Marked therapeutic efficacy of a novel polyethylene glycol-SN38 conjugate, EZN-2208, in xenograft models of B-cell non-Hodgkin's lymphoma

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ABSTRACT

Examination of the clinical utility of SN38 (10-hydroxy-7-ethyl-camptothecin), the active metabolite of CPT-11, has not been possible to date due to poor solubility of SN38. Here we evaluated the activity of EZN-2208, a water-soluble polyethyleneglycol-SN38 conjugate, in pre-clinical models of Burkitt's non-Hodgkin's lymphoma (NHL) (Raji and Daudi), and follicular NHL (DoHH2). *In vitro*, the IC_{50} of EZN-2208 ranged from 3-24 nM, which was 30- to 45-fold lower than CPT-11 or 2.5- to 3.5-fold higher than SN38. In both an early-disease Raji model and an advanced-disease Daudi model, treatment with multiple doses of EZN-2208 resulted in 90% and 100% cures of animals, respectively (cure defined as no sign of tumors by gross observations at the termination of study). The activity of EZN-2208 was dramatically superior to that of CPT-11 in all three models. The excellent therapeutic efficacy of EZN-2208 in several B-cell NHL xenograft models merits its evaluation in the clinic for lymphoid malignancies.

Key words: SN38, polyethylene glycol, drug conjugate, B-cell non-Hodgkin's lymphoma, CPT-11, *in vivo*.

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Introduction

Despite recent advances in the treatment of B-cell non-Hodgkin's lymphoma (NHL), novel therapeutics with improved efficacy and safety are needed. Although a wide variety of chemotherapeutic options are available for patients with B-cell NHL, these drugs, even when combined with radiation therapy, have not resulted in long-term disease-free survival in the majority of the patients.[1,2] Over the last decade, rituximab treatment has revolutionized the therapy of NHL. However, as these patients progress after receiving multiple rituximab-based regimens, alternative treatment options are needed for this group of patients as well.[3]

CPT-11 (Camptosar®, irinotecan), a DNA topoisomerase I inhibitor, is widely used for the treatment of patients with advanced colorectal cancer and some other solid tumors. Although in some Phase I or II trials a response rate of 25-62% has been reported for patients with relapsed or refractory aggressive lymphomas who received CPT-11,[4-8] the small study sizes, the wide spectrum of histological types of tumors, and the diverse dosage and schedule options have made it difficult to assess the efficacy of CPT-11 in this patient population. In addition, the high unpredictable gastrointestinal toxicity can compromise the clinical utility of CPT-11. Furthermore, CPT-11 as a drug has several limitations, including the requirement of an endogenous enzyme to convert the pro-drug (CPT-11) to the active form (SN38), leading to inter-individual variability in drug safety profile and antitumor acitivity. Therefore, novel topoisomerase I inhibitors that can mitigate these limitations are sought. SN38 (10-hydroxy-7-ethyl-camptothecin) is the active moiety of CPT-11. Hydrolysis of the water-soluble pro-drug, CPT-11, to SN38 by carboxylesterase 2 is associated with a 100- to 1,000-fold increase in cytotoxicity.[9] Despite promising anti-cancer potential in the laboratory, currently SN38 itself has not been used as an anticancer drug in humans due to its poor solubility in any pharmaceutically acceptable excipient. The solubility of SN38 can be vastly improved by PEGylation. EZN-2208 (polyethylene glycol [PEG]-SN38) is a water-soluble PEGylated conjugate with approximately 3.5 to 4.0 SN38 molecules attached to the multi-arm PEG backbone.[10] We have previously shown in pre-clinical models using multiple solid tumors (including a model of *in vivo* CPT-11 resistance), that EZN-2208 has a significantly enhanced therapeutic index compared with CPT-11.[11] In these studies, EZN-2208 provides higher exposure of tumors to SN38 via the preferential accumulation of EZN-2208 in the solid tumors (enhanced permeability and retention [EPR] effect) compared with CPT-11.[11]

In this paper, we demonstrate that treatment with EZN-2208 can cure animals in pre-clinical models of B-cell NHL (Raji, Daudi, DoHH2). The high anti-tumor activity may be attributed, in part, to improved pharmacokinetics as reported by us previously.[11]

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The online version of this article contains a supplementary appendix.

Design and Methods

Materials, cell lines and animals

EZN-2208 was prepared as described previously.[10,11] Irinotecan (CPT-11, Camptosar®), was obtained from Bell Medical Services, NJ, USA. The human B-cell lymphoma cancer cells, Raji (Burkitt's) and Daudi (Burkitt's), were obtained from American Type Culture Collection (Manassas, VA, USA), and the DoHH2 (follicular) cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). Seven- to eight-week old female BALB/c or CB17 SCID mice were purchased from Harlan, Indianapolis, IN, USA. All animal studies were approved by the University of Medicine and Dentistry New Jersey Institutional Animal Care and Use Committee.

Antiproliferation assay and in vitro apoptosis analysis

The antiproliferative activity of EZN-2208 was determined using the Cell Titer 96 Aqueous One Solution (MTS) (Promega, Madison, WI, USA) as described previously.[11] Apoptosis analysis was performed using the annexin V-FITC/propidium iodide assay. (Invitrogen/Molecular Probes, Eugene, OR, USA) (*further details are included in the Online Supplementary Appendix*).

In vivo therapeutic efficacy in B-cell NHL xenografts

The therapeutic efficacy of EZN-2208 was evaluated in three human xenograft models of B-cell NHL. Female CB17 SCID (n=6-10/group) mice were injected i.v. with Raji, DoHH-2 and Daudi cells at 2.5×10^6 cells/mouse, 10^7 cells/mouse, and 1.5×10^7 cells/mouse, respectively. For early-disease models, the mice received treatments of CPT-11 or EZN-2208 either at the single or multiple (q2d×5) MTD doses on day 1 (Raji and Daudi) or day 2 (DoHH2) post-administration of cells. For the advanced-disease models, mice received EZN-2208 or CPT-11 on day 6 (Daudi) or day 11 (DoHH2) post-administration of cells. The single MTD of EZN-2208 and CPT-11 in SCID mice is 30 mg/kg and 60 mg/kg, respectively. The multiple (q2d×5) MTD of EZN-2208 and CPT-11 in SCID mice is 10 mg/kg and 40 mg/kg, respectively. In these models, tumors grow systemically and mice become paralyzed when tumor cells infiltrate the spinal cord, resulting in hind-leg paralysis. Mice were sacrificed at onset of paralysis or when they lost 20% of their pre-treatment body weight. The observation period lasted five to seven times the median survival time (MST) of control animals, at which time all mice were sacrificed and a gross necropsy was performed. *In vivo* survival studies were analyzed using Kaplan-Meier plots, with GraphPad Prism software. Cure of animals was defined as no evidence of tumor by gross observation at the termination of the study. It is important to note that the doses or concentrations of EZN-2208 stated in this paper refer to SN38 equivalents. For example, a dose of 30 mg/kg of EZN-2208 is equivalent to a dose containing 30 mg/kg of SN38, or 829 mg/kg (28-fold higher) of whole conjugate, when loading SN38 in the whole EZN-2208 conjugate is 3.62%.

Results and Discussion

We show here excellent therapeutic efficacy of EZN-2208 in three models of pre-clinical B-cell NHL. *In vitro*, EZN-2208, as well as SN38, demonstrated potent cytotoxicity compared to CPT-11 in a panel of human lymphoma cells lines. The IC_{50} of EZN-2208 ranged from 3-24 nM, which was 30- to 45-fold more potent than CPT-11 (Table 1). The cytotoxicity of EZN-2208 was 2.5- to 3.5-fold less than that of SN38 (1.2 to 5 nM). The impressive low nM IC_{50} of SN38 or EZN-2208 in various lymphoma lines suggest the utility of this agent in the clinic for patients with lymphoid malignancies. In terms of apoptosis assays, no significant difference in apoptosis-inducing effects of EZN-2208 versus SN38 was observed (*Online Supplementary Appendix*). The major mechanism of cell killing at 24 h was rapid progression to late apoptosis and cell necrosis. From 24 to 48 h, any residual viable cells underwent a slower progression to early apoptosis before progressing to necrosis.

Since experiments performed *in vitro* typically do not capture the advantages of PEGylating SN38, such as improved pharmacokinetics, they may underestimate the efficacy of EZN-2208. In fact, excellent *in vivo* therapeutic efficacy of EZN-2208 was obtained in all three models of B-cell NHL (Raji, Daudi and DoHH2). The first study was performed in the Raji model (Figure 1). Whereas control animals treated with saline had a median survival time (MST) of 16 days, treatment with a single MTD of EZN-2208 resulted in cure for 50% of animals (Figure 1A). In contrast, CPT-11 dosed at its single MTD resulted in only a 25% increase in life span (ILS) (Figure 1A). In the same model, treatment of animals with multiple doses of EZN-2208 resulted in cures of 90% of animals in contrast to no cures and 169% ILS observed for multiple dose CPT-11 group (Figure 1B).

We then evaluated the therapeutic efficacy of EZN-2208 in relation to tumor burden in two other B-cell NHL xenograft models, Daudi and DoHH2. For the Daudi model, treatments were given either one day or six days post-administration of cells, thus establishing an early-disease model or a late-disease model, respectively (Figure 2A). In the early-disease model, where control animals had an MST of 35 days, a single MTD of EZN-2208 resulted in cures of 6 of 9 animals in contrast to only a 66% ILS and no cures observed for the group that received a single MTD of CPT-11. In this setting, multiple injections of EZN-2208 cured 100% of animals. Significant cures (5 of 9) were also obtained when CPT-11 was given as multiple injections in an early-disease setting. However, in the

Table 1. ***In vitro*** **cytotoxicity.**[a]

Cell line	SN38	EZN-2208[b]	CPT-11
Raji	5±1	18±1	843±309
Daudi	9±7	24±9	722±367
DoHH2	1.2±0.9	3.4±1.9	144±50

[a]IC50 (nM) of EZN-2208 is expressed in terms of SN38 equivalents; [b]Values shown are average ± SD (n=3 independent experiments).

advanced-disease setting, CPT-11 efficacy was significantly diminished. A single MTD of CPT-11 was completely ineffective, and multiple injections of CPT-11 only caused 137% ILS with no cures. Conversely, EZN-2208 had a dramatic effect in the advanced-disease model. A single MTD of EZN-2208 cured 5 of 9 animals, and multiple injections of EZN-2208 cured 100% of animals. Another experiment was performed in DoHH2 model where the efficacy of EZN-2208 was begun two days after tumor injection or with an even more advanced-disease setting compared to the Daudi model above (treatment was initiated 11 days after cell injection). Where control animals had MST of 19 days, multiple-dose EZN-2208 treatment started two days after cell injection resulted in cures of 72% of animals; however, CPT-11 treatment resulted in a MST of 66 days and 42% cures. When treatment was initiated 11 days post-administration of cells, CPT-11 or EZN-2208 treatment resulted in MST of 43 days or 77 days, respectively. We do realize that these models are representative of Burkitt's (Raji and Daudi) or follicular (DoHH2) B-cell NHL and hence general claim of utility of EZN-2208 in all settings of lymphoid malignancies may not be applicable. However, the impressive pre-clinical efficacy shown here in these models does serve as proof of concept and merits the investigation of this agent in patients with lymphoid

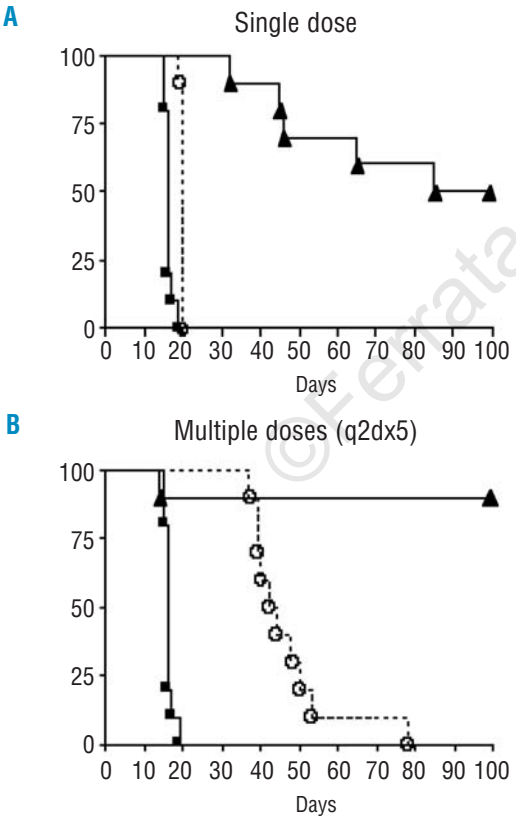

Figure 1. Therapeutic efficacy of EZN-2208 in a xenograft model of NHL (Raji). CB17 SCID mice (6-10 mice per group) were inoculated i.v. with 2.5×10^6 Raji cells per mouse. After one day, mice were treated with either a single (qd×1) or multiple (q2d×5) injections of EZN-2208 or CPT-11 at MTD. (A) Single MTD doses: Control (■), EZN-2208 at 30 mg/kg (▲), CPT-11 at 60 mg/kg (○) (B) Multiple MTD doses (q2d×5): Control (■), EZN-2208 at 10 mg/kg (▲), CPT-11 at 40 mg/kg (○).

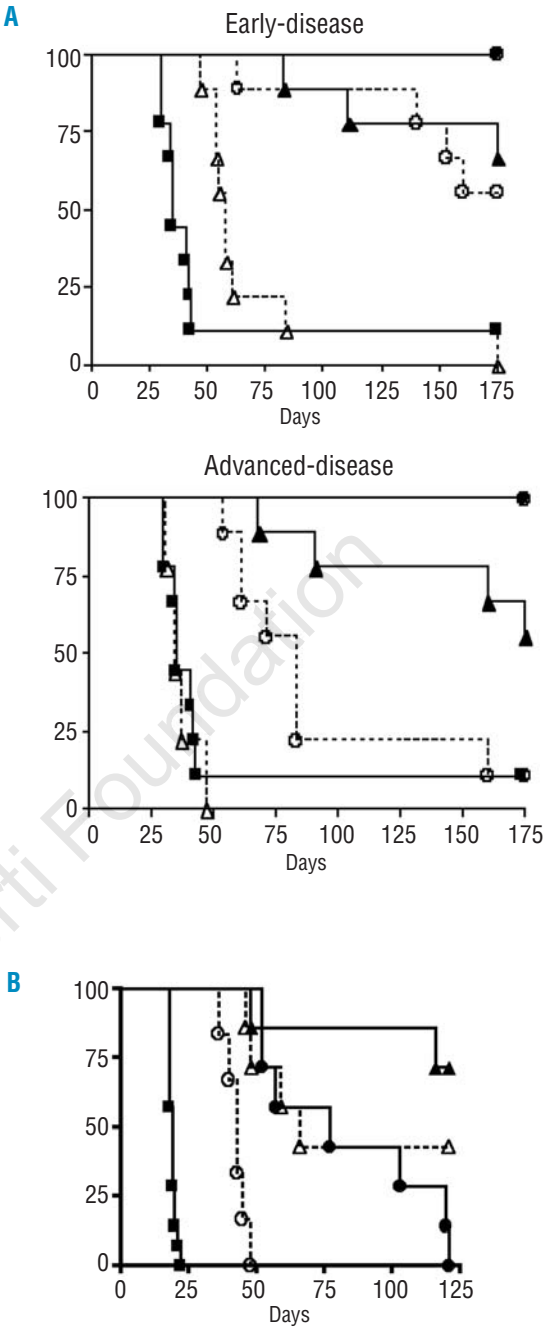

Figure 2. (A) Therapeutic efficacy of EZN-2208 in an early- (*first graph*) versus advanced- (*second graph*) disease xenograft model of NHL (Daudi). CB17 SCID mice (6-10 mice per group) were inoculated i.v. with 1.5×10^7 Daudi cells per mouse. EZN-2208 or CPT-11 was given either as single (qd×1) or multiple (q2d×5) MTD doses. Early-disease model treatment started on day 1, and advanced-disease model treatment started on day 6. Control (■), EZN-2208 at 30 mg/kg qd×1 (▲), CPT-11 at 60 mg/kg qd×1 (△), EZN-2208 at 10 mg/kg q2d×5 (●), CPT-11 at 40 mg/kg q2d×5 (○). (B) Therapeutic efficacy of EZN-2208 in an early-versus advanced-disease xenograft models of NHL (DoHH2). CB17 SCID mice (6-10 mice per group) were inoculated i.v. with 1.0×10^7 DoHH2 cells per mouse. EZN-2208 or CPT-11 was given as multiple (q2d×5) MTD doses starting on day 2 or on day 11 post-administration of cells. Control (■), EZN-2208 at 10 mg/kg q2d×5 starting on day 2 (▲), CPT-11 at 40 mg/kg q2d×5 starting on day 2 (△), EZN-2208 at 10 mg/kg q2d×5 starting on day 11 (●), or CPT-11 at 40 mg/kg q2d×5 starting on day 11 (○).

cancers. The excellent therapeutic efficacy of EZN-2208 is attributed, in part, to the favorable pharmacokinetic profile achieved by the drug. We have shown previously in solid tumor xenograft models that EZN-2208 provides longer circulation times to the active moiety (SN38) compared with CPT-11.[11]

Besides giving an improved pharmacokinetic advantage, EZN-2208 may also have a novel mechanism of action. We have preliminary evidence using a hypoxia-inducible factor-1 (HIF-1) reporter cell line that treatment with a single dose of EZN-2208 results in potent, sustained downmodulation of HIF-1α (~80% by day 5), whereas CPT-11 has no effect; CPT-11 has minor effects only when the drug is given in multiple doses.[12] This observation is in agreement with studies published by Rapisarda *et al*. in which topotecan, another topoisomerase I inhibitor, administered on a daily but not an intermittent schedule, resulted in down-regulation of HIF-1α and tumor growth inhibition.[13,14] Thus, a prolonged exposure of SN38 by EZN-2208 can down-regulate HIF-1α in a more potent and sustained manner compared to CPT-11. Besides being associated with poor prognosis in many types of solid tumors, the literature also suggests that HIF-1 is frequently activated in lymphoma and may contribute to disease progression.[15] In one study, 44% of diffuse large B-cell lymphoma versus 11% of follicular lymphoma biopsies had moderate-to-high expression of both HIF-1α and HIF-2α.[16]

A significant limitation of CPT-11 in the clinic is frequent and unpredictable gastrointestinal toxicity. In an ongoing Phase I program in solid tumors and lymphoid malignancies, where EZN-2208 was administered as a one hour intravenous infusion every three weeks, no early late gastrointestinal toxicities were observed, and neutropenic fever was the dose-limiting toxicity.[17]

In conclusion, we have developed a novel PEGylated SN-38 drug conjugate which demonstrates significant therapeutic efficacy in several models of B-cell NHL and may have a distinct mechanism of action (via potent and sustained inhibition of HIF-1α) compared to CPT-11. Excellent pre-clinical data and a favorable safety profile of EZN-2208 in Phase I studies support further evaluation of this compound in patients with B-cell NHL.

Authorship and Disclosures

PS conceived and designed the experiments, analyzed and interpreted the data and wrote the manuscript; PK, MM and JM performed the *in vitro* and *in vivo* experiments and analyzed the data; HZ developed the chemistry of EZN-2208; LG and IH contributed in the design of experiments, interpretation of the data and writing of the manuscript; IH conceived the concept of developing EZN-2208.

All authors are full time employees of Enzon Pharmaceuticals and own company stock options and/or units.

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