Tumor regression and curability of preclinical neuroblastoma models by PEGylated SN38 (EZN-2208), a novel topoisomerase I inhibitor

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\textbf{Running title:} PEGylated SN38 therapeutic efficacy in neuroblastoma

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Translational relevance

Human neuroblastoma represents a major therapeutic challenge due to the lack of drug effective at achieving disease eradication. Approximately a half of neuroblastoma patients present with metastatic disease at diagnosis, and only 25% of them survive at five years in spite of high dose chemotherapy and autologous hematopoietic stem cells rescue.

This study describes for the first time an innovative therapeutic treatment for neuroblastoma, based on the use of the novel DNA topoisomerase-I inhibitor, EZN-2208, that, due to the extraordinary results obtained in various biologically and clinically relevant preclinical settings, led to the rapid translation to clinical trials in patients with various pediatric cancers, including neuroblastoma.
ABSTRACT

Purpose

Treatment of neuroblastoma (NB) is successful in less than half of patients with high-risk disease. The anti-tumor activity of a water soluble pegylated SN38 drug conjugate (EZN-2208), was compared with CPT-11 (a pro-drug for SN38) in preclinical models of human NB.

Experimental Design

The in vitro cytotoxicity of EZN-2208 was tested by counting trypan blue dye- and annexin-V-positive cells, while its therapeutic efficacy evaluated, in terms of survival, anti-tumor, and anti-angiogenic activities, in subcutaneous luciferase-transfected, pseudometastatic and orthotopic NB animal models.

Results

EZN-2208 was about 100-fold more potent than CPT-11 in vitro, by inducing apoptosis/necrosis and p53 expression and by reducing HIF-1α/ HIF-2α expression. EZN-2208 gave superior antitumor effects compared to CPT-11 in NB xenografts. EZN-2208 treatment always resulted in lack of tumor detection at the end of trials whereas only small therapeutic effects were observed with CPT-11, as assessed by luciferase assay, tumor size, or even staining histological sections of tumors with antibodies recognizing NB cells and cell proliferation. In NB model resistant to Doxorubicin (D), Cisplatin, Vincristine (V), Fenretinide and Topotecan (T), EZN-2208 induced 100% curability. It also blocked tumor relapsed after TVD-combined treatment. Mechanistic experiments showed statistically significant enhanced TUNEL and Histone H2ax staining and decreased VEGF, CD31, MMP-2 and MMP-9 expression in tumors removed from EZN-2208-treated mice and radiating vessels invading the tumor implanted onto the chorioallantoic membranes.
Conclusions

EZN-2208 should be considered a most promising novel anti-NB agent and ongoing phase I study in pediatric patients should identify the optimal dose for Phase II study.

INTRODUCTION

Treatment of neuroblastoma (NB), the second most common solid tumor in childhood, is successful in less than half of patients with high-risk disease (1). The effective treatment of NB, either at advanced stages or at minimal residual disease, remains indeed one of the major challenges in pediatric oncology. The use of intensive therapeutic interventions have marginally prolonged the overall long-term disease-free survival rates, mainly due to the dose-limiting toxicity associated with systemic delivery of cytotoxic drugs in vivo (2). Moreover, the incidence of fatal relapses is still high and long-term survival remains very low. Innovative therapies are thus required to eradicate residual disease after chemotherapy and surgery.

Camptothecin and its analogs irinotecan (CPT-11) and topotecan, have hold great promise in the treatment of NB, for several reasons (3-6). First, they have impressive activity against NB cell lines resistant to standard anti-neuroblastoma agents. These findings imply that they might provide a novel non-cross-resistant chemotherapeutic addition to the standard drug armamentarium for NB. Second, their non-hematological toxicities are manageable and transient. Such limitations are major consideration in NB patients who often undergo nephrectomy, are treated with cardiotoxic (doxorubicin) and nephrotoxic (platinum compounds) agents, and who receive irradiation. Third, cytotoxicity occurs at relatively nonmyelosuppressive dosages, suggesting that they can be safely given with other anticancer drugs (5, 6).

On the other hand, CPT-11 has several limitations, such as the limited and variable conversion by endogenous enzymes to convert the pro-drug (CPT-11) to the active form (SN38) (no better than approximately 4% of the total drug), an inter-individual variability in drug safety profile, the
induction of diarrhea (associated, in part, with the parent molecule), and the limited anti-tumor activity as a single agent (7, 8) including in the treatment of NB (6). These limitation suggest that direct delivery of the prodrug, SN-38 may improve the efficacy and/or safety of this compound. However, SN-38 is insoluble in pharmaceutical acceptable vehicles and therefore cannot be given by itself. This problem has been overcome with the development of a pegylated SN38, designated EZN-2208.

EZN-2208 is a water soluble pegylated SN38 drug conjugate, composed of a four-arm 40 KDa polyethylene glycol (PEG) linked via glycine residue to SN38 (9). This highly soluble molecule shows favorable prolonged circulation time leading to a preferential accumulation in the solid tumors and has shown considerably more efficacy than CPT-11 in various preclinical models of solid and hematologic tumors (10, 11). Moreover, in Phase I trials in adult patients, EZN-2208 was well tolerated, with dose-limiting toxicity (DLT) of isolated, short-lived neutropenia with or without fever. This is in distinction to irinotecan where grade ¾ diarrhea is frequently encountered at therapeutic doses (12, 13). In on-going Phase II trials with EZN-2208, prolonged stable disease, sometimes associated with tumor shrinkage, was also observed.

In this report, the activity of EZN-2208 has been evaluated in experimental models of pediatric NB. However, unlike previous compounds that have been evaluated in experimental model of pediatric NB tumor where the tumor is grown as subcutaneous xenograft in nude mice, we have rigorously evaluated EZN-2208 in this tumor model as well as pseudometastatic and orthotopic models of human NB. Collectively, these models better reflect the growth of advanced NB in children (i.e. large adrenal gland tumors and multiple small metastatic lesions), which have been difficult to control.

EZN-2208 demonstrated excellent and clearly superior antitumor activity compared with CPT-11 as well as with many others clinically used anti-neoplastic drugs. In all tested models, EZN-2208-
treated tumor-bearing mice survived, with complete regression of implanted tumors. Therefore, EZN-2208 is a most promising, novel anti-tumor agent for the treatment of NB.

METHODS

Materials, cell lines and animals

EZN-2208 was prepared as described previously (9, 10). Irinotecan (CPT-11, CAMPTOSAR®) was purchased from Pfizer (Pfizer Italia S.r.l.; Borgo S. Michele, LT, Italy); Topotecan (HYCAMTIN®) from Glaxo (GlaxoSmithKline S.p.A.; Verona, Italy); Cisplatin and Doxorubicin from Ebeve Italia S.r.l., Roma, Italy; Vincristine from Sigma Chemical Co. (St. Louis, MO). Fenretinide (N-4-hydroxyphenyl retinamide, HPR) was kindly provided by Dompè (Dompè Farmaceutici S.p.A., Milan, Italy). Deferoxamine (Desferal, DFX) was purchased from Novartis Pharma (Stein, Switzerland).

To broadly cover the phenotypes exhibited by neuroblastoma (NB) cells in vitro and in vivo, five human (GI-LI-N, HTLA-230, IMR-32, SK-N-BE2c and SH-SY5Y) and one murine (NXS2) NB cell lines were used (14, 15). In some experiments, the SH-SY5Y cell line was infected with retrovirus expressing the firefly luciferase gene and luciferase activity of retrovirally-transduced cells confirmed by bioluminescence imaging (IVIS Caliper Life Sciences, Hopkinton, MA) after a 10 min. incubation with 150 μg/mL of D-luciferin (Caliper Life Sciences), as described (16). All cell lines were grown in complete DMEM or RPMI-1640 medium, supplemented with 10% heat-inactivated FCS, as described (14, 15). All cell lines were tested for mycoplasma contamination, and characterized by cell proliferation morphology evaluation and multiplex short tandem repeat profiling test, both after thawing and within four passages in culture.

All animals were purchased from Harlan Laboratories (Harlan Italy, S.Pietro al Natisone, Italy) and housed under specific pathogen-free conditions. Experiments involving animals were reviewed and
approved by the licensing and ethical committee of the National Cancer Research Institute, Genoa, Italy, and by the Italian Ministry of Health. All experiments in vivo were performed with 5-8 mice per group and were repeated twice.

**In vitro cytotoxicity and apoptosis**

The in vitro cytotoxicity of EZN-2208 was evaluated in a panel of NB cell lines by counting trypan blue dye-stained cells. Briefly, adherent cells were plated in 75-cm³ flasks and incubated overnight at 37°C. The day after cells were treated with serial dilutions of EZN-2208 and further incubated for 24 hours. At the end of the incubation period, cells were detached with trypsin, stained with trypan blue dye, and counted microscopically. Apoptosis analysis was performed using the annexin V-FITC/propidium iodide assay, as described (11).

**Immunoblotting analysis**

Western blotting analysis from whole cell lysates was performed as described (17). Briefly, cultured NB cells were treated for 24 and 48 hours with the same concentration of either CPT-11 or EZN-2208. In some experiments, as positive control for HIF-1α induction, cells were pre-incubated with 0.15 mM of Desferal (DFX) for 6 hours, washed and treated with CPT-11 and EZN-2208 for a total of 24 hours-incubation. Monoclonal anti-p53 (clone PAb 1801) and anti-HIF-1α (clone 54) were purchased from BD Biosciences (Buccinasco, MI, Italy), anti-HIF-2α (clone ep190b) an anti-GAPDH (clone 14c10) antibodies were from Novus Biologicals, Inc (Cambridge, UK) and Cell Signaling Technology (Danvers, MA, US), respectively.

**Animal models**
For the subcutaneous (s.c.) animal model, 2.0 x 10^7 luciferase-transfected, SH-SY5Y cells were inoculated in the mid-dorsal region of five-week-old female SCID mice (5 mice/group). Tumor expansion over time, as well as the response to treatment have been readily visualized by bioluminescence imaging (BLI) and quantified by highly sensitive, cooled CCD camera mounted in a light-tight specimen box (IVIS™), as described (15, 16).

For the pseudometastatic animal model, four-week-old female, athymic (nu/nu) mice (8 mice/group) were injected intravenously (i.v.) in the tail vein with 4 x 10^6 HTLA-230 tumor cells, as described (18). Body weight and general physical status of the animals were recorded daily, and mice were sacrificed by cervical dislocation after the administration of xilezine (Xilor 2%, Bio98 Srl, Milan, Italy), when they showed signs of poor health, such as abdominal dilatation, dehydration, or paraplegia.

In the orthotopic animal model, five-week-old female, athymic (8 mice/group) or immunocompetent (A/J mice, 5/group) animals were anesthetized with ketamine (Imalgene 1000, Merial Italia SpA., Milan, Italy), subjected to laparotomy, and injected with either 1 x 10^6 GI-LI-N or 5 x 10^4 NXS2 cells, respectively, in the capsule of the left adrenal gland, as previously described (14, 19). No mice died as a result of this treatment. Mice were monitored at least two times weekly for evidence of tumor development and quantification of tumor size, and were sacrificed by cervical dislocation after being anesthetized with xilezine, when they showed signs of poor health, such as abdominal dilation, dehydration, or paraplegia.

In vivo therapeutic efficacy

Mice were treated i.v. every other day for 5 total doses with 10 mg/kg of CPT-11 (CAMPTOSAR) or with the SN38 equivalents of EZN-2208 (for details, see reference number 10), in the first set of in vivo experiments, or with MTD doses, for multiple-dose regimen therapies (10) of both
CAMPTOSAR (40 mg/kg) and EZN-2208 in a second set of in vivo experiments. In every experiments, a group of control mice received HEPES-buffered saline.

For the s.c. animal model, tumors were allowed to grow for 7 (first set of experiments) and 13 (second set of experiments) days, reaching a size of about 100 and 200 mm³ before i.v (tail vein) treatment commenced. For the pseudometastatic model, mice were treated i.v. either 24 or 72 hours post-inoculation of tumor cells. For the orthotopic animal models, tumors were allowed to grow from the injected cells for 21 and 3 days, in immunodeficient and immunocompetent mice, respectively, before treatments started. For the tumor regression studies, mice were sacrificed and tumors were measured with calipers. Tumor volumes were calculated by the formula \( \pi/6 \times (w_1 \times (w_2)^2) \), where \( w_1 \) represented the largest tumor diameter and \( w_2 \) represented the smallest tumor diameter, as described (19). The anti-tumor EZN-2208 activity was also evaluated either in comparison with MTD doses of different, clinically used, anti-tumor compounds (Topotecan, CPT-11, Cisplatin, Doxorubicin, Vincristine and Fenretinide), or as a second-line treatment in Topotecan-Vincristine-Doxorubicin (TVD)-resistant, orthotopic NB-bearing mice. For these experiments, tumors were allowed to grow from the injected cells for 21 days in nude mice (6 mice/group), before randomly treated with: EZN-2208, i.v., 10 mg/kg every other day for 5 total doses; CAMPTOSAR, i.v., 40 mg/kg, every other day for 5 total doses; Topotecan, intraperitoneally (i.p.), 10 mg/kg, every 4 days x 3 times; Cisplatin, i.v., 5 mg/kg, once per week, 3 weeks total; Doxorubicin, i.v., 5 mg/kg, once per week, 5 weeks total; Vincristine, i.v., 1 mg/kg, once per week, 7 weeks total; fenretinide, i.v., 1.5 mg/kg, twice per week, 6 weeks total, as previously reported (10, 17, 19-22).

Another group of animals (7/group) was treated 21 days after tumor challenge with a combination of Topotecan (3 mg/kg, i.p., daily from day 21 to day 25), Vincristine (50 μg/kg, i.v., at day 25) and Doxorubicin (1.5 mg/kg, i.v., at day 25), mimicking schedule and treatment doses performed in patients suffering from high-risk NB (23). When signs of poor health, such as abdominal dilation,
became evident (day 80), half of TVD-treated mice, randomly chosen, were then inoculated with EZN-2208, 10 mg/kg every other day for 5 total doses.

**Chorioallantoic membrane assay**

Chorioallantoic membrane (CAM) experiments were performed according to the method of Ribatti et al. (24). NB (GI-LI-N and HTLA-230 tumor cell lines) biopsy fragments (1-2 mm³) taken from untreated mice were grafted onto the CAM and then treated with either PBS (control), EZN-2208, or CPT-11 (SN38 equivalents and at MTD). CAMs were examined daily until day 12 and photographed *in ovo* with a stereomicroscope equipped with a camera and an image analyzer system (Olympus Italia, Rozzano Italy). On day 12, the angiogenic response, as determined by the number of vessels converging toward the grafts, was evaluated with an image analyzer. CAMs were then processed for light microscopy, as reported (17). Microvessels density was expressed as the percentage of the total number of intersection points occupied by CD31-positive vessels cut transversely (diameter, 3-10 μm). Mean values ± SD were determined for each analysis.

**Histological analysis**

In the first set of experiments, histological evaluation of primary tumors was performed 50 days after GI-LI-N cell inoculation. Briefly, tumors were orthotopically implanted in athymic mice. After 21 days, animals were treated with 10 mg/kg of CPT-11 (CAMPTOSAR) or with the SN38 equivalents of EZN-2208 every other day for 5 total doses. In every experiments, a group of control mice received HEPES-buffered saline.

Three weeks after the end of the treatment, mice were anaesthetized with xylezine and killed by cervical dislocation. Tumors were collected and then embedded in optimum cutting temperature
(O.C.T.) (Miles Chemical Co., Elkhart, IN) compound. Tissue sections (5 μm thick) were examined after staining with Mayer’s Hematoxylin-Eosin (H&E) (Sigma).

Tumor frozen sections were then washed twice in phosphate buffered saline (PBS) and saturated with 2% bovine serum albumin (BSA) in PBS before staining with primary antibodies against anti-human NB (NB84a, Dako) and Ki-67 proliferation antigen (mouse anti-human Ki-67, clone Ki-55, Dako), as described (17).

In the second set of experiments, histological evaluation of primary tumors was performed at 44 days after GI-LI-N cell inoculation. Briefly, orthotopically implanted athymic mice were implanted. After thirty-five days, animals were treated with 10 mg/kg of CPT-11 (CAMPTOSAR) or with the SN38 equivalents of EZN-2208 or with MTD doses of CAMPTOSAR (40 mg/kg), every other day for 5 total doses. In every experiment, a group of control mice received HEPES-buffered saline. One day after the end of the treatment, mice were anaesthetized with xylazine and killed by cervical dislocation. Tumors were collected, divided in two, and processed as stated above.

Tumor frozen sections were stained with primary antibodies against Histone H2ax (H2AFX, clone EP854(2)Y, Lifespan Biosciences, Prodotti Gianni, Milan, Italy) to detect DNA-damage dependent histone phosphorylation (Huang X Cell Cycle 2003); VEGF (Thermo Fisher Scientific, Fremont, CA, USA) and CD31 (clone SC-1506, Santa Cruz Biotechnology, D. B. A. Italia S. R. L., Segrate, Milan, Italy) to detect inhibition of angiogenesis. TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated end labeling) staining was performed using a commercially available apoptosis detection kit (In situ Cell Death Detection, POD; Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer’s instructions, as described (17).

Paraffin-embedded tissue sections were de-paraffinized by a xylene-ethanol sequence, re-hydrated in a graded ethanol solutions, and TRIS-buffered saline (TBS, pH 7.6), and then processed for antigen retrieval by boiling tissue sections for 10 min in 1 mM EDTA, pH 8.0, in a microwave oven. The sections were then washed twice in PBS and saturated with 2% BSA in PBS before
staining with primary antibodies against MMP-2 (clone 36006, R & D System, Abingdon, UK) and MMP-9 (clone 443, R & D System) to detect inhibition of tumor invasiveness-related markers. Morphometric analysis was performed on 9 randomly selected fields every 3 sections, observed at 200x magnification with an Olympus photomicroscope, using Image Analysis software (Olympus Italia). VEGF and MMP-2/MMP-9 labeled areas were evaluated.

**Statistical analysis**

Results are expressed as mean ± 95% Confidence Intervals (95% CI). The statistical significance of differential findings between experimental groups and controls was determined by ANOVA, with the Tukey’s multiple comparison test (GraphPad Software, Inc., El Camino Real, San Diego, CA). The significance of the differences between experimental groups in the survival experiments was determined by Kaplan-Meier curves by the use of Chi square log-rank test (Graph-Pad Prism 3.0). In the morphometric analysis, the mean value in each image from the section, the final mean value for all the images and the SEM were calculated. The statistical significance of the differences between the mean values of the different experimental conditions was determined by Student’s t test (GraphPad software). Findings were considered significant at P values < 0.05 for all statistical evaluations.

**RESULTS**

**In vitro studies**

EZN-2208 showed potent *in vitro* cytotoxicity against NB cells, with IC$_{50}$ values ranging from 0.002 to 0.15 µmol/L (Table 1, supplementary). Consistent with previous results (11), EZN-2208 induced a rapid induction of annexin-V-positive NB cells, followed by massive necrosis (data not
shown). This cell death were accompanied by a rapid and strong induction of p53 and a down-modulation of HIF-2α proteins expression (Figure 1S, A-B). Noteworthy, compared with CAMPTOSAR, EZN-2208 led also to a strong down-modulation of both constitutive (Figure 1S, C) and DFX-induced (Figure 1S, D) HIF-1α protein levels.

**Therapeutic effects of CPT-11 and SN38 equivalents of EZN-2208**

In the subcutaneously injected NB-bearing mice, the effects of EZN-2208 and CAMPTOSAR were compared against a luciferase-transfected human NB cell line (SH-SY5Y) inoculated in the right flank of animals. Treatment started seven days after cell injection and the anti-tumor efficacy evaluated by bioluminescence (BLI). As showed in Figure 1,A, while CAMPTOSAR led to a partial anti-tumor response, compared to control mice that were sacrificed at day 40 for excessive tumor mass, a complete regression of primary tumor growth in NB-bearing mice were observed after treatment with EZN-2208.

In the pseudometastatic model, mice were treated either 24 or 72 hours after intravenous tumor cells (HTLA-230) inoculation. As showed in Figure 1,B, animals treated with EZN-2208 displayed significant increased life span compared to control mice or those treated with CAMPTOSAR. After 150 days post cell implantation, all EZN-2208-treated mice were still disease-free, while control and CAMPTOSAR-treated animals had already died with metastatic disease.

In the orthotopic NB model, the anti-tumor efficacy of EZN-2208 was evaluated in both immunocompetent (A/J mice) and immunodeficient (nude mice) NB-bearing animals. A/J mice were inoculated with the very aggressive NB cell line NXS2 and treated after three days post cell inoculation, while nude mice were inoculated with GI-LI-N cells and treated after 21 days post cell inoculation. In both models, mice treated with EZN-2208 showed a dramatic arrest in primary tumor growth compared to control mice. Moreover, while CAMPTOSAR-treated mice died with widespread tumor masses within 50 (CAMPTOSAR-resistant animal model) and 80
(CAMPTOSAR-sensitive animal model) days, respectively, long term survival was seen in 100% of EZN-2208-treated animals (Figure 1,C and Figure 2,A). Furthermore, GI-LI-N-bearing, EZN-2208-treated mice showed a dramatic regression of the tumor mass, as showed in Figure 2,B.

To assess the impact of EZN-2208 on tumor cell proliferation, viability, and apoptosis, we stained tumor cryosections taken from NB xenografts (50 days after cells inoculation), and examined them at low and medium magnification for tumor changes. Histopathological analysis of tumors showed that both CAMPTOSAR and EZN-2208 inhibited tumor cell proliferation, as assessed by the drastic decrease in NB84- and Ki-67-positive cells. However, EZN-2208 resulted in a statistically significant increased anti-proliferative effect compared to CAMPTOSAR ($p<0.001$), with GI-LI-N tumors almost disappeared, as assessed by staining histological sections of the tumors with antibodies recognizing NB cells (NB84) and the cell proliferation marker, Ki-67 (Figure 2,C-D).

In the previous IHC analysis it has been not possible to perform tumor parenchyma- and angiogenesis-related evaluations because, at the time examined, EZN-2208-treated tumors had almost disappeared. Thus, we decided to study the anti-tumor, the anti-angiogenic and the anti-invasive capabilities of EZN-2208 by performing IHC analysis on tumors derived from mice in which treatments have started at later time point.

GI-LI-N tumors were thus allowed to orthotopically grow for 35 days before treatments started. Mechanistic experiments performed one day after the end of the treatment showed statistically significant ($P<0.001$) enhanced TUNEL and Histone H2AX staining in tumors removed from mice treated with EZN-2208, indicating its much potent effect on tumor cell apoptosis, compared with CAMPTOSAR (Figure 3).

**Therapeutic effects of MTD doses of EZN-2208 and CPT-11**

In the s.c. NB model, luciferase-transfected SH-SY5Y cells were allowed to grow in the right flank of mice for 13 days before the treatment begun and the anti-tumor efficacy evaluated at different
time points by BLI. As showed in Figure 4,A, while CAMPTOSAR treatment showed a partial and temporary arrest of tumor growth, EZN-2208 led to a complete regression of primary tumors.

In the very aggressive, orthotopically implanted, syngeneic animal model of NB (NXS2 cells), CAMPTOSAR did not exert any anti-tumor effect, while EZN-2208 led to 100% of long term survivors (Figure 4,B). In immunodeficient orthotopic NB (GI-LI-N cells) animal model, CAMPTOSAR at MTD dose led to a partial increased in long term survival. In contrast, EZN-2208-treated, GI-LI-N-bearing mice were 100% cured after 180 days post cells implantation (Figure 4,C). Concordantly with previous results \(^8,9\), EZN-2208 was well-tolerated with neither obvious toxicities (i.e. no weight loss and no skin rush) nor acute and chronic, liver and renal toxicities (Table 2, supplementary).

Effect of EZN-2208 on angiogenic responses in the CAM assay

The differences in the anti-angiogenic activity between EZN-2208 and CPT-11 (CAMPTOSAR) \textit{in vivo}, using the CAM assay, is shown in Figure 5, A-B. Tumor xenografts derived from NB-bearing, untreated mice, were grafted onto CAMs. CAMs incubated with CAMPTOSAR showed a decrease in the number of allantoic vessels radiating in a “spoked wheel” pattern towards the xenografts, when compared to those incubated with PBS. However, incubation of the CAMs with EZN-2208 significantly reduced the number of radiating vessels that invaded the implant compared to either specimens alone or CAMs incubated with CAMPTOSAR, as shown by morphometric assessment of microvessel density \((P<0.01)\) (Figure 5,A-B).

Effect of EZN-2208 on tumor cell angiogenesis \textit{in vivo}
The impact of EZN-2208 on angiogenesis was also evaluated by IHC on tumors derived from either untreated or CAMPTOSAR- and EZN-2208-treated, orthotopically-implanted (GI-LI-N cells), mice. As showed in Figure 5,C-D, both treatments exerted their anti-angiogenic activities, which resulted in decreased VEGF and CD31 expression in primary NB tumors. However, in terms of CD31-positive endothelial cell staining, EZN-2208, resulted in a statistically significant increased anti-angiogenic effect compared to CAMPTOSAR ($P<0.05$).

Finally, studies on the tumor invasiveness-related markers, performed in parallel sections, demonstrated a pronounced, statistically significant inhibition of MMP-2 and MMP-9 expression in tumors derived from EZN-2208-treated mice ($P<0.05$), when compared to tissue sections from CAMPTOSAR-treated animals (Figure 5,E-F).

Taken together, the IHC results obtained clearly indicate a pivotal role of EZN-2208 in inhibiting tumor angiogenesis and, potentially, the systemically tumor spreading.

**Clinical impact of EZN-2208**

Once demonstrated the enhanced anti-tumor effect of EZN-2208 compared with Irinotecan in many biologically relevant xenograft models of NB, we next evaluated the efficacy of EZN-2208 compared to other anti-tumor compounds, either in Phase II/III or clinically used in NB therapy. These drugs were administered as single-agent therapy or in a combined schedule treatment. Mice were orthotopically inoculated in the adrenal gland with GI-LI-N cells and then treated 21 days after tumor challenge. The results are shown in Figure 6. Mice treated with Doxorubicin and Fenretinide as single agents, behaved as control mice, dying from progression of the primary tumor and metastatic disease within 75 days post cell inoculation. Cisplatin, Topotecan and Vincristine, as single agents, led to a partial increased anti-tumor effect compared with control, while Irinotecan, used at MTD dose, led to a partial increased long term survival. In comparison, EZN-2208-treated GI-LI-N-bearing mice were 100% cured after 150 days post cells implantation (Figure 6,A).
Furthermore, enhanced life span was also observed in TVD-treated mice, but without any long term survivors. However, when TVD-treated mice, showing signs of poor health, were subsequently treated with EZN-2208 (see arrow), they showed a significant increased life span compared with animals treated only with TVD ($P<0.001$) (Figure 6,B). From these results, we conclude that EZN-2208 may be an excellent primary and second line therapy for patients with NB.

**DISCUSSION**

This work demonstrates the extraordinary therapeutic efficacy of an SN38 drug conjugate (EZN-2208) in NB xenografts. This novel drug led to a complete tumor regression in all the NB animal models tested and significantly outperformed CPT-11. Based on these data, exploration of EZN-2208 for the treatment of NB is warranted.

Better therapies to treat advanced cases of NB have been sought for many years. Currently, high-risk NB patients treated with radiotherapy, intensive chemotherapy, autologous stem-cell rescue and with 13-cis-retinoic acid or with monoclonal antibodies targeting NB-specific proteins (25) have a 3-year event-free survival rate of 34% (2, 26). Moreover, due to limiting factors for intensive induction therapy such as acute and chronic toxicity and the development of secondary neoplasias (27), the overall outcome for high-risk refractory or recurrent NB patients remains very poor.

The DNA topoisomerase-I inhibitor, Irinotecan (CPT-11; Camptosar) is approved for the treatment of metastatic colorectal cancer (28), and for some other solid tumors including NB (29). However, particularly when administered as a single agent in NB, patients given CPT-11 have demonstrated limited partial responses, disease stabilization (29), or no objective response(6), and to the Camptothecin analogue Topotecan (5). When given in combination with other agents, camptothecin analogs have been demonstrated efficacy in preclinical NB models (30, 31) and in
Phase I clinical trials (32, 33). More interestingly, an orally available formulation of Irinotecan, in combination with Temozolomide, led to partial response in a small number of relapsed high-risk NB patients and was well tolerated (34).

The lack of remarkable efficacy of CPT-11 is associated with many limitations including limited conversion of the CPT-11 prodrug to the highly active moiety, SN38 (7, 8), deactivation of SN38 or CPT-11 by opening of the lactone “E” ring (7, 8), and induction of gastrointestinal side-effects mediated by the bispiperizane moiety unique to CPT-11, intact CPT-11, or SN38 (35). Pegylated SN38 overcomes some or all of these limitations since only the highly active moiety, SN38, is released from the drug conjugate, the pegylation at the C20 position preserves the lactone ring, and only SN38 is available to possibly induce GI side-effects (9). In addition, it has been experimental determined in tumor-bearing animals that the pegylated molecule accumulates at the tumor providing sustained release of SN38, consistent with the proposed “enhanced permeation retention” (EPR) effect observed with other pegylated molecules (36, 37). However, the basis of the superior efficacy achieved with PEG-SN38 compared to CPT-11 is incompletely understood. In our previous studies in mice we have demonstrated that the conversion of CPT-11 to its more active metabolite, SN38 is low (1.5%) and SN38 derived from PEG-SN38 will be approximately 50-fold higher and for a longer duration than that derived from CPT-11 (10). Therefore, PEG-SN38 delivers a higher and sustained level of SN38 in the plasma and the tumor compared with CPT-11 in our mouse models. This should be compared with others where the conversion of CPT-11 to SN38 in rodents after a single dose has been reported to vary between 3 to 45% (38, 39) as well as the 2-5% conversion rate of CPT-11 to SN38 in cancer patients. The basis for this difference in animal models may be related to methodological details, routes of administration, the exact species and strains of animals used, and variable carboxyesterase activity that would produce SN38 from CPT-11 (8).
We have observed that EZN-2208 was highly efficacious and cured not only subcutaneous-implanted NB tumors-bearing mice, but exerted its anti-tumor effect also against both pseudometastatic and orthotopic NB xenografts. These models are particularly relevant since they closely model advanced cases of NB. In the case of the pseudometastatic model, with NB cells injected intravenously in mice, this xenograft model mimics the metastatic spread observed in advanced stage NB patients (18). Circulating NB cells in the blood and micrometastases in the bone marrow at the time of primary surgery of NB patients is a strong predictor of relapse (40). Since bone marrow micrometastases are a direct measurement of the ability of tumor cells to spread systemically, the establishment of a model that closely mimics the clinical situation allows a more realistic evaluation of anti-tumor therapies. Thus, the pseudometastatic model employed in these studies provides a consistent test for the potential use of EZN-2208 in human metastatic disease. The schedule of treatment performed, beginning either 24 or 72 hours after NB cells injection, was deliberately chosen to allow evaluation of the effects of EZN-2208 during the metastatic cascade (18). While CPT-11 exerted a partial anti-tumor response, EZN-2208, at both schedule treatments used, displayed 100% of tumor-free mice for more then five months, suggesting a complete inhibition of both circulating cells and residual disease.

We have also compared the efficacy of EZN-2208 and CPT-11 in orthotopic tumor models of NB, since such models closely mimic tumor progression, angiogenesis, invasion, and metastasis (41). In the case of NB, orthotopic models mimic the large adrenal gland tumors and multiple small metastatic lesions observed in patients (19). Interestingly, EZN-2208 led to a dramatic tumor regression and to a complete curability of both early-stage, CPT-11-resistant, and more established, CPT-11-sensitive, NB models (Figure 4), highlighting the overall enhanced anti-tumor activity of this pegylated SN38 formulation, compared with the pro-drug CPT-11, independently from tumor stage, sensitivity and schedule of treatment.
EZN-2208 also had superior anti-tumor effect with respect to several clinically used anti-neoplastic drugs, such as Topotecan, Cisplatin, Doxorubicin and Vincristine. More clinically relevant, EZN-2208 led to a statistically significant enhanced anti-tumor effects ($P<0.001$) also in mice that resulted sensitive, but not cured, to a Topotecan-Vincristine-Doxorubicin (TVD) combination treatment, which is clinically used as first line treatment for stage IV NB patients (23). These mice, which were treated with EZN-2208 when signs of poor health became evident as a consequence of TVD treatment failure (day 80 post cell implantation), displayed a dramatic arrest and regression of tumor growth, and a long-term survival of about 85% of mice. From these excellent results we conclude that EZN-2208 could be also used as second-line therapy for refractory NB tumors.

Several molecular mechanisms of action seem to be involved in the EZN-2208-based treatment. Compared to CPT-11, EZN-2208 was able to induce more DNA damage, apoptosis/necrosis and p53 expression. Moreover, EZN-2208 exerted its superior potential in inhibiting tumor angiogenesis. Several recent studies implicate angiogenesis as an essential mechanism regulating NB growth and anti-angiogenic therapies have been reviewed (42). Sprouting of new blood vessels from pre-existing capillaries under the influence of pro-angiogenic growth factor expression, such as VEGF, has been reported (43). HIF-1α mediates angiogenesis by induction of VEGF(44) and regulates tumor angiogenesis and invasion (45). Moreover, hypoxia-induced decrease in p53 protein level is known to result in cancer cell resistance to DNA damage-induced apoptosis (46). Thus, the inhibition of HIF-1α could decrease HIF-1α-mediated VEGF production, leading to apoptosis-mediated cell death and a subsequent block of tumor neo-angiogenesis.

In this work we demonstrated that EZN-2208 was extremely potent in down modulating HIF-1α and HIF-2a expression, whereas little effect was observed with Irinotecan (Figure 1S, and Sapra P. et al, manuscript in preparation). It is likely that the reduction in HIF-1α led to an increase of p53 protein, and to a statistically significant decrease in CD31, VEGF, MMP-2, and MMP-9, thus validating the anti-angiogenesis and the anti-invasion effect of EZN-2208 on NB tumors.
Previously, other topoisomerase I and II inhibitors have been shown to inhibit HIF-1α expression (47, 48); it is likely that SN38 is a more potent inducer of HIF-1α degradation. In addition, since HIF-2α is also strongly correlated with high tumor vascularization (49) and it has been demonstrated promoting aggressive NB phenotypes (50), it is striking that EZN-2208 dramatically decrease HIF-2α protein level compared with CPT-11. Hence, the anti-angiogenic potential of this novel drug may further explaining in more details its superior efficacy with respect to CPT-11. Thus, this anti-angiogenic response could be also wished for the inhibition of secondary neoplasias, after anti-NB therapies (27).

Collectively, the results obtained provided experimental evidence to strongly support the beginning of Phase-I for EZN-2208 in pediatric patients with relapsed or refractory high-risk NB patients. There is ongoing Phase I study of EZN-2208 in a pediatric patients with relapsed solid tumors.

**AUTHOR’S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**

P. Sapra, L.M. Greenberger and I.D. Horak own stock in Enzon Pharmaceuticals, Inc

**Acknowledgements**

We thank A. Daga A., H. Zhao, G. Taverniti, and A. Rapisarda for expert technical assistance. Work supported by Enzon Pharmaceuticals, Associazione Italiana per la Ricerca sul Cancro (AIRC, MFAG and IG), and the Fondazione Italiana per la Lotta al Neuroblastoma.

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**LEGENDS**

**Figure 1. Therapeutic effects of EZN-2208 and CPT-11, at SN38 equivalents, against NB-bearing mice.** Mice were treated every other day for 5 total doses with 10 mg/kg of CPT-11 (CAMPTOSAR) or EZN-2208. Control mice received HEPES buffered saline. A) SCID mice were subcutaneously (s.c.) injected in the right flank with luciferase (luc)-transfected, human NB cells, SH-SY5Y, and treated one week after tumor cell implantation. The anti-tumor response was evaluated for BLI intensity over time (12-50 days). Histograms quantified BLI imaging showing complete regression of primary tumor growth in mice treated with EZN-2208. Control mice were sacrificed for excessive tumor mass. Columns depicts the mean of expression of bioluminescent and errors bars show 95% CI. B) Mice were intravenously (i.v.) inoculated with human NB cells, HTLA-230, and treated after either 24 or 72 hours from cell inoculation. C) A/J mice orthotopically implanted in the left adrenal gland with murine NB cells (NXS2) were allowed to form tumors of approximately 200 mm$^3$ in size and treated 3 days after cells implantation.

**Figure 2. Therapeutic effects of EZN-2208 and CPT-11, at SN38 equivalents, against NB-bearing mice and impact on tumor cell proliferation.** Mice were treated every other day for 5 total doses with 10 mg/kg of CPT-11 (CAMPTOSAR) or EZN-2208. Control mice received
HEPES buffered saline. **A**) Nude mice orthotopically implanted in the left adrenal gland with human NB cells, GI-LI-N, were allowed to form tumors of approximately 200 mm$^3$ in size and treated 21 days after cells implantation. **B**) Dramatic arrest and regression of tumor growth in EZN-2208-treated mice. Each point represents the mean ± 95% CI. of eight replicates. **C**) IHC analysis of NB primary tumors removed from untreated mice (control) and mice treated either with CAMPTOSAR or EZN-2208. Tumors were harvested on day 50 and tissue sections were immunostained for NB84, to show NB cells and Ki-67, to show tumor proliferating cells. Cell nuclei were stained with DAPI. Scale bar 100 μm. **D**) Columns, mean of NB84 and Ki-67 expressions; errors bars show 95% CI. *, p<0.05; **, p<0.01;***, p<0.001.

**Figure 3. Impact of EZN-2208 on in vivo tumor cell apoptosis.** Nude mice orthotopically implanted in the left adrenal gland with human NB cells, GI-LI-N, were allowed to form tumors of approximately 400 mm$^3$ in size (day 35) and then injected with 10 mg/kg CPT-11 (CAMPTOSAR) or EZN-2208, every other days for 5 total doses. Control mice received HEPES buffered saline. Histological analysis have been performed on primary NB tumors removed from untreated (control) and CAMPTOSAR- or EZN-2208-treated mice (44 days after cell inoculation), 24 h after the last treatment (day 43). Tissue sections were immunostained with TUNEL, to detect apoptosis, and with primary antibody against Histone H2a.x (H2AFX) to detect DNA-damage depending Histone phosphorylation. Scale bars 150 μm. Pictures are representative images. Columns, mean of TUNEL and H2AFX expressions; errors bars show 95% CI. *, p<0.05; **, p<0.01;***, p<0.001.

**Figure 4. Therapeutic effects of EZN-2208 and CPT-11, at MTD doses, against NB-bearing mice.** Mice were treated every other day for 5 total doses with 40 mg/kg of CPT-11 (CAMPTOSAR) or with the 10 mg/kg of EZN-2208. Control mice received HEPES buffered saline. **A**) SCID mice were subcutaneously (s.c.) injected in the right flank with luciferase (luc)-
transfected, human NB cells, SH-SY5Y, and treated 13 days after tumor cell implantation. The anti-tumor response was evaluated for BLI intensity before (12 day) and after treatment over time (20-75 days). Histograms quantified BLI imaging showing complete regression of primary tumor growth in mice treated with EZN-2208. Control mice were sacrificed for excessive tumor mass. Columns depicts the mean of expression of bioluminescent and errors bars show 95% CI. A/J (B) and nude (C) mice, orthotopically implanted in the left adrenal gland with NXS2 or GI-LI-N NB cells, respectively, were allowed to form tumors of approximately 200 mm³ in size and then treated as in Figures 1-2.

Figure 5. Impact of EZN-2208 on angiogenesis and tumor invasiveness-related markers. A-B) Chorioallantoic membrane (CAM) assay. A) Biopsy fragments, 1-2 mm³, from xenografts derived from NB (GI-LI-N and HTLA-230) cells injected in athymic mice were then grafted onto the CAM either alone (specimens control) or together with CAMPTOSAR or EZN-2208. CAMs were examined daily until day 12 and photographed in ovo with a stereomicroscope equipped with a camera and image analyzer system (Olympus Italia, Italy). Original magnification, 50X. B) Morphometric assessment of microvessel area. Columns depicts the mean of suppression of vessel density with respect to control (% area = 100%) and errors bars show SEM. C-F) IHC evaluation of primary NB tumors. Nude mice orthotopically implanted in the left adrenal gland with human NB cells, GI-LI-N, were allowed to form tumors of approximately 400 mm³ in size (day 35) and then injected with 40 mg/kg CPT-11 (CAMPTOSAR) or EZN-2208, every other days for 5 total doses. Tumors were harvested one day after the end of the treatment and tissue sections were immunostained for VEGF and CD31 (C), to show tumor angiogenesis and for MMP-2 and MMP-9 (E) to show tumor invasion capability. Cell nuclei were stained with DAPI. Scale bar 200 μm.

Pictures are representative images. D, F) Columns, mean value of VEGF and CD31 and MMP-2
and 9 expressions; errors bars show 95% CI. n.s., not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

**Figure 6. Clinical relevance of the therapeutic effects of EZN-2208.** Nude mice orthotopically implanted in the left adrenal gland with the human NB cells, GI-LI-N, were allowed to form tumors of approximately 200 mm$^3$ in size. Treatments started 21 days after cells implantation. **A** ) Mice were treated every other day for 5 total doses with 40 mg/kg of CPT-11 (CAMPTOSAR) or with the 10 mg/kg of EZN-2208. Other groups of mice were treated with Topotecan, Cisplatin, Doxorubicin, Vincristine, Fenretinide at the doses and schedules of treatment showed in M&M section. **B** ) Mice were treated with a combination of Topotecan (T, daily from day 21 to day 25), Vincristine (V, at day 25) and Doxorubicin (D, at day 25) with schedule and doses showed in M&M section. When signs of poor health became evident (arrow), half of TVD-treated mice, randomly chosen, were then inoculated with EZN-2208, 10 mg/kg every other day for 5 total doses.
Figure 1

A) Whole animal bioluminescence (ROI unit x 10^6) for control, CAMPTOSAR, and EZN-2208 over 50 days after s.c. (luc-transfected SH-SY5Y) tumor injection.

B) Survival rates for control, CAMPTOSAR, and EZN-2208 after 72h following s.c. tumor injection.

C) Survival rates for control, CAMPTOSAR, and EZN-2208 following i.v. (HTLA-230) tumor challenge and days after orthotopic (NXS2) tumor challenge.
Figure 2

A) Graph showing the percentage of cell survival in control, CAMPTOSAR, and EZN-2208 groups over time.

B) Graph showing the tumor size in control, CAMPTOSAR, and EZN-2208 groups over time.

C) Images showing NB84-positive cells in control, CAMPTOSAR, and EZN-2208 groups.

D) Images showing Ki-67-positive cells in control, CAMPTOSAR, and EZN-2208 groups.
Figure 3

TUNEL expression (% area)

H2AFX expression (% area)

control CAMPTOSAR EZN-2208

control

EZN-2208

TUNEL

H2AFX

0 10 20 30

0 10 20 30 40 50

∗∗∗

∗∗

∗∗∗

∗∗∗

∗∗∗

∗∗∗
Figure 4

A) Whole animal bioluminescence (ROI, photons/sec) over time for s.c. (luc-transfected SH-SY5Y) tumor implantation. The figure shows the comparison between control, CAMPTOSAR, and EZN-2208 treatments.

B) Survival rates over days after orthotopic (NXS2) tumor challenge. The graph compares survival outcomes between control, CAMPTOSAR, and EZN-2208 treatments.

C) Survival rates over days after orthotopic (GI-LI-N) tumor challenge, showing the efficacy of different treatments.
Figure 5
Figure 6

A) % survival versus days after orthotopic (GI-LI-N) tumor challenge for different treatments:
- control
- EZN-2208
- Topotecan
- CAMPTOSAR
- Cisplatin
- Doxorubicin
- Vincristine
- Fenretinide

B) % survival versus days after orthotopic (GI-LI-N) tumor challenge:
- control
- TVD (T21-25)
- TVD → EZN-2208 (T80)
Tumor regression and curability of preclinical neuroblastoma models by PEGylated SN38 (EZ-N2208), a novel topoisomerase I inhibitor

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Clin Cancer Res Published OnlineFirst August 11, 2010.