Tumor Regression and Curability of Preclinical Neuroblastoma Models by PEGylated SN38 (EZN-2208), a Novel Topoisomerase I Inhibitor

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Abstract

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

Experimental Design: The in vitro cytotoxicity of EZN-2208 was tested by counting trypan blue dye- and Annexin V–positive cells, whereas its therapeutic efficacy was evaluated, in terms of survival, and antitumor and antiangiogenic activities, in s.c. luciferase-transfected, pseudometastatic, and orthotopic neuroblastoma 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 hypoxia-inducible factor (HIF)-1α/HIF-2α expression. EZN-2208 gave superior antitumor effects compared with CPT-11 in neuroblastoma 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 or tumor size, or even by staining histologic sections of tumors with antibodies recognizing neuroblastoma cells and cell proliferation. In a neuroblastoma model resistant to doxorubicin, cisplatin, vincristine, fenretinide, and topotecan, EZN-2208 induced 100% curability. It also blocked tumor relapse after topotecan-vincristine-doxorubicin combined treatment. Mechanistic experiments showed statistically significantly enhanced terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and Histone H2ax staining as well as decreased vascular endothelial growth factor, CD31, matrix metalloproteinase (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 antineuroblastoma agent. An ongoing phase I study in pediatric patients should identify the optimal dose for a phase II study.

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EZN-2208, prolonged stable disease, sometimes associated with tumor shrinkage, was also observed. This is in distinction to irinotecan, in which grade 3/4 diarrhea is frequently encountered at or without fever.

In on-going phase II trials with adult patients, EZN-2208 was well tolerated, with dose-limiting toxicity of isolated, short-lived neutropenia with or without fever. This is in distinction to irinotecan, in which grade 3/4 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 neuroblastoma. However, unlike previous compounds that have been evaluated in experimental models of pediatric neuroblastoma, tumor where the tumor is grown as a s.c. xenograft in nude mice, we rigorously evaluated EZN-2208 in this tumor model as well as in pseudometastatic and orthotopic models of human neuroblastoma. Collectively, these models better reflect the growth of advanced neuroblastoma in children (i.e., large adrenal gland tumors and multiple small metastatic lesions), which have been difficult to control.

EZN-2208 showed excellent and clearly superior antitumor activity compared with CPT-11 as well as with many other clinically used antineoplastic 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 antitumor agent for the treatment of neuroblastoma.

Materials and Methods

Materials, cell lines, and animals
EZN-2208 was prepared as described previously (9, 10). Irinotecan (CPT-11, Camptosar) was purchased from Pfizer Italia S.r.l., topotecan (Hycamtin) from Glaxo-Smithkline S.p.A., cisplatin and doxorubicin from Ebeve Italia S.r.l., and vincristine from Sigma Chemical Co. Fenretinide (N-4-hydroxyphenyl retinamide) was kindly provided by Dompé Farmaceutici S.p.A. Deferoxamine (Desferal) was purchased from Novartis Pharma.

To broadly cover the phenotypes exhibited by neuroblastoma 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) neuroblastoma 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) after a 10-minute 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 were 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) 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 done with 5 to 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 neuroblastoma cell lines by counting trypan blue dye–stained cells. Briefly, adherent cells were plated in
Immunoblotting analysis

Western blotting analysis from whole cell lysates was done as described (17). Briefly, cultured neuroblastoma 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 hypoxia-inducible factor (HIF)-1α induction, cells were preincubated with 0.15 mmol/L of Desferal (deferoxamine) 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 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, anti-HIF-2α (clone ep190b) and anti-GAPDH (clone 14c10) antibodies were from Novus Biologicals, Inc. and Cell Signaling Technology, respectively.

Animal models

For the s.c. animal model, 2.0 × 10^7 luciferase-transfected SH-SY5Y cells were inoculated in the mid-dorsal region of 5-week-old female severe combined immunodeficient mice (5 mice/group). Tumor expansion over time, as well as the response to treatment were readily visualized by bioluminescence imaging (BLI) and quantified by a highly sensitive, cooled charge-coupled device camera mounted in a light-tight specimen box (IVIS), as described (15, 16).

For the pseudometastatic animal model, 4-week-old female, athymic (nu/nu) mice (8 mice/group) were injected i.v. in the tail vein with 4 × 10^6 HTLA-230 tumor cells, as described (18). The 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), when they showed signs of poor health, such as abdominal dilatation, dehydration, or paraplegia.

In the orthotopic animal model, 5-week-old female, athymic (8 mice/group) or immunocompetent (A/J mice, 5/group) animals were anesthetized with ketamine (Imalgene 1000, Merial Italia SpA.), subjected to laparotomy, and injected with either 1 × 10^8 GI-LI-N or 5 × 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. The 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 five total doses with 10 mg/kg of CPT-11 (Camptosar) or with the SN38 equivalents of EZN-2208 (for details, see ref. 10), in the first set of in vivo experiments, or with maximum tolerated doses (MTD) 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 experiment, 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 mm^3 before i.v (tail vein) treatment commenced. For the pseudometastatic model, mice were treated i.v. either 24 or 72 hours postinoculation 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: \[ V = \pi \left(\frac{w_1 \times w_2}{2}\right)^2 \], where \(w_1\) represents the largest tumor diameter and \(w_2\) the smallest tumor diameter, as described (19). The antitumor EZN-2208 activity was also evaluated either in comparison with MTDs of different, clinically used, antitumor compounds (topotecan, CPT-11, cisplatin, doxorubicin, vincristine, and fenretinide), or as a second-line treatment in topotecan-vincristine-doxorubicin (TVD)-resistant, orthotopic neuroblastoma-bearing mice. For these experiments, tumors were allowed to grow from the injected cells for 21 days in nude mice (6 mice/group) before being randomly treated with EZN-2208, i.v., 10 mg/kg every other day for five total doses; Camptosar, i.v., 40 mg/kg, every other day for five total doses; topotecan, i.p., 10 mg/kg, every 4 days × 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 (n = 7) 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 done in patients suffering from high-risk neuroblastoma (23). When signs of poor health, such as abdominal dilatation, became evident (day 80), half of TVD-treated mice, randomly chosen, were inoculated with EZN-2208, 10 mg/kg every other day for five total doses.

Chorioallantoic membrane assay

Chorioallantoic membrane (CAM) experiments were done according to the method of Ribatti et al. (24). Neuroblastoma (GI-LI-N and HTLA-230 tumor cell lines) biopsy fragments (1-2 mm^3) 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 vivo with a stereomicroscope equipped with a camera and an image analyzer system (Olympus Italia). 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). Microvessel 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.

**Histologic analysis**

In the first set of experiments, histologic evaluation of primary tumors was done 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 five total doses. In every experiment, a group of control mice received HEPES-buffered saline.

Three weeks after the end of the treatment, mice were anesthetized with xylezine and sacrificed by cervical dislocation. Tumors were collected and then embedded in optimum cutting temperature compound (Miles Chemical Co.). Tissue sections (5 μm thick) were examined after staining with Mayer's H&E (Sigma).

Tumor frozen sections were then washed twice in PBS and saturated with 2% bovine serum albumin in PBS before staining with primary antibodies against antihuman neuroblastoma (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, histologic evaluation of primary tumors was done at 44 days after GI-LI-N cell inoculation. Briefly, orthotopically implanted athymic mice were implanted. After 35 days, the animals were treated with 10 mg/kg of CPT-11 (Camptosar) or with the SN38 equivalents of EZN-2208 or with MTDs of Camptosar (40 mg/kg) every other day for five total doses. In every experiment, a group of control mice received HEPES-buffered saline. One day after the end of the treatment, the mice were anesthetized with xylezine and sacrificed 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] to detect DNA damage–dependent histone phosphorylation (Huang X Cell Cycle 2003), and vascular endothelial growth factor (VEGF; Thermo Fisher Scientific) and CD31 (clone SC-1506, Santa Cruz Biotechnology, D.B.A. Italia S. R. L.) to detect inhibition of angiogenesis. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done using a commercially available apoptosis detection kit (In situ Cell Death Detection, POD, Roche Molecular Biochemicals) according to the manufacturer's instructions, as described (17).

Paraffin-embedded tissue sections were deparaffinized by a xylene-ethanol sequence, rehydrated in a graded ethanol solution and TBS (pH 7.6), and then processed for antigen retrieval by boiling tissue sections for 10 minutes in 1 mmol/L EDTA (pH 8.0) in a microwave oven. The sections were then washed twice in PBS and saturated with 2% bovine serum albumin in PBS before staining with primary antibodies against matrix metalloproteinase 2 (MMP-2; clone 36006, R & D System) and MMP-9 (clone 443, R & D System) to detect inhibition of tumor invasiveness–related markers.

Morphometric analysis was done on nine randomly selected fields every three sections, observed at ×200 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.). The significance of the differences between experimental groups in the survival experiments was determined by Kaplan-Meier curves with the use of $\chi^2$ 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 SE 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 $P < 0.05$ for all statistical evaluations.

**Results**

**In vitro studies**

EZN-2208 showed potent in vitro cytotoxicity against neuroblastoma cells, with IC_{50} values ranging from 0.002 to 0.15 μmol/L (Supplementary Table S1). Consistent with previous results (11), EZN-2208 induced a rapid induction of Annexin V–positive neuroblastoma cells, followed by massive necrosis (data not shown). This cell death was accompanied by a rapid and strong induction of p53 and a downmodulation of HIF-2α protein expression (Supplementary Fig. S1A and B). Noteworthy, compared with Camptosar, EZN-2208 led also to a strong downmodulation of both constitutive (Supplementary Fig. S1C) and deferoxamine-induced (Supplementary Fig. S1D) HIF-2α protein levels.

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

In the s.c. injected neuroblastoma-bearing mice, the effects of EZN-2208 and Camptosar were compared against a luciferase-transfected human neuroblastoma cell line (SH-SY5Y) inoculated in the right flank of animals. Treatment started seven days after cell injection and the antitumor efficacy was evaluated by bioluminescence (BLI). As shown in Fig. 1A, although Camptosar led to a partial antitumor response, compared with control mice that were
sacrificed at day 40 for excessive tumor mass, a complete regression of primary tumor growth in neuroblastoma-bearing mice was observed after treatment with EZN-2208. In the pseudometastatic model, the mice were treated either 24 or 72 hours after i.v. tumor cell (HTLA-230) inoculation. As shown in Fig. 1B, animals treated with EZN-2208 displayed significantly increased life span compared with control mice or those treated with Camptosar. After 150 days post–cell implantation, all EZN-2208–treated mice were still disease-free, whereas control and Camptosar-treated animals had already died with metastatic disease.

In the orthotopic neuroblastoma model, the antitumor efficacy of EZN-2208 was evaluated in both immunocompetent (A/J mice) and immunodeficient (nude mice) neuroblastoma-bearing animals. A/J mice were inoculated with the very aggressive neuroblastoma cell line NXS2 and treated after three days post–cell inoculation, whereas 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 with control mice. Moreover, whereas 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 (Figs. 1C and 2A). Furthermore, GI-LI-N–bearing, EZN-2208–treated mice showed a dramatic regression of the tumor mass, as shown in Fig. 2B.

To assess the impact of EZN-2208 on tumor cell proliferation, viability, and apoptosis, we stained tumor cryosections taken from neuroblastoma xenografts (50 days after cell inoculation), and examined them at low and medium magnification for tumor changes. Histopathologic 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 significantly increased antiproliferative effect compared with Camptosar (P < 0.001), with GI-LI-N tumors almost disappearing, as assessed by staining histologic sections of the tumors with antibodies recognizing neuroblastoma cells (NB84) and the cell proliferation marker Ki-67 (Fig. 2C and D).

In the previous immunohistochemical analysis it was not possible to carry out tumor parenchyma- and angiogenesis-related evaluations because, at the time examined, EZN-2208–treated tumors had almost disappeared. Thus, we decided to study the antitumor, the antiangiogenic, and the anti-invasive capabilities of EZN-2208 by doing immunohistochemical analysis on tumors derived from mice in which treatments had started at a later time point. GI-LI-N tumors were thus allowed to orthotopically grow for 35 days before treatments started. Mechanistic experiments done one day after the end of the treatment showed statistically significantly (P < 0.001) enhanced TUNEL and Histone H2AX staining in tumors removed from mice treated with EZN-2208, indicating its much

![Fig. 1. Therapeutic effects of EZN-2208 and CPT-11, at SN38 equivalents, against neuroblastoma-bearing mice. Mice were treated every other day for five total doses with 10 mg/kg of CPT-11 (Camptosar) or EZN-2208. Control mice received HEPES-buffered saline. A, severe combined immunodeficient mice were s.c. injected in the right flank with luciferase (luc)-transfected SH-SY5Y human neuroblastoma cells and treated one week after tumor cell implantation. The antitumor 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, mean of expression of bioluminescent; errors bars, 95% CI. ROI, region of interest. B, mice were i.v. inoculated with HTLA-230 human neuroblastoma cells and treated after either 24 or 72 hours from cell inoculation. C, A/J mice orthotopically implanted in the left adrenal gland with NXS2 murine neuroblastoma cells were allowed to form tumors of approximately 200 mm³ in size and treated three days after cells implantation.](image-url)
potent effect on tumor cell apoptosis, compared with Camptosar (Fig. 3).

Therapeutic effects of MTDs of EZN-2208 and CPT-11

In the s.c. neuroblastoma model, luciferase-transfected SH-SY5Y cells were allowed to grow in the right flank of mice for 13 days before the treatment began, and the antitumor efficacy was evaluated at different time points by BLI. As shown in Fig. 4A, whereas 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 neuroblastoma (NXS2 cells), Camptosar did not exert any antitumor effect, whereas EZN-2208 led to 100% of long-term survivors (Fig. 4B). In the immunodeficient orthotopic neuroblastoma (GI-LI-N cells) animal model, Camptosar at MTD 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–cell implantation (Fig. 4C). Concordantly with previous results (10, 11), 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 (Supplementary Table S2).

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

The differences in the antiangiogenic activity between EZN-2208 and CPT-11 (Camptosar) in vivo, using the CAM assay, are shown in Fig. 5A and B. Tumor xenografts derived from neuroblastoma-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 with those incubated with PBS. However, incubation of the CAMs with EZN-2208 significantly reduced the number of radiating vessels that invaded the implant compared with either specimens alone or CAMs

Fig. 2. Therapeutic effects of EZN-2208 and CPT-11, at SN38 equivalents, against neuroblastoma-bearing mice and impact on tumor cell proliferation. Mice were treated every other day for five 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 GI-LI-N human neuroblastoma cells were allowed to form tumors of approximately 200 mm³ in size and treated 21 days after cells implantation. B, dramatic arrest and regression of tumor growth in EZN-2208–treated mice. Each point, mean ± 95% CI of eight replicates. C, immunohistochemical analysis of neuroblastoma 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 neuroblastoma cells and Ki-67 to show tumor proliferating cells. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole. Scale bar, 100 μm. D, columns, mean of NB84 and Ki-67 expressions; errors bars, 95% CI; *, P < 0.05; **, P < 0.01;***, P < 0.001.
incubated with Camptosar, as shown by morphometric assessment of microvessel density \((P < 0.01; \text{Fig. 5A and B}).\)

**Effect of EZN-2208 on tumor cell angiogenesis in vivo**

The impact of EZN-2208 on angiogenesis was also evaluated by immunohistochemistry on tumors derived from either untreated or Camptosar- and EZN-2208–treated, orthotopically implanted (GI-LI-N cells) mice. As shown in Fig. 5C and D, both treatments exerted their antiangiogenic activities, which resulted in decreased VEGF and CD31 expression in primary neuroblastoma tumors. However, in terms of CD31-positive endothelial cell staining, EZN-2208 resulted in a statistically significantly increased antiangiogenic effect compared with Camptosar \((P < 0.05).\)

Finally, studies on the tumor invasiveness–related markers, done in parallel sections, showed 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 with tissue sections from Camptosar-treated animals (Fig. 5E and F).

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

**Clinical impact of EZN-2208**

Once the enhanced antitumor effect of EZN-2208 compared with irinotecan in many biologically relevant xenograft models of neuroblastoma was shown, we evaluated the efficacy of EZN-2208 compared with other antitumor compounds, either in phase II/III or in clinical use in neuroblastoma 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 Fig. 6. Mice treated with doxorubicin and lenalidomide 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,
MTD, 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–cell implantation (Fig. 6A).

Furthermore, enhanced life span was 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 significantly increased life span compared with animals treated only with TVD ($P < 0.001$; Fig. 6B). From these results, we conclude that EZN-2208 may be an excellent primary and second-line therapy for patients with neuroblastoma.

**Discussion**

This work shows the extraordinary therapeutic efficacy of a SN38 drug conjugate, EZN-2208, in neuroblastoma xenografts. This novel drug led to a complete tumor regression in all the neuroblastoma animal models tested and significantly did better than CPT-11. Based on these
data, exploration of EZN-2208 for the treatment of neuroblastoma is warranted.

Better therapies to treat advanced cases of neuroblastoma have been sought for many years. Currently, high-risk neuroblastoma patients treated with radiotherapy, intensive chemotherapy, autologous stem-cell rescue, and with 13-cis-retinoic acid or with monoclonal antibodies targeting neuroblastoma-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 neuroblastoma 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 neuroblastoma (29). However, particularly when administered as a single agent in neuroblastoma, patients given CPT-11 have shown limited partial responses.

**Fig. 5.** Impact of EZN-2208 on angiogenesis and tumor invasiveness–related markers. A and B, CAM assay. A, biopsy fragments, 1 to 2 mm³, from xenografts derived from neuroblastoma (GI-LI-N and HTLA-230) cells injected in athymic mice were 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). Original magnification, ×50. B, morphometric assessment of microvessel area. Columns, mean of suppression of vessel density with respect to control (% area = 100%); errors bars, SE. C to F, immunohistochemical evaluation of primary neuroblastoma tumors. Nude mice orthotopically implanted in the left adrenal gland with GI-LI-N human neuroblastoma cells 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 day for five 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 4′, 6-diamidino-2-phenylindole. Scale bar, 200 μm. Pictures are representative images. D and F, columns, mean value of VEGF and CD31 and MMP-2 and MMP-9 expressions; errors bars, 95% CI; n.s., not significant; *, *P < 0.05; **, **P < 0.01; ***, **P < 0.001.
disease stabilization (29), or no objective response (6), as in treatment with the camptothecin analogue topotecan (5). When given in combination with other agents, camptothecin analogs have shown efficacy in preclinical neuroblastoma 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 neuroblastoma 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 because 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 gastrointestinal side effects (9). In addition, it has been experimentally 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” effect observed with other pegylated molecules (36, 37). However, the basis of the superior efficacy achieved with PEG-SN38 compared with CPT-11 is not completely understood. In our previous studies in mice we had shown that the conversion of CPT-11 to its more active metabolite SN38 is low (1.5%) and that 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% and 45% (38, 39) as well as the 2% to 5% conversion rate of CPT-11 to SN38 in cancer patients. The basis for this difference in animal models may be related to methodologic 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 observed that EZN-2208 was highly efficacious and cured not only in s.c. implanted neuroblastoma tumor-bearing mice, but also exerted its antitumor effect against both pseudometastatic and orthotopic neuroblastoma xenografts. These models are particularly relevant because they closely model advanced cases of neuroblastoma. In the case of the pseudometastatic model, with neuroblastoma cells injected i.v. in mice, this xenograft model mimics the metastatic spread observed in advanced-stage neuroblastoma patients (18). Circulating neuroblastoma cells...
in the blood and micrometastases in the bone marrow at the time of primary surgery of neuroblastoma patients is a strong predictor of relapse (40). Because 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 antitumor 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 done, beginning either 24 or 72 hours after neuroblastoma cells injection, was deliberately chosen to allow evaluation of the effects of EZN-2208 during the metastatic cascade (18). Although CPT-11 exerted a partial antitumor response, EZN-2208, at both schedule treatments used, displayed 100% of tumor-free mice for more than five months, suggesting a complete inhibition of both circulating cells and residual disease.

We also compared the efficacy of EZN-2208 and CPT-11 in orthotopic tumor models of neuroblastoma, because such models closely mimic tumor progression, angiogenesis, invasion, and metastasis (41). In the case of neuroblastoma, 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 the more established, CPT-11–sensitive neuroblastoma models (Fig. 4), highlighting the overall enhanced antitumor activity of this pegylated SN38 formulation, compared with the pro-drug CPT-11, independent of tumor stage, sensitivity, and schedule of treatment.

EZN-2208 also had superior antitumor effect with respect to several clinically used antineoplastic drugs, such as topotecan, cisplatin, doxorubicin, and vincristine. More relevant clinically, EZN-2208 led to statistically significantly enhanced antitumor effects (P < 0.001) in mice that where shown to be sensitive, but not cured, to a TVD combination treatment, which is clinically used as first-line treatment for stage IV neuroblastoma 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 neuroblastoma tumors.

Several molecular mechanisms of action seem to be involved in the EZN-2208–based treatment. Compared with 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 neuroblastoma growth, and antiangiogenic therapies have been reviewed (42). The sprouting of new blood vessels from preexisting capillaries under the influence of proangiogenic 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 neoangiogenesis.

In this work we showed that EZN-2208 was extremely potent in downmodulating HIF-1α and HIF-2α expression, whereas little effect was observed with irinotecan (Supplementary Fig. S1).5 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 antiangiogenesis and the anti-invasion effect of EZN-2208 on neuroblastoma tumors. Previously, other topoisomerase I and II inhibitors had 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, because HIF-2α is also strongly correlated with high tumor vascularization (49) and has been shown to promote aggressive neuroblastoma phenotypes (50), it is striking that EZN-2208 dramatically decreases HIF-2α protein level compared with CPT-11. Hence, the antiangiogenic potential of this novel drug may further explain in more detail its superior efficacy with respect to CPT-11, and this antiangiogenic response could be also desired for the inhibition of secondary neoplasias, after antineuroblastoma therapies (27).

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

Disclosure of Potential Conflicts of Interest


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