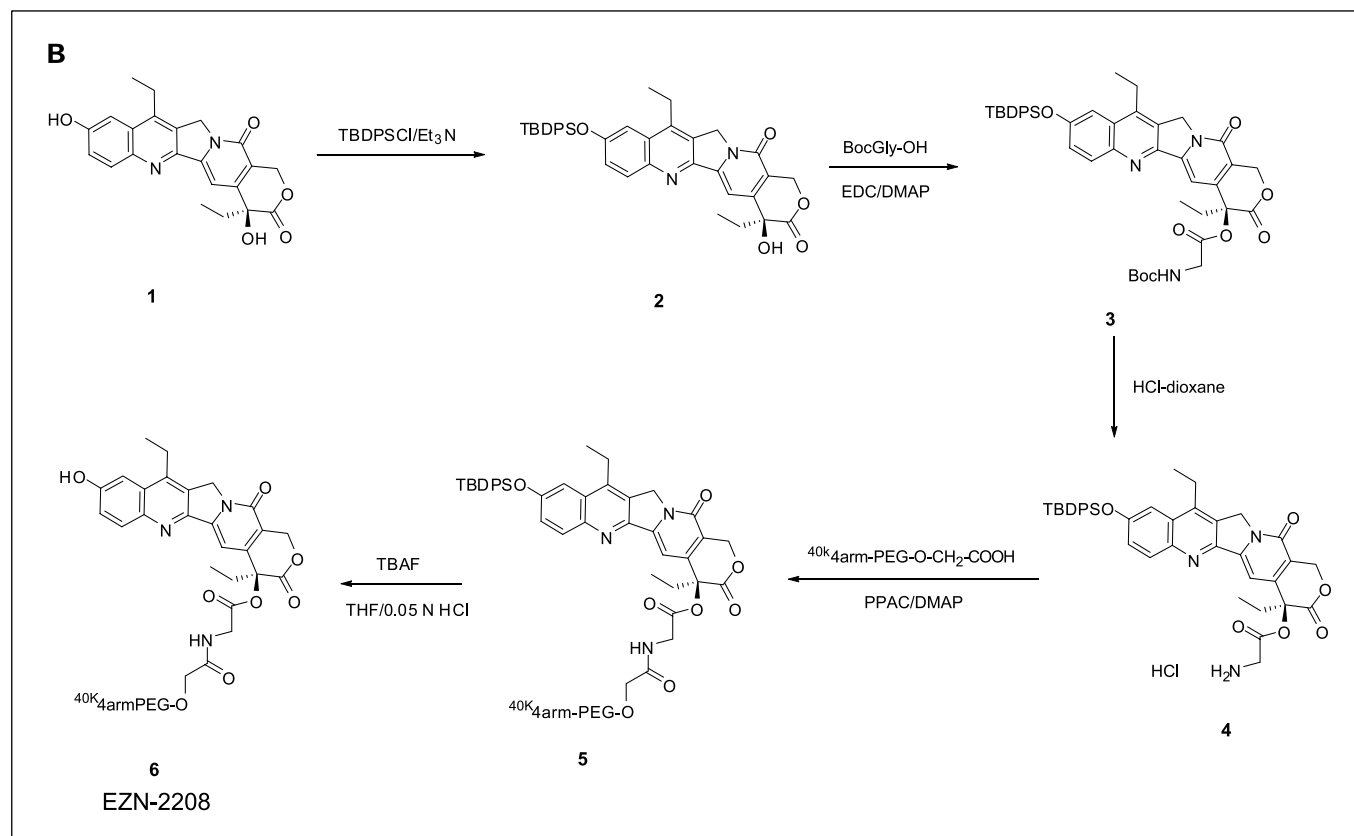
B

Fig. 1 *Continued. B,* synthetic scheme for EZN-2208. TBDPS, *t*-butyldiphenylsilyl; TBAF, tetrabutylammonium fluoride.

A549 (Ham's F12K with 10% FBS); and OV-90 (Medium 199 with 15% FBS). OVCAR-8 (obtained from the Division of Cancer Treatment and Diagnosis, Tumor/Cell Line Repository, National Cancer Institute, Bethesda, MD) and A2780 (obtained from the European Collection of Cell Cultures, Sigma-Aldrich Corp.) were grown in RPMI 1640 with 10% FBS. All cell lines were maintained at 37°C (humidified, 5% CO_2). The medium was changed every 3 to 4 d and the cultures were trypsinized and recultured when they reached 85% confluence.

Preparation of EZN-2208. SN38 was first reacted with *t*-butyldiphenylsilyl chloride to selectively protect the 10-OH with *t*-butyldiphenylsilyl group (Fig. 1B). Subsequent acylation of 20-OH with Boc-Gly (t-butylcarbonyl-glycine) gave the glycinate of SN38 (compound 3, Fig. 1B). The Boc group was removed by HCl in dioxane without affecting the *t*-butyldiphenylsilyl group. The resulting intermediate (compound 4, Fig. 1B) was conjugated with four-arm PEG acid using propane phosphonic acid anhydride as the coupling agent. Finally, the *t*-butyldiphenylsilyl group was removed with tetrabutylammonium fluoride to give the final product PEG-Gly-(20)-SN38 or EZN-2208 (compound 6, Fig. 1B).

In vitro *cytotoxicity.* The *in vitro* cytotoxicity of EZN-2208 was determined in colorectal, lung, pancreatic, and ovarian cell lines using a cell proliferation [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] tetrazolium dye reduction assay. Briefly, adherent cells (10,000-20,000 per well) were plated in 96-well plates and incubated overnight at 37°C. The next morning, the cells were treated with serial dilutions of EZN-2208, CPT-11, or SN38 dissolved in DMSO and further incubated for 3 to 4 d at 37°C. At the end of the incubation period, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium dye was added, and formation of a colored product, formazan, was measured at 490 nm using a SpectraMax 340PC reader (Molecular Devices).

In vivo *studies.* Seven- to 8-wk-old female BALB/c homozygous and 4- to 5-wk-old female athymic nude mice were purchased from Harlan. All animal studies were approved by the University of Medicine and Dentistry of New Jersey Institutional Animal Care and Use Committee.

Maximum tolerated dose in nude animals. Female naive athymic nude mice were injected i.v. with either a single dose or multiple injections [every 2 d (q2d) × 5] of EZN-2208 or CPT-11. Doses ranged from 10 to 35 mg/kg for EZN-2208 and from 40 to 125 mg/kg for CPT-11. Mice were monitored daily for visible signs of toxicity and weighed biweekly. The maximum tolerated dose (MTD) was defined as the highest dose at which no death occurred, and body weight loss was ≤20% of pretreatment animal weight (~ 20 g).

In vivo *therapeutic efficacy.* S.c. tumor xenograft models were established in the right axillary flank region of female nude mice (4-5 wk) by injecting either human cancer cells or tumor fragments. MX-1 tumors were established by implanting a 4- to 5-mm³ tissue fragment of MX-1 tumor collected from donor mice into the axillary flank of recipient nude mice. HT-29 and MiaPaCa-2 tumors were established by injecting 1 × 10⁶ HT-29 cells per mouse or 2.5 × 10⁶ MiaPaCa-2 cells per mouse into the right axillary flank.

Treatment with MTDs of EZN-2208 or CPT-11 given as a single dose or as multiple doses (q2d × 5) was initiated when tumors reached an average volume of 75 to 100 mm³. Mice also were treated with CPT-11 at corresponding dose levels of EZN-2208 (30 mg/kg for single injection and 10 mg/kg for multiple doses). In one MX-1 study, treatment was started when tumors reached an average volume of 415 mm³. In the observation phase, mice were monitored for tumor sizes and body weights biweekly and euthanized either when individual tumor volumes reached >1,650 mm³ or at the end of the experiment (>15 wk). It is important to note that the doses or concentrations of EZN-2208 stated in this article refer to SN38 equivalents. For example, a dose of 25 mg/kg of EZN-2208 means that the dose contains 25 mg/kg

of SN38 and 725 mg/kg (29-fold higher) of whole conjugate, assuming that the loading of SN38 in the whole EZN-2208 is 3.45%.

Therapeutic efficacy in CPT-11–sensitive and CPT-11–refractory tumors. HT-29 human colorectal tumors were established in nude mice by s.c. injection of 1×10^6 cells per mouse into the right axillary flank. When tumors reached an average volume of ~100 mm^3, mice were treated with CPT-11 (40 mg/kg, q2d × 4). Mice were monitored for tumor growth. On day 16, the mice that had tumor volumes less than three times the initial tumor volume were considered CPT-11 sensitive, and the mice that had tumor volumes at least three times the initial tumor volume at the start of CPT-11 therapy were considered CPT-11 resistant. Both CPT-11–sensitive or CPT-11–refractory mice were selected, randomized, and divided further into two groups each. One group was treated with the MTD of CPT-11 (40 mg/kg, q2d × 5), and the second group was treated with the MTD of EZN-2208 (10 mg/kg, q2d × 5) starting from day 16.

Plasma and tumor distribution in tumor-bearing mice. MX-1 tumors were established by implanting a 4- to 5-mm^3 tissue fragment of MX-1 tumor collected from donor mice into the axillary flank of recipient nude mice. When tumors reached an average volume of 360 mm^3, mice were given a single injection of EZN-2208 or CPT-11 at their respective MTDs. Mice (three per group) were sacrificed at various time points, and blood and tumor samples were obtained. Blood samples were collected into EDTA-containing tubes, and plasma was harvested. The plasma fraction was frozen on dry ice and stored at -80°C until analyzed. Tumors were excised, weighed, and cut into small pieces (~4-10 mm^3). The pieces were homogenized in 9 mL/g of 20 mmol/L ammonium acetate (pH 3.5) and centrifuged; the supernatant was collected and stored at -80°C. The concentrations of plasma and tumor EZN-2208 and released SN38 and SN38 metabolite SN38 glucuronide were determined using high-performance liquid chromatography analysis and expressed as peak area ratios using 7-ethyl camptothecin as an internal standard.

High-performance liquid chromatography. Analytic high-performance liquid chromatography was done using a C18 reverse-phase column (Jupiter, 5 μm, 150 mm × 2 mm; Phenomenex) under gradient conditions. To 100 μL of frozen plasma was added 1 μL of 20% trifluoroacetic acid and the plasma was thawed unassisted. Then, 100 μL acidified plasma or tumor homogenate was deproteinated with 200 μL ice-cold acetonitrile containing 0.5% acetic acid and 0.1 μg/mL 7-ethyl camptothecin (internal standard). The extraction mixture was clarified by centrifugation at $14,000 \times g$ for 5 min and 200 μL of the supernatant were transferred to a high-performance liquid chromatography sample vial. The sample was evaporated to dryness in a SpeedVac (Thermo Scientific) and reconstituted in 200 μL of 20 mmol/L ammonium acetate (pH 3.5) containing 15% acetonitrile and 1% tetrahydrofurane. Sample (100 μL) was applied to a column preequilibrated with 85% of 20 mmol/L ammonium acetate (pH 3.5; mobile phase A) and acetonitrile (mobile phase B). The column was initially eluted at 25°C at a flow rate of 0.3 mL/min for 4 min at 15% B. The column was eluted for an additional 6 min at 25% B followed by a 5-min linear gradient to 100% B. The column was held at 100% B for 2 min before being reequilibrated by eluting the column for 2 min with a linear gradient to 15% B and held at 15% B for an additional 6 min. Eluted peaks were detected using fluorescence detection (excitation, 368 nm; emission, 515 nm; Agilent Technologies, Inc.). Plasma concentrations were calculated from a linear standard curve of peak area-internal standard ratios. SN38 glucuronide, SN38, internal standard, and EZN-2208 had retention times of 3.7, 10.7, 15.8, and 16.7 min, respectively.

Data analysis. For *in vitro* cytotoxicity studies, dose-response curves were generated from the mean of triplicate determinations, and IC$_{50}$ values were obtained using the GraphPad Prism software (Advanced Graphics Software). Pharmacokinetic variables for EZN-2208, CPT-11, or free SN38 were estimated using noncompartmental analysis (WinNonlin, version 4.1; Pharsight). For efficacy studies, percent tumor growth inhibition (%TGI) was calculated using the

Table 1. *In vitro* cytotoxicity study (IC$_{50}$, μmol/L)				
Cancer type	Cell line	SN38	EZN-2208	CPT-11
Colorectal	Colo 205	0.2 ± 0.2	1.0 ± 0.6	11 ± 1.4
	HT-29	0.1 ± 0.04	0.5 ± 0.3	27 ± 8.6
Lung	A549	1.0 ± 0.1	2.7 ± 0.4	67 ± 17
Pancreatic	PANC-1	0.5 ± 0.3	0.6 ± 0.11	37 ± 18
	AsPC-1	0.5 ± 0.1	1.4 ± 0.9	37 ± 1.1
	BxPC-3	0.4 ± 0.5	0.2 ± 0.03	49 ± 48
Ovarian	OVCAR-3	0.1 ± 0.02	0.3 ± 0.2	20 ± 7.1
	OV-90	0.1 ± 0.02	0.6 ± 0.7	18 ± 4.8
	OVCAR-8	0.03 ± 0.01	0.5 ± 0.1	9.0 ± 1.3
	A2780	0.02 ± 0.01	0.2 ± 0.2	8.0 ± 3.5
	SK-OV-3	0.2 ± 0.1	0.9 ± 0.01	52 ± 8.5

NOTE: IC$_{50}$ of EZN-2208 is expressed in terms of SN38 equivalents. Values shown are average ± SD ($n = 3$ independent experiments).

following formula: $[(C - T) / C] \times 100$, where C is the mean tumor volume of the control group at a specified time and T is the mean tumor volume of the treatment group at the same time. Differences between treatments were compared using ANOVA with statistical significance $P < 0.05$.

Results

In vitro *studies.* EZN-2208 showed potent *in vitro* cytotoxicity with IC$_{50}$ values ranging from 0.2 to 2.7 μmol/L (Table 1). The mean IC$_{50}$ value in a panel of 11 cell lines was 809 ± 725 nmol/L (mean ± SD; $n = 11$). EZN-2208 was 10- to 245-fold more potent than CPT-11 and 1.2- to 17-fold less potent than free SN38, except in BxPC-3 cells, in which it was 2-fold more potent than SN38.

In vivo *studies—MTD determination in mice.* The MTD in nude and severe combined immunodeficient mice was determined to support xenograft therapeutic efficacy models. The MTD of EZN-2208 and CPT-11 was 30 and 80 mg/kg, respectively, when given as a single dose. When given in multiple-dose regimens, the MTD of EZN-2208 and CPT-11 was 10 and 40 mg/kg, respectively.

Antitumor efficacy in xenograft models of breast, pancreatic, and colorectal tumors. The efficacy of EZN-2208 was compared with CPT-11 at their respective MTDs or at equivalent doses on both a single- and multiple-dose schedule in xenograft models of breast (MX-1), colorectal (HT-29), and pancreatic (MiaPaCa-2) xenografts. In an initial experiment in MX-1 xenografts, treatment with EZN-2208 below the MTD, either as a single dose of 20 mg/kg or multiple doses of 5 mg/kg (q2d × 6), led to 100% TGI and complete cures of all animals up to 16 weeks, after which the mice were humanely sacrificed (data not shown). At equivalent dose levels (20 mg/kg), treatment with CPT-11 caused no significant inhibition of tumor growth and 44% TGI when given as a single dose or multiple injections, respectively (data not shown). In a subsequent study in the same MX-1 model, EZN-2208 was highly efficacious when tested on large bulky tumors (initial tumor volume approximately four times larger than stated above: 415 mm^3). Treatment with a single MTD of EZN-2208 (30 mg/kg) led to dramatic tumor reduction within 10 days after treatment (Fig. 2A, *left*). The animals were essentially "cured" because no tumor was evident by gross observation at

>100 days after dosing, except in one animal that had an abnormal growth in a site distinct from the tumor injection site (Fig. 2A). In contrast, treatment with a single MTD of CPT-11 resulted in 56% TGI on day 13. However, after this period, tumor growth rapidly resumed, and all animals had to be sacrificed by day 20 due to excessive tumor burden. Treatment with a single dose of CPT-11 at the dose equivalent to EZN-2208 (30 mg/kg or ~40% of the MTD for CPT-11) had no effect on tumor growth. In the same model, treatment with multiple doses of EZN-2208 at its MTD led to cures of 100% of animals (Fig. 2A, *right*). In contrast, CPT-11, given at its MTD, was partially effective because tumor regrowth resumed by day

45, and no animal was tumor-free when the study was terminated (Fig. 2A, *right*).

The efficacy of EZN-2208 was evaluated in a pancreatic xenograft model (MiaPaCa-2). On this cell line, EZN-2208 was 614-fold more potent than CPT-11. Treatment with a single MTD of EZN-2208 resulted in 71% TGI (on day 69) and 100% survival of animals (Fig. 2B, *left*). However, a single dose of CPT-11 given at the MTD had no effect on tumor growth. Multiple-dose treatment of EZN-2208 caused 95% TGI, and by day 147 (last day of study), 66% of animals were cured (no evidence of tumors by gross observation; Fig. 2C, *right*). In contrast, multiple-dose CPT-11 treatment resulted in 47% and
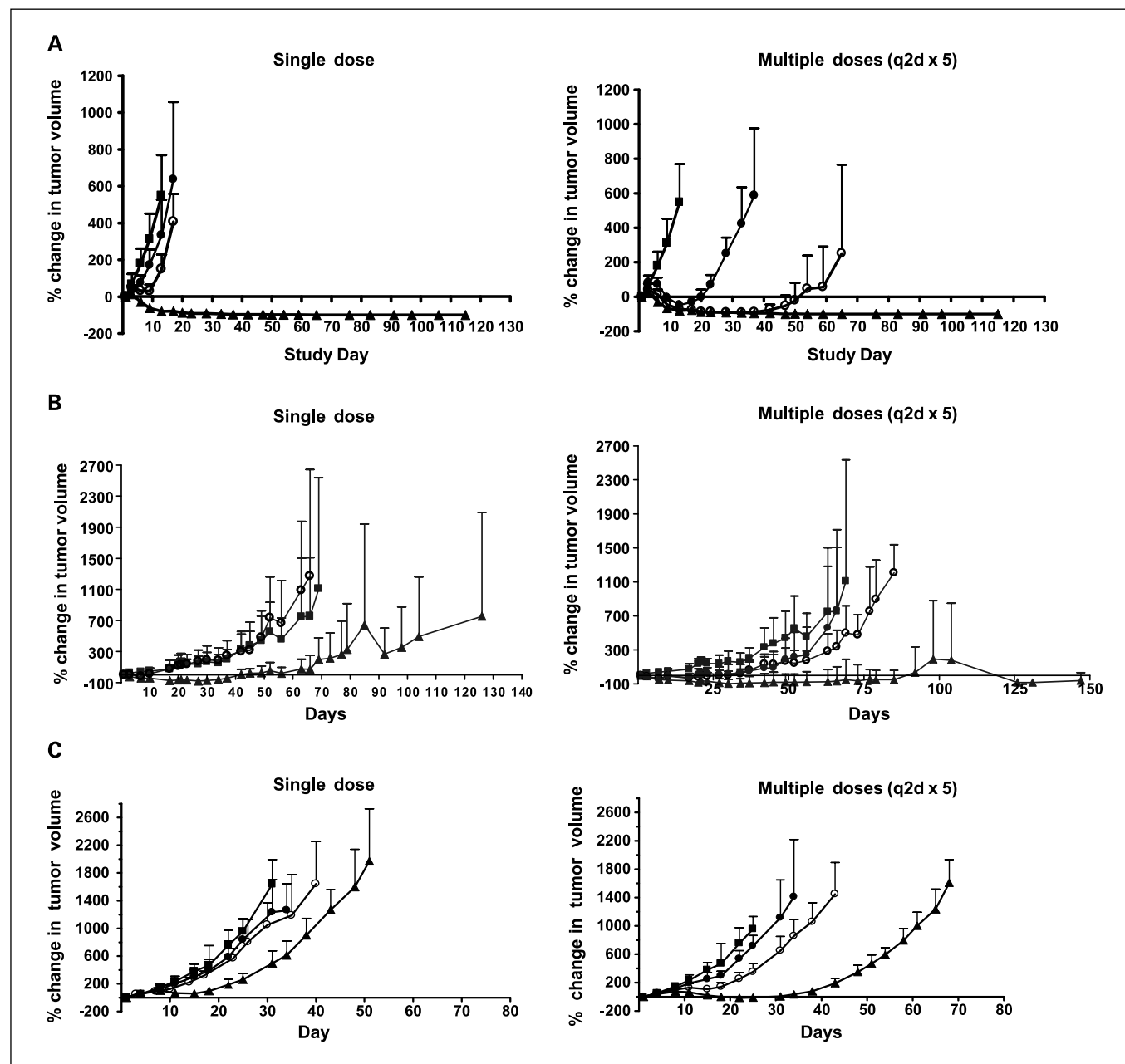

Fig. 2. Therapeutic efficacy of EZN-2208 in xenograft models of solid tumors. Female athymic nude mice (6-10 mice per group) were inoculated s.c. with 4- to 5-mm³ tumor fragments of MX-1 breast tumors (*A*), 1 × 10⁶ HT-29 colorectal cells (*B*), or 2.5 × 10⁶ MiaPaCa-2 pancreatic cells (*C*). After 1 day, mice were treated with either a single (*left*) or multiple (q2d × 5) injections (*right*) of saline (■), EZN-2208 at MTD (▲), CPT-11 at MTD (○), or CPT-11 at equivalent MTD of EZN-2208 (●).

20% TGI when dosed at MTD or at corresponding dose level as EZN-2208 (10 mg/kg, q2d × 5), respectively (Fig. 2B, *right*).

In a model of colorectal cancer (HT-29), treatment with either a single dose or multiple injections of EZN-2208 was significantly more effective than CPT-11 at their respective MTDs or corresponding dose levels ($P < 0.01$). On day 26, treatment with a single MTD of EZN-2208 resulted in 68% TGI, in contrast with 17% TGI observed for CPT-11 (Fig. 2C, *left*). When given as multiple doses, EZN-2208 treatment resulted in 92% TGI. However, CPT-11 dosed at the MTD caused 57% TGI and when given at corresponding dose compared with EZN-2208 caused 27% TGI (Fig. 2C, *right*). As a follow-up to this model, a study was done to evaluate if retreatment with EZN-2208 could maintain the tumor growth-inhibitory effects. Animals were initially treated with multiple injections of MTD of EZN-2208 as described above. When tumor growth resumed, animals were retreated with three cycles of EZN-2208 on the multidose regimen. In particular, tumors were treated on days 1 to 9, 40 to 48, and 77 to 86 (Fig. 3). As evident from the graph, HT-29 tumors retained the ability to respond to repeated cycles of EZN-2208. The tumors did not grow as reported in Fig. 2B; instead, the tumors were stabilized as long as cycles of EZN-2208 treatment were given.

Antitumor efficacy in CPT-11–resistant mice. Because inherent or acquired resistance to CPT-11 is a common phenomenon (11), we explored the utility of EZN-2208 in a CPT-11–resistant tumor model. EZN-2208 had exceptional therapeutic efficacy in "CPT-11–resistant" mice (mice that failed to respond to an initial course of multiple-dose therapy with CPT-11). In particular, mice with CPT-11–resistant tumors did not respond to further multiple-dose CPT-11 treatment initiated ~8 days after termination of the first round of therapy. Rather, tumor growth continued, and a 255% increase in tumor volume was observed (Fig. 4A). In contrast, EZN-2208 given to the mice with CPT-11–resistant tumors resulted in a 25% decreased tumor volume by day 41. Furthermore, in the group retreated with CPT-11, all animals were sacrificed by day 54 due to excessive tumor burden (>1,650 mm^3). In contrast, in the EZN-2208–treated group, by day 72, 58% of animals had tumors <1,650 mm^3 (Fig. 4A).

In addition, EZN-2208 also was more effective than CPT-11 when given to mice with tumors that had initially responded

Fig. 4. Activity of EZN-2208 in CPT-11–resistant (*A*) and CPT-11–sensitive (*B*) colorectal tumor xenografts. Nude mice bearing human colorectal (HT-29) xenografts were separated into CPT-11–resistant and CPT-11–sensitive groups based on their response to initial CPT-11 treatment. In both these groups, animals were randomized and treated with either EZN-2208 (▲) or CPT-11 (●), (*n* = 7). Arrow, time of administration of the indicated drugs.

to CPT-11 therapy (Fig. 4B). In this case, ~32 days after the termination of the second round of therapy, treatment with EZN-2208 resulted in only moderate increased tumor volume (193%) compared with treatment with second-line CPT-11, which resulted in 1,298% increased tumor volume (Fig. 4B). Similar results were obtained in a second experiment (data not shown).

Pharmacokinetics and tissue biodistribution of EZN-2208 and CPT-11 in breast (MX-1) tumor xenografts. To help understand why treatment with EZN-2208 was significantly more effective than with CPT-11, the tumor and plasma distribution of both the drugs were examined. CPT-11 had a very rapid clearance from the circulation ($t_{1/2}\ \chi = 1.7$ h) compared with EZN-2208 ($t_{1/2}\ \chi = 11.7$ h; Fig. 5A). Accordingly, SN38 released from CPT-11 was cleared from the circulation much faster ($t_{1/2}\ \chi = 2.1$ h) compared with EZN-2208 ($t_{1/2}\ \chi = 26$ h; Fig. 5A). The levels of CPT-11 or SN38 released from CPT-11 were undetectable 24 h after injection. The longer circulation half-life of EZN-2208 resulted in high exposure of either EZN-2208 [area under the curve (AUC) = 107,064.8 h * μg/mL]
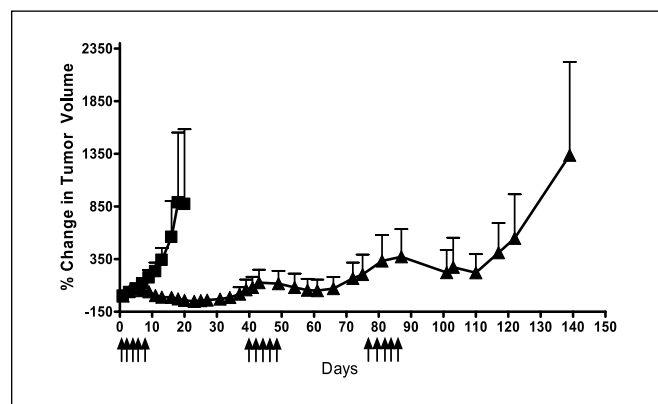

Fig. 3. Effect of multiple cycles of EZN-2208 treatment. ▲, nude mice bearing human colorectal xenografts were treated with multiple cycles of MTD of EZN-2208 (q2d × 5; *n* = 10). ■, animals treated with saline served as controls. Arrow, time of treatment.
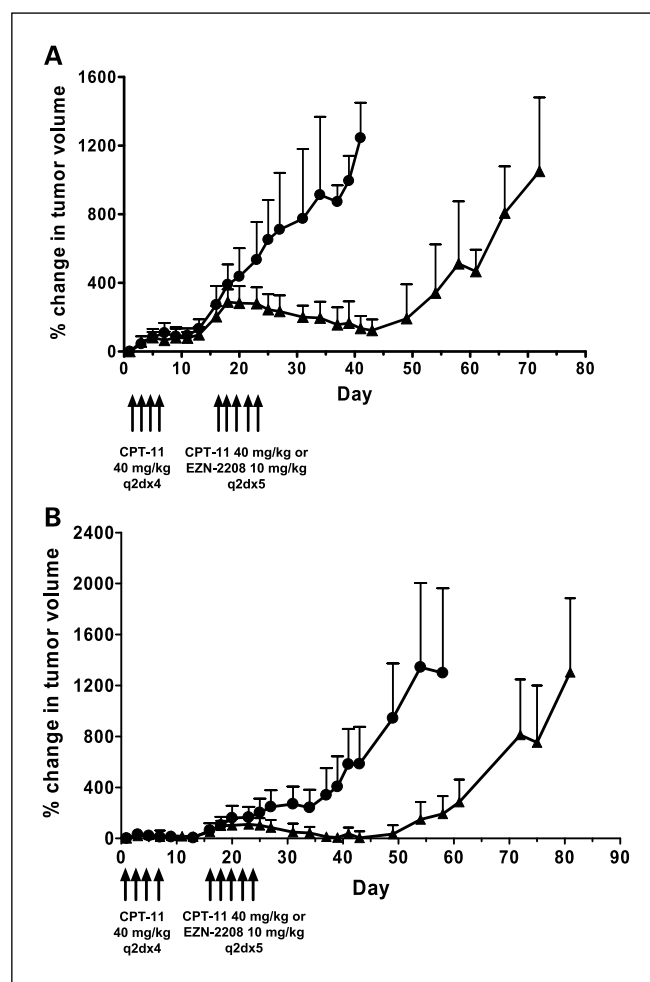
or released SN38 (AUC = 128.9 h * μg/mL). In contrast, exposure (AUC) of CPT-11 was only 193.6 and 2.9 h * μg/mL for SN38 equivalents (Fig. 4A). In tumors, exposure of EZN-2208 was 468-fold higher than CPT-11 (AUC of EZN-2208 = 38,824.5 h * μg/g versus AUC of CPT-11 = 83 h * μg/g). This resulted in 207-fold higher exposure to free SN38 when injected as EZN-2208 compared with CPT-11 (AUC of released SN38 from EZN-2208 = 227.8 h * μg/g versus AUC of released SN38 from CPT-11 = 1.1 h * μg/g; Fig. 5B).

Discussion

In this study, we show improved therapeutic efficacy of EZN-2208 over CPT-11 in preclinical tumor xenografts, including a CPT-11–resistant model. *In vitro*, EZN-2208 showed potent

Fig. 5. Plasma (*A*) and tumor (*B*) concentration-time curves of drugs after an i.v. bolus MTD treatment of EZN-2208 and CPT-11 to MX-1 xenografts (*n* = 3). Mice were sacrificed at various time points, and blood and tumor samples were obtained. The concentrations of EZN-2208 (▲), CPT-11 (■), and released SN38 [from EZN-2208 (Δ) or from CPT-11 (□)] were determined using high-performance liquid chromatography analysis.

effects on a panel of tumor cell lines; however, there existed difference in sensitivity to various cell lines. This could be due to differences in either rate of release of free SN38, intracellular delivery of SN38, or resistance of cells to SN38. *In vitro* experiments in tissue culture do not capture the advantages of PEGylating SN38, such as improved pharmacokinetics, and hence may underestimate the efficacy of EZN-2208. This was confirmed *in vivo*, where EZN-2208 showed excellent efficacy in xenograft animal models of solid tumors. The superior effects of EZN-2208 compared with CPT-11 were observed when the compounds were compared at equivalent doses, at the MTDs, with single- or multiple-dose schedules using (*a*) tumors derived from three solid tumor xenografts, (*b*) small and large established tumors, and (*c*) tumors that were either sensitive or refractory to CPT-11 therapy.

Although the exact mechanism of action that explains the enhanced tumor activity of EZN-2208 compared with CPT-11 is not completely understood, two factors help to explain the result. First, the pharmacokinetics and biodistribution of EZN-2208 is favorable with prolonged circulation in the blood leading to increased accumulation in the tumors. Treatment of animals with EZN-2208 resulted in a 207-fold higher exposure to free SN38 compared with treatment with CPT-11. Further, the tumor to plasma concentration ratio of EZN-2208 or SN38 released from EZN-2208 increased over time, suggesting that EZN-2208 may accumulate in the tumor via the "enhanced permeation retention" effect (data not shown). On the other hand, the tumor to plasma concentration of CPT-11 did not improve over time (data not shown). A second factor contributing to the enhanced antitumor efficacy of EZN-2208 is PEGylation at the C20 position of SN38 (within the lactone ring) that has been shown for other camptothecins to stabilize the lactone ring (21). Because the closed lactone ring is the active form of SN38 (16, 22), and metabolism of CPT-11 creates an open lactone ring that is less tumoricidal, PEGylation should increase the residence time of the closed lactone ring and increase efficacy compared with CPT-11.

The most striking observation of this study was the effectiveness of EZN-2208 in an animal model of CPT-11–refractory tumors. Resistant to CPT-11 was defined as a failure to respond to CPT-11 *in vivo*, and a cell line was not developed *in vitro*. Although the basis for resistance *in vivo* is much harder to study, it is likely to be much more clinically relevant. It is possible that the efficacy of EZN-2208 in CPT-11–refractory mice also may be attributed to good pharmacokinetic and biodistribution properties of EZN-2208; however, it is also likely that EZN-2208 may have a novel mechanism of action. It has been shown that topotecan, another TOP1 inhibitor, inhibits hypoxia-inducible factor-1α, leading to marked decrease of angiogenesis and significant tumor growth inhibition (23). Consistent with this observation, it may be possible that in CPT-11–sensitive mice CPT-11 induces a decrease in hypoxia-inducible factor-1α in cells, which then accumulate EZN-2208 due to an enhanced permeation retention effect. However, in CPT-11–refractory mice, CPT-11 fails to induce a decrease in hypoxia-inducible factor-1α, leading to even more angiogenesis. These highly vascular tumors may further favor accumulation of EZN-2208 due to enhanced permeation retention effects. In fact, another polymeric SN38 conjugate, NK012, was shown to have high antitumor activity in vascular
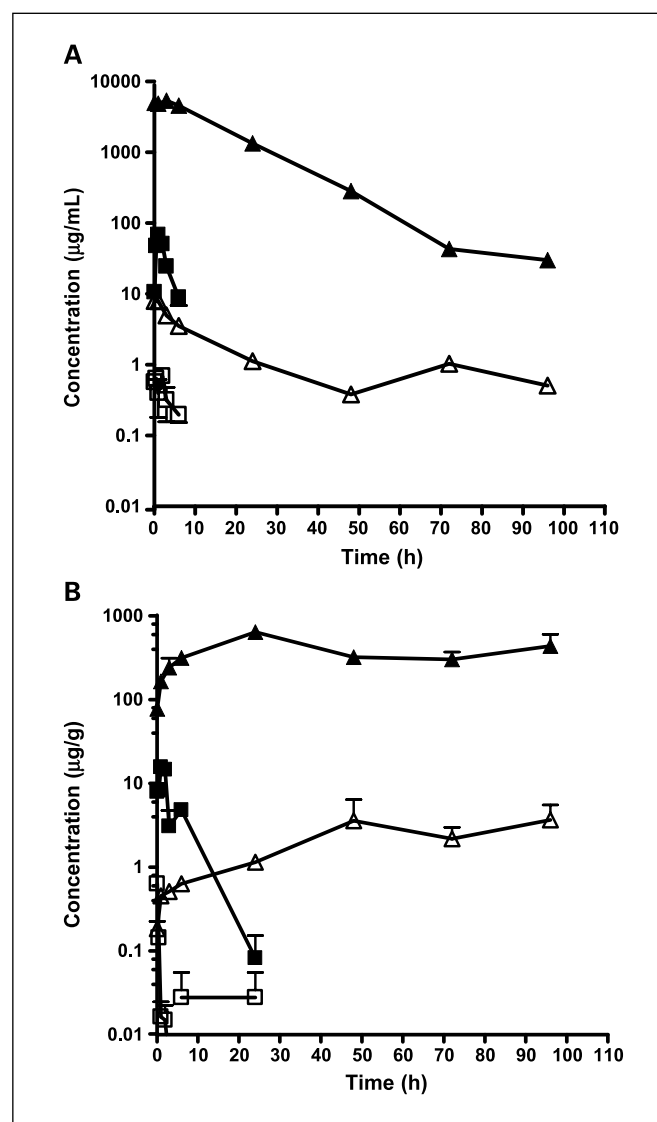
endothelial growth factor–secreting tumors, thus favoring the concept of polymer-based drugs accumulating in angiogenic tumors (24). Once EZN-2208 has accumulated in angiogenic tumors, it can function as a sustained SN38 delivery system, and thus possibly mimicking the antiangiogenic nature of "metronomic" therapy (25).

Alternatively, CPT-11–resistant tumors may have lower levels of TOP1 because low levels of TOP1 have been linked to CPT-11 resistance in tissue culture. As EZN-2208 provides higher exposure of SN38 than CPT-11, drug concentrations may be sufficient to kill cells even with low levels of TOP1. Further, variable levels of carboxylesterase are another contributing factor to CPT-11 resistance, and this enzyme is not required for release of SN38 from EZN-2208. Another possibility is that CPT-11–resistant tumors have elevated expression of ABCG2. However, tumor cell lines that overexpress ABCG2 are cross-resistant to SN38, or EZN-2208 (data not shown), and thus we do not favor the last theory.

It has previously been reported that the growth of CPT-11–resistant HT-29 or DLD-1 colorectal carcinoma xenograft models, which have been established with similar dosing regimens compared with the one reported here, is dramatically inhibited when an epidermal growth factor receptor antibody (C-225) is combined with CPT-11 therapy (26), whereas either agent alone did not control tumor growth. Although the basis for the effect remains incompletely understood, it was hypothesized that synergy was because the C-225/CPT-11 therapy both influence cell cycle, apoptosis, or possibly antiangiogenic mechanisms. Our data suggest that EZN-2208 works effectively even as a single agent in these resistant tumors and therefore combination therapy of EZN-2208 with epidermal growth factor receptor inhibitors may provide additional benefit for CPT-11 relapsed patients.

Besides imparting improved efficacy over CPT-11, EZN-2208 also may have an improved safety profile in humans compared with that of CPT-11. It has been suggested that the bis-piperidine group on CPT-11 induces cholinergic diarrhea in animals (11). As the bis-piperidine group does not exist in EZN-2208, this PEGylated compound may have an improved gastrointestinal safety profile in humans. The safety profile of EZN-2208 currently is being evaluated in phase I studies.

There has been a lot of interest to solubilize SN38, and various formulations have been developed, including a liposome-based formulation of SN38 (LE-SN38; refs. 27, 28), a polymeric micellar formulation (NK012; ref. 24), and a tocopherol conjugate (SN2310; ref. 29). All existing formulations of SN38 rely either on noncovalent bond formulation (LE-SN38) or conjugate SN38 through the 10-OH group (LE-SN38, NK012, and SN2310), which have no effect on the lactone E-ring in the active conformation. Therefore, EZN-2208 provides a novel approach to preserve the activity of SN38 during circulation and to release the active molecule on cleavage.

In conclusion, we have shown that EZN-2208 is a novel, water-soluble prodrug of SN38 that enables significantly increased solubility, parenteral delivery of SN38, longer apparent half-life, higher exposure, and significantly enhanced therapeutic index in several preclinical xenograft models, including a model of CPT-11 resistance, compared with CPT-11. Thus, EZN-2208 seems to be an anticancer agent with a novel mechanism of action, and its efficacy should be explored in a wide variety of tumors in populations including patients refractory to CPT-11–containing treatment.

Acknowledgments

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