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EZN-2208, A NOVEL PEGYLATED SN-38 DRUG CONJUGATE, MARKEDLY INHIBITS TUMOR GROWTH AND METASTATIC SPREADING IN PRECLINICAL MODELS OF HUMAN NEUROBLASTOMA

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Treatment of neuroblastoma (NB), the second most common solid tumor in childhood, is successful in less than half of patients with high-risk disease. The 5 year survival for metastatic disease is still less than 60% and, consequently, novel therapeutic approaches are needed. Camptothecin and its analog irinotecan (CPT-11), hold great promise in the treatment of NB but several limitations (i.e. early and late gastrointestinal toxicity, short half-life of the CPT-11 active moiety, SN38, and drug resistance) suggest that chemical modifications may further improve the therapeutic index. EZN-2208 is a water soluble pegylated SN38 drug conjugate, composed of a four-arm 40 KDa polyethylene glycol (PEG) linked via a glycine residue to SN38. In various preclinical models of solid tumors, with cells implanted subcutaneously, EZN-2208 was significantly more efficacious compared to CPT-11.

In this work, the anti-tumor activities of EZN-2208 have been first evaluated against a pseudometastatic and an orthotopic mouse models of human NB, with tumor cells injected intravenously and in the adrenal gland, respectively. Both models are biologically and clinically relevant because they address different stages of the NB disease such as minimal residual disease, the former and primary mass with metastases, the latter. Mice were treated every other day for 5 total doses with 10 mg/kg of CPT-11 (Sigma) or with the SN38 equivalents of EZN-2208. In the pseudometastatic model, mice treated with EZN-2208, both 24 and 72 hours after NB cell injection, displayed significant increased life span compared to control mice or those treated with CPT-11. After 150d post cell implantation, all EZN-2208-treated mice were still disease-free, while control and CPT-11-treated animals died with metastatic disease within 50 and 85d, respectively **(Figure 1)**. In the orthotopic model, mice treated with EZN-2208, 21d after tumor cells implantation, showed a dramatic arrest and regression in primary tumor growth compared to control mice. While CPT-11-treated mice died with widespread tumor masses within 80 days, long term survival was seen in 100% of EZN-2208-treated animals **(Figure 2,A-B)**. In addition, 21 days after the end of treatment (T_{50}) tumors had almost disappeared, as assessed by staining histological sections of the tumors with antibodies recognizing human NB cells and the cell proliferation marker, Ki-67 **(Figure 2,C-D)**. In a second set of *in vivo* experiments, MTD doses of both CPT-11 and EZN-2208 were compared in immunodeficient (GI-LI-N cells) and immunocompetent (NXS2 cells) orthotopic NB animal models. While CPT-11 at MTD dose (40 mg/kg) led to a partial increased in long term survival, EZN-2208-treated, GI-LI-N-bearing mice were 100% cured after 180 days post cells implantation **(Figure 3)**. In the very aggressive syngeneic NB animal model (NXS2 cells), while CPT-11 did not exert any anti-tumor effect, EZN-2208 led to 100% and 40% of long term survivors, in mice challenged with $5x10^4$ and $5x10^5$ tumor cells, respectively **(Figure 4, A-B)**. The differences in the anti-angiogenic activity between CPT-11 and EZN-2208 *in vivo* using the CAM assay, is shown in **Figure 5**. Tumor xenografts derived from neuroblastoma (GI-LI-N and HTLA-230 models) were grafted onto CAMs. CAMs incubated with CPT-11 (at both 10 and 40 mg/kg) showed a decrease in the number of allantoic vessels radiating in a "spoked wheel" pattern towards both the xenografts, when compared to those incubated with control **(Figure 5, A)**. 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 CPT-11, as shown by morphometric assessment of microvessel area **(Figure 5, A-B)**. In the last set of *in vivo* experiments, EZN-2208-anti-tumor effect was compared to that obtained by CAMPTOSAR, the clinical grade, CPT-11, formulation. In a subcutaneous NB animal model, in which luciferase-trasfected human NB cells (SH-SY5Y) were inoculated in the right flank of SCID mice, the treatment with EZN-2208 led to a significant tumor regression compared to CAMPTOSAR **(Figure 6, A-B)**. Finally, mechanistic experiments performed in the orthotopic animal model of human NB (GI-LI-N cells), allowed to growth for 5 weeks before the beginning of the treatment, showed enhanced TUNEL and Histone H2ax staining in tumors removed from mice treated with EZN-2208, indicating its effect on tumor cell apoptosis **(Figure 7)**.

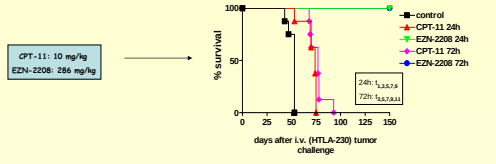

Figure 1. Therapeutic effects of EZN-2208 against pseudometastatic NB-bearing mice. Nude mice were *i.v.* inoculated with human NB cells (HTLA-230) and then treated *i.v.* with CPT-11 or EZN-2208, with schedules and doses reported in the panels.

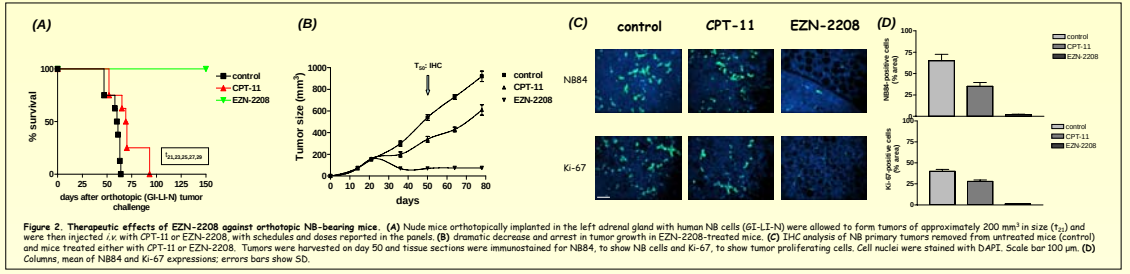

Figure 2. Therapeutic effects of EZN-2208 against orthotopic NB-bearing mice. **(A)** Nude mice orthotopically implanted in the left adrenal gland with human NB cells (GI-LI-N) were allowed to form tumors of approximately 200 mm^3 in size (t_{21}) and were then injected *i.v.* with CPT-11 or EZN-2208, with schedules and doses reported in the panels. **(B)** dramatic decrease and arrest in tumor growth in EZN-2208-treated mice. **(C)** IHC analysis of NB primary tumors removed from untreated mice (control) and mice treated either with CPT-11 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 SD.

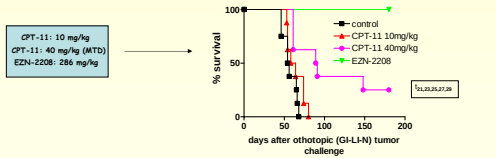

Figure 3. Comparison of CPT-11 and EZN-2208 MTD's doses against orthotopic NB-bearing mice. Nude mice orthotopically implanted in the left adrenal gland with human NB cells (GI-LI-N) were allowed to form tumors of approximately 200 mm^3 in size (t_{21}) and were then injected *i.v.* with CPT-11 (at both 10 and 40 mg/kg) or EZN-2208, with schedules and doses reported in the panels.

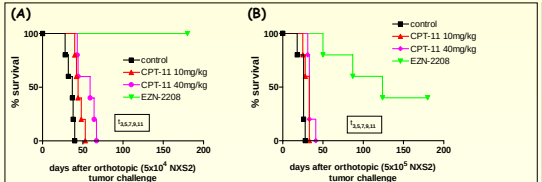

Figure 4. Comparison of CPT-11 and EZN-2208 MTD's doses against orthotopic NB-bearing immunocompetent mice. A/J mice orthotopically implanted in the left adrenal gland with **(A)** $5x10^4$ or **(B)** $5x10^5$ NB cells (NXS2) were allowed to form tumors of approximately 200 mm^3 in size (t_3) and were then injected *i.v.* with CPT-11 or EZN-2208, with schedules and doses reported in the panels.

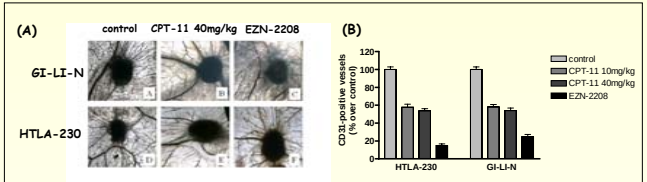

Figure 5. Chorioallantoic membrane (CAM) assay. **(A)** Biopsy fragments, 1-2 mm^3, from xenografts derived from neuroblastoma (GI-LI-N and HTLA-230) cells injected in athymic mice were then grafted onto the CAM either alone (specimens control) or together with CPT-11 or EZN-2208. CAMs were examined 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 and errors bars show SD.

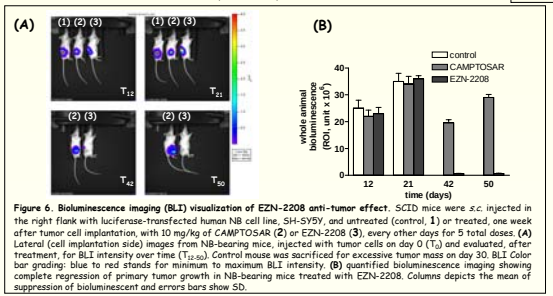

Figure 6. Bioluminescence imaging (BLI) visualization of EZN-2208 anti-tumor effect. SCID mice were *s.c.* injected in the right flank with luciferase-transfected human NB cell line, SH-SY5Y, and untreated (control, **1**) or treated, one week after tumor cell implantation, with 10 mg/kg of CAMPTOSAR (**2**) or EZN-2208 (**3**), every other days for 5 total doses. **(A)** Lateral (cell implantation side) images from NB-bearing mice, injected with tumor cells on day 0 (T_0) and evaluated, after treatment, for BLI intensity over time ($T_{12\text{-}50}$). Control mouse was sacrificed for excessive tumor mass on day 30. BLI Color bar grading: blue to red stands for minimum to maximum BLI intensity. **(B)** quantified bioluminescence imaging showing complete regression of primary tumor growth in NB-bearing mice treated with EZN-2208. Columns depicts the mean of suppression of bioluminescent and errors bars show SD.

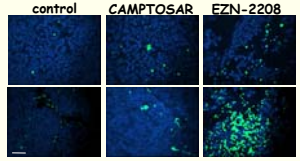

Figure 7. Impact of EZN-2208 on tumor cell apoptosis. Nude mice orthotopically implanted in the left adrenal gland with human NB cells (GI-LI-N) were allowed to form tumors of approximately 400 mm^3 in size (T_{35}) and were then injected *i.v.* with 10 mg/kg of CAMPTOSAR or EZN-2208, every other days for 5 total doses. Histological analysis have been performed on primary neuroblastoma tumors removed from untreated (control) and CAMPTOSAR- or EZN-2208-treated mice (44 days after cell inoculation), 24 h after the last treatment (T_{43}). Tissue sections were immunostained with TUNEL, to detach apoptosis, and with primary antibody against Histone H2a.x (H2AFX) to detach DNA-damage depending Histone phosphorylation. Scale bars 100 μm.

In conclusion,

EZN-2208 is of great interest as a new, promising anti-neuroblastoma agent, to be administered alone and/or in combination with traditional chemotherapeutics. In an ongoing Phase I trial, EZN-2208 was well tolerated on a q3-week schedule, with neutropenia as dose limiting toxicity.

Italian Foundation for Neuroblastoma Research