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Nanoparticle Delivery of an SN38 Conjugate is More Effective Than Irinotecan in a Mouse Model of Neuroblastoma

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Abstract

Neuroblastoma (NB) is the most common and deadly solid tumor in children. The majority of NB patients have advanced stage disease with poor prognosis, so more effective, less toxic therapy is needed. We developed a novel nanocarrier-based strategy for tumor-targeted delivery of a prodrug of SN38, the active metabolite of irinotecan. We formulated ultrasmall-sized (< 100 nm) biodegradable poly(lactide)-poly(ethylene glycol) based nanoparticles (NPs) containing SN38 conjugated to tocopherol succinate (SN38-TS). Alternative dosing schedules of SN38-TS NPs were compared to irinotecan. Comparison of SN38-TS NPs (2 doses) with irinotecan (20 doses) showed equivalent efficacy but no cures. Comparison of SN38-TS NPs (8, 8, and 16 doses, respectively) to irinotecan (40 doses) showed that all SN38-TS NP regimens were far superior to irinotecan, and “cures” were obtained in all NP arms. SN38-TS NP delivery resulted in 200x the amount of SN38 in NB tumors at 4 hr post-treatment, compared to SN38 detected for the irinotecan arm; no toxicity was seen with NPs. We conclude that this SN38-TS NP formulation improved delivery, retention, and efficacy, without causing systemic toxicity.

Keywords

irinotecan; nanoparticles; neuroblastoma; SN38; tocopherol succinate

1. INTRODUCTION

Neuroblastoma (NB) is the most common and deadly solid tumor of childhood. NB, a tumor of the sympathetic nervous system, accounts for 8–10% of all childhood cancers, and 15% of deaths from cancer in children (1). Some infants experience spontaneous regression, whereas other patients have maturation of their tumor into benign ganglioneuromas.

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Unfortunately, the majority of patients have metastatic disease, and many progress relentlessly despite intensive multimodality treatment. So, despite dramatic improvements in the cure rate for other pediatric neoplasms, the survival rate for patients with NB has lagged behind. Recent advances in understanding the molecular pathogenesis of NB have provided considerable insight into the genetic and biochemical mechanisms underlying these seemingly disparate behaviors (2, 3). These, in turn, have identified the genes, proteins, and pathways that should be effective targets for biologically based therapy (4).

The development of targeted agents for NBs and other tumors is being actively pursued, and they hold great promise for future treatment strategies. We are also investigating a targeted delivery approach for chemotherapeutic and biological agents that are suitable for encapsulation in nanoparticles (NPs). Nanoparticles can preferentially deliver drugs to tumors by taking advantage of the enhanced permeability and retention (EPR) effect (5). An additional advantage of using NP formulations is in providing a biocompatible vehicle for water-insoluble therapeutics and stabilizing labile molecules, thus enabling delivery of drugs otherwise unsuitable for therapeutic use. This approach permits enhanced drug delivery to tumor tissue, while simultaneously reducing systemic exposure and toxicity. There are a variety of different types of NPs (6), but we have formulated poly(lactide)-poly(ethylene glycol) (PLA-PEG)-based polymeric NPs containing a conjugate of SN38, the active metabolite of irinotecan. SN38 is 1,000 times more active than irinotecan, but it has toxicity and solubility issues that make it unsuitable for patient administration. However, NP encapsulation overcomes these problems and allows for safe IV administration.

We have compared the efficacy and toxicity of conventionally administered irinotecan to NP delivery of SN38 formulated as a prodrug by conjugation to tocopherol succinate in a mouse xenograft model of NB. This approach allows for greater drug retention in circulating NPs. This, in turn, results in dramatically increased tumor drug delivery and retention, as well as greater antitumor efficacy at reduced doses in this xenograft model.

2. MATERIALS AND METHODS

2.1 Compounds

SN-38 was developed and evaluated as a chemotherapeutic agent, but it was both toxic and not water soluble, so irinotecan was later introduced as its water-soluble prodrug. Irinotecan (Camptosar, Pfizer) is a commercially available, orally bioavailable topoisomerase I inhibitor that is used clinically for a variety of tumor types. It is converted by carboxylesterase converting enzyme into SN38, its active metabolite. Irinotecan (20 mg/ml) was diluted in 0.9% normal saline and administered by oral gavage once daily at 10 mg/kg, Monday through Friday, for either 4 weeks or 8 weeks. Saline was used as the control. The SN38-Tocopherol Succinate (SN38-TS) NPs were administered at an effective dose of 10 mg/kg SN38. The SN38-TS NPs were given via tail vein injection either once every other week, once per week, or twice per week (Monday/Wednesday), for 4 or 8 weeks. Irinotecan was obtained from the pharmacy at The Children's Hospital of Philadelphia. The doses used in this study were based on prior published studies (7). Human brain-derived neurotrophic factor (BDNF; PeproTech, Rock Hill, NJ) was reconstituted in distilled water at 2 µg/ml. For

long-term storage in -20°C , the reconstituted BDNF was further diluted to $1\mu\text{g/ml}$ and used at a final concentration of 100 ng/ml .

2.2 Nanoparticle Formulation

The SN38-TS conjugate was synthesized from SN-38 (AK Scientific, Union City, CA) and D- α -tocopherol hemisuccinate (Sigma-Aldrich, St. Louis, MO, USA) by direct coupling in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride with 4-dimethylaminopyridinium 4-toluenesulfonate as a catalyst. The structure and purity were confirmed by ^1H NMR. SN38-TS loaded NPs were formulated by nanoprecipitation using poly(D,L-lactide)-block-poly(ethylene glycol) (Advanced Polymer Materials, Dorval, Canada) and Pluronic F-68 (Sigma-Aldrich, St. Louis, MO) as the particle-forming polymer and stabilizer, respectively. The particle size was analyzed by dynamic light scattering, and the drug loading was determined by UV-Vis spectrophotometry after SN38-TS extraction in sec-butanol. For more information about the formulation, optimization, release kinetics and other information related to these nanoparticles, please see the recently accepted manuscript by Alferiev et. al., in *Biomaterials* (8).

2.3 Cell Lines

Trk-null SH-SY5Y cells were stably transfected with TrkB (SY5Y-TrkB), and these cells were used for all in vitro and in vivo studies. The cells were grown in RPMI-1640 containing 10% fetal bovine serum and 0.3 mg/mL G418. Cells were maintained in culture flasks at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were harvested using 0.2% tetrasodium EDTA in PBS.

2.4 In Vitro Experiments

Sulphorhodamine B (SRB) assays were performed to determine the effect of irinotecan and SN38-TS NPs on the survival and growth of the TrkB-expressing neuroblastoma cells. 5×10^3 cells per well were plated in 96 well plates and exposed to drug at different concentrations (1 nM, 3 nM, 5 nM, 10 nM) for one hour followed by addition of 100 ng/mL of BDNF. Plates were harvested at 24, 48, and 72 hours following addition of drug. The plates were processed via standard SRB assay protocol. All in vitro experiments were performed in triplicate and repeated at least 3 times.

2.5 Animals

Six-week-old athymic nu/nu mice were obtained from Jackson Laboratories. Mice were maintained at five per cage under humidity- and temperature-controlled conditions in a light/dark cycle that was set at 12-hour intervals. The Institutional Animal Care Committee of the Joseph Stokes, Jr. Research Institute at CHOP approved the animal studies described herein.

2.6 In Vivo Experiments

For the xenograft studies, animals were injected subcutaneously in the flank with 1×10^7 SY5Y-TrkB cells in 0.1 ml of Matrigel (BD Bioscience, Palo Alto, CA). Tumors were measured 2 times per week in 3 dimensions, and the volume calculated as follows: $[(0.523\times L\times W\times W)/1000]$. For the image analysis studies, SN38-TS NPs labeled with the red

fluorescent dye BODIPY_{650/665} (8) were injected via tail vein when the average tumor size reached 1 cm³. Animals were imaged using the IVIS Spectrum Pre-clinical In Vivo Imaging system, at ex/em wavelengths of 640/700 nm at 4, 24, 48, 144, and 192 hours post injection. Dorsal, side, and supine images were taken for each animal. Fluorescence counts were normalized to a mouse not injected with any dye, to correct for auto fluorescence from mouse tissues.

For the first series of tumor inhibition studies, animals were treated with the compounds for 4 weeks. Irinotecan was given as an oral gavage at 10 mg/kg QD, 5x/week. SN38-TS NPs were injected via tail vein, either 1x/2 weeks, 1x/week, or 2x/week. The control group was injected with blank NPs 2x/week. For the next set of studies, animals were treated for 8 weeks (with the exception of one group for 4 weeks). Irinotecan was given orally at 10 mg/kg QD, 5x/week. The control group received oral doses of saline. SN38-TS NPs were injected via tail vein, either 1x/week or 2x/week. A fifth group was included with a treatment regimen of 2x/week for 4 weeks to validate the findings of the previous study and allow for study comparison. We used PO dosing of irinotecan, as this is the route used clinically, and there are published data that the PO route has similar efficacy and pharmacokinetics as IV dosing (7, 9).

Body weights were obtained once a week, and the dose of compound was adjusted accordingly. Blood counts were checked regularly. Mice were sacrificed when tumor volume reached 3 cm³. Retro-orbital and terminal bleeds were obtained for blood counts and pharmacokinetic studies. Animals were given a single dose of irinotecan or SN38-TS NPs and tumor, spleen, and liver were harvested post-sacrifice (at 4, 24 and 72 hours) after heart perfusion with cold saline, (performed to minimize organ blood content for drug concentration analysis).

2.7 Pharmacokinetics/pharmacodynamics analysis of mouse tissues

Tissues were homogenized using a Biologics Inc., Model 3000 ultrasonic homogenizer. We added 20:80 methanol:water with 1% formic acid to a known weight of tissue to obtain a ratio of 4 ml/gram sample. Samples were homogenized on ice and frozen until analysis. SN38 pro-drug spiked mouse plasma and tissue homogenate were hydrolyzed using sodium hydroxide (1 M, 15 µl) and incubated for 15 minutes at 37°C in a Thermo electron incubator. The reaction was stopped by adding 98% formic acid (10 µl). Analysis confirmed complete hydrolysis of SN38 pro-drug under these conditions. Standards were prepared in CD-1 mouse plasma containing sodium heparin as an anticoagulant. A nine-point calibration curve was prepared at different concentrations by spiking a working stock. Plasma and tissue homogenate samples were extracted via acetonitrile precipitation in a 96-well format. Electrospray ionization in the positive ion mode was utilized for the tandem mass spectrometric detection of SN38 (m/z 393.2–349.0) and irinotecan (m/z 587.3–123.9) using AB Sciex 4000 mass spectrometer. Separation was accomplished utilizing Kinetex PFP (50 4.1 mm id, 2.6 µm) column with Shimadzu LC 20AD HPLC system with a run time of 4.5 min. Assay was linear over the range of 1 ng/ml to 1000 ng/ml for both SN38 and irinotecan in mouse plasma. The matrix factors of the mouse tissues (tumor, kidney, spleen and liver)

obtained using tissue homogenates spiked with 100 ng of SN38 pro-drug per ml (n=3) against mouse plasma calibration curves were applied in the drug assay calculations.

2.8 Statistical Analysis

A linear mixed effects model was used to test the difference in the rate of tumor volume change over time between groups. The model included group, day, and group-by-day interaction as fixed effects, and included a random intercept and a random slope for each mouse. Separate models and tests were constructed for the on-treatment period and off-treatment period. Event - free survival (EFS) curves were estimated using Kaplan-Meier method and compared using a log-rank test.

3. RESULTS

3.1 Effect of irinotecan and SN38-TS NPs on growth of cells in vitro

An SRB assay was performed to assess the growth inhibition and toxicity of irinotecan and SN38 on cell growth. A range of irinotecan concentrations (1, 3, 5, 10 nM and 3 μ M) was used. There was no apparent inhibition of cell growth observed at concentrations from 1–10 nM. At 3 μ M of Irinotecan, the cell survival pattern looked similar to that of control cells grown without ligand (Fig. 1). SN38-TS NPs showed an increased inhibition of growth with an increase in NP drug concentration. To rule out the role of TS in cell growth inhibition, SRB assays were performed with SN38 (free drug) alone, TS alone, and the combination of SN38 (free drug) and TS, at the same concentrations as the NP formulations. No significant effect was seen on cell growth with TS alone at any concentration, whereas SN38 alone exhibited complete inhibition of cell growth at all of the concentrations studied, with or without TS.

3.2 Biodistribution of SN38-TS NPs in mice

SN38-TS NPs labeled with a red fluorophore, BODIPY_{630/650} were injected into mice via tail veins. The maximum amount of fluorescence in tumors was seen at 4 hours, with a steady decline in fluorescent intensity in NB tumors at later time points (Fig. 2A). Side and supine views of the mice revealed that there was also accumulation of NPs on the dorsal side of the body due to concentration of particles in the 10 cervical and brachial lymph nodes, as well as in the liver. Similar high fluorescence is seen in the inguinal and lumbar lymph nodes, as seen in the supine view (Fig. 2B).

3.3 Effect of irinotecan and SN38-TS NPs on in vivo xenografts

The ability of irinotecan and SN38-TS NPs to inhibit the growth of SY5Y-TrkB cells in vivo was tested using xenograft models. For the first study, treatment was carried out for 4 weeks. All the animals in the control group were removed once they reached 1 cm³ for a subsequent study described below. Tumor growth curves for mice treated with irinotecan 5x per week for four weeks and mice treated with the SN38-TS NPs 1x every other week, for four weeks overlapped, demonstrating equivalent efficacy in tumor inhibition despite a 10-fold dose reduction with SN38-TS NPs. The group treated with SN38-TS NPs 2x/week maintained stable tumor growth control up to 66 days post-cessation of treatment, and had the greatest inhibition of tumor growth when compared to the other treatment groups (Fig.

3A). Mice treated with NPs 2x/week exhibited a significant survival advantage compared to those with a 1x/week treatment regimen, with a 100% survival through day 130 (100 days post-cessation of treatment), (Fig. 3B).

There was significant difference between the group treated with irinotecan 5x/wk versus those treated with SN38-TS NPs 1x/2 weeks (Log-rank test, $p=0.3105$). However there was a significant difference, for SN38-TS NP 1x/week versus irinotecan 5x/week ($p=0.0014$), for SN38-TS NP 1x/2 weeks versus SN38-TS NP 1x/ week ($p=0.0034$), for SN38-TS NP 2x/week versus irinotecan 5x/week ($p < 0.0001$), for SN38-TS NP 2x/week versus SN38-TS NP 1x/2 weeks ($p < 0.0001$), and for SN38-TS NP 2x/week versus SN38-TS NP 1x/week ($p < 0.0001$).

Because our SN38-TS NPs exhibited promising control over tumor growth, we next investigated whether NP treatment for 8 weeks would provide a more protracted survival advantage. Mice treated with oral irinotecan displayed inhibited tumor growth and decreased tumor size over the course of treatment. However, their tumors regrew within 4 weeks of treatment cessation, with some mice reaching a tumor volume of over 3 cm³ by day 37 post-treatment. All of the mice treated with SN38-TS NPs had negligible tumor volumes (below 0.1 cm³) for at least 60 days following cessation of therapy. Furthermore, the tumor regrowth patterns of the NP-treated mice were significantly slower than untreated or irinotecan-treated mice (Fig. 4A). A significant survival advantage was observed in the groups treated with SN38-TS NPs compared to the irinotecan group treated 5x/week/8 weeks. Mice treated with NPs 2x/week/8 weeks exhibited significant survival advantage over the 1x/week/8 weeks and 2x/week/4 weeks groups, with almost 100% survival through day 180 (120 days post cessation of treatment, Fig. 4B). Log-rank tests revealed $p < 0.0001$ for SN38-TS NP 1x/week/8 weeks group versus irinotecan 5x/week/8 weeks, $p=0.0007$ for SN38-TS NP 2x/week/4 weeks versus irinotecan 5x/week/8 weeks, $p=0.0753$ for SN38-TS NP 2x/week/4 weeks versus SN38-TS NP 1x/week/8 weeks, $p < 0.0001$ for SN38-TS NP 2x/week/8 weeks versus irinotecan 5x/week/8 weeks, $p=0.0142$ for SN38-TS NP 2x/week/8 weeks versus SN38-TS NP 1x/week/8 weeks, and $p=0.0001$ for SN38-TS NP 2x/week/8 weeks versus SN38-TS NP 2x/week/4 weeks.

3.4 Effect of SN38-TS NP treatment on large tumors

Because this NP formulation provided significant control over moderately-sized NB xenografts (average 0.2 cm³), we wanted to determine whether SN38-TS NPs could effectively control larger NB tumors, which mimic more advanced-stage disease. NB xenografts were allowed to grow untreated until they reached an average of 1 cm³. Then, tumor-bearing mice were treated intravenously with a total of 16 doses of SN38-TS NPs at 2x per week. Over the course of treatment, all tumors regressed to approximately 0.2 cm³ and remained in stable remission for an average of 60 days, similar to the smaller NB tumors (data not shown). Interestingly, when these tumors began to recur (at 60–90 days after treatment cessation), their growth was consistently slow and protracted (Fig. 5A). This finding led us to investigate the histology of the tumors at the time of sacrifice. Hematoxylin & Eosin staining of the SN38-TS NP-treated tumors harvested at about approximately 18 weeks (~120 days) post last treatment showed dramatic maturation towards a

ganglioneuroma phenotype in all of the treated tumors examined (Fig. 5B). Interestingly, these tumors also expressed significantly higher levels of neuronal differentiation markers, such as tyrosine hydroxylase, consistent with neuronal maturation (Fig. 5B).

3.5 Pharmacokinetics of NP distribution in mouse tissues

We also performed pharmacokinetic analyses of mice treated with irinotecan or SN38-TS NPs to determine the biodistribution of SN38 at different time points post-treatment. Blood, liver, spleen, and tumor samples taken from mice at 4, 24, and 72 hours after oral irinotecan administration or NP injection were analyzed for drug content via LC-MS/MS. Average irinotecan and SN38 levels in irinotecan-treated mice were 16.4 ± 4.7 ng/g (SD) and <10 ng/g of tissue, respectively, at 4 hours post-treatment, whereas SN38 levels were 200-fold higher in NP-treated samples (Table 1). Furthermore, although mice treated with irinotecan had undetectable levels of irinotecan and SN38 in tumors at 24 or more hours after treatment (< 10 ng/g), NP-treated tumors retained very high levels of SN38 at 24 hours post-treatment (1482 ± 354.6 ng/g). This level decreased at 72 hours (558.3 ± 190.7 ng/g), but remained significantly elevated compared to irinotecan-treated mice at 72 hours post-treatment (Table 1).

4. DISCUSSION

NBs are characterized by heterogeneous clinical behavior, including spontaneous regression or differentiation into benign ganglioneuromas (1). Additionally, NB patients under 12–18 months of age tend to have a better outcome than older patients. Unfortunately, over half of all NBs are older with unresectable or metastatic disease at the time of diagnosis, and are considered high-risk. Even with very intensive, multimodality therapy, including chemotherapy, radiation therapy, stem cell transplantation, and immunotherapy, over half of these patients do not survive (10). Furthermore, we have reached the limits of acute and long-term toxicity with this intensive approach, so more effective, less toxic approaches are greatly needed. Targeted agents show promise in select subsets of patients that express the target protein, but responses may be short-lived, and they do not work for all high-risk patients (4, 11, 12).

Given the current limitations of intensive, multimodality therapy for high-risk NBs, we have taken the approach of more targeted drug delivery that could treat these tumors more effectively, while maintaining reduced toxicity. NP encapsulation of chemotherapeutic agents takes advantage of the EPR effect to deliver more drug to tumors than conventional administration, which should increase the efficacy of these agents, while simultaneously reducing systemic exposure (5, 13–15). NP formulations also provide a biocompatible vehicle for water-insoluble agents, as well as stability for labile molecules. Irinotecan, a commonly used topoisomerase I inhibitor, is a weak or inactive pro-drug that is metabolized to SN38, its active agent (16). The conversion of irinotecan to SN38 is inefficient and subject to significant interpatient variability (17, 18). However, 40–60% of administered irinotecan was in the form of SN38 in blood and tissues at 4 hr in our animal model (Table 1). SN38 itself is 1,000 times more potent than irinotecan, but it has toxicity and solubility issues that make it unsuitable for systemic administration (19, 20). However, SN38 is an

attractive agent for NP drug delivery because this approach obviates the inherent disadvantages of SN38 as a free drug. In this study, we examined the efficacy of NP delivery of SN38 versus oral administration of irinotecan in a mouse NB xenograft model.

Others have encapsulated SN38 in NPs (21–23), because of its poor solubility. Pal and coworkers (22) tested liposome entrapped SN38 had antitumor efficacy and low toxicity in mouse and dog tumor models. Atyabi and colleagues (21) used pegylated liposomes of 150–200 nm containing SN38 to test their biodistribution in mice and found that the distribution in liver, spleen, kidney and lung was less with pegylated liposomes, and they persisted longer in the circulation. Zhang and coworkers (23) developed an oligoethylene glycol SN38 codrug that formed micelles of 25–30 nm and required esterase activation. This formulation exhibited favorable antitumor efficacy against human xenografts. Preclinical as well as phase I studies have been conducted of an SN38-incorporating 20 nm polymeric micelles (NK012) that show improved efficacy over irinotecan (24–26), and Marier showed that an SN38 pro-drug formulated as emulsion (SN2310) had a better safety profile than irinotecan (27). In the present studies, we employed a biodegradable NP formulation where TS-derivatized SN38 was incorporated in pegylated polymeric PLA nanoparticles of 70–80 nm designed to provide improved encapsulation efficiency and NP-drug association stability

Nanoencapsulated SN38 conjugated to TS acted as a potent inhibitor of cell growth in SH-SY5Y-TrkB cells, whereas TS proved to be ineffective at inhibiting cell growth, suggesting that only the SN38 component of the NP formulation contributes to cell death in vitro (Fig. 2). We also observed no toxicity when NB tumor-bearing mice were treated with tocopherol succinate alone (data not shown). However, the levels of TS in the tumor that were achieved with NP delivery were likely far greater than those achieved by oral administration, so it is possible that the resultant high local levels could be sufficient to exert an anti-tumor effect. Furthermore, even if TS had little or no effect alone, it may have enhanced the efficacy of SN38 when delivered as a conjugate.

Next, to determine whether these NPs could safely and effectively reach tumor tissue, we analyzed the biodistribution of fluorescently conjugated SN38-TS NPs. Over the first 4 to 24 hours post-injection, NPs were visualized throughout the circulatory system, but after 24 hours post-injection, NPs preferentially accumulated in the tumor, as well as in the liver, spleen, and lymph nodes. NP-treated mice showed no evidence of toxicity, as demonstrated by normal mouse weights, blood counts, and behavior throughout the course of treatment. Furthermore, pharmacokinetic analysis of tumor tissue showed that NP delivery of SN38 had a ~200-fold advantage at 4 hr over oral administration of irinotecan, and sustained levels in tumors for at least 72 hr post-treatment (Table 1). This suggests that, compared to conventional irinotecan, NP administration of SN38-TS NPs can significantly increase the exposure of tumor tissue to the cytotoxic effects of SN38 while preventing system exposure to its inherent toxicities.

We next tested the ability of SN38-TS NPs to control NB tumor growth over time. We observed significantly greater tumor control after cessation of treatment, as well as protracted long-term survival in NP-treated mice when compared to oral irinotecan treatment with substantially more total drug delivered. Furthermore, we determined that

mice treated with the NP formulation just once every two weeks (2 doses) had survival curves equivalent to mice treated with oral irinotecan 5x/week for 4 weeks (20 doses). Together, these results show that the SN38-TS NP formulation is safe, as well as significantly more effective at controlling NB tumor growth and recurrence than conventional irinotecan therapy.

SN38-TS NPs were also effective at controlling larger, more progressive NB tumors, which mimic more advanced stage disease. In a pilot study, we showed that SN38-TS NPs induced tumor regression from an average of 1 cm³ to 0.1 cm³ when administered twice a week for 8 weeks (data not shown). These mice remained in remission for an average of 60 days post-cessation of treatment, analogous to our previous mouse studies. Interestingly, when tumors recurred after a period of remission, they grew at a slower pace than recurrences in irinotecan-treated mice, and they exhibited a dramatically altered morphology. All recurrent tumors examined in the SN38-TS NP treated group resembled ganglioneuromas (Fig. 5A and B).

Santos and colleagues treated neuroblastoma xenografts with CPT-11 (irinotecan), and they found that the tumors differentiated during treatment to ganglioneuroblastomas, but then reverted to an immature phenotype when treatment was discontinued (28). Although the mechanism for this differentiation is unknown, it was associated with a decrease in *MYCN* expression. Our findings of differentiation were similar, but unlike the Santos study with irinotecan, the differentiation persisted months after treatment was discontinued. SN38 is the major active metabolite of irinotecan and inactivates topoisomerase 1 (29), so the effects in both the Santos study and ours are presumably mediated by the same mechanism. However, the durability of the differentiation in our study is presumably related to the higher intratumoral concentrations of SN38 we achieved (Table 1) and/or the protracted duration of exposure afforded by NP delivery. It is possible that the sustained SN38 exposure killed proliferating NB cells and left a more differentiated population of cells that promoted Schwann cell invasion. The Schwann cells could then have provided the neurotrophic factors that led to neuronal differentiation (30).

Other groups have synthesized PEGylated, nanoparticulate, or nanoprecipitate formulations of SN38 to overcome the issues of poor solubility and high toxicity (23, 31, 32). These approaches showed superiority over conventionally delivered irinotecan, but most were designed primarily to address the poor solubility of SN38 and did not take full advantage of the EPR effect. Others have used lipid or chitosan nanocapsules for oral or parental administration (33–35). Finally, another study developed polymeric NPs encapsulating SN38 using poly lactic-co-glycolic acid (36). In the present study, we utilized biodegradable, PEGylated polymeric nanoparticles in combination with a pro-drug derivatization approach for delivery of SN38. Our SN38-TS NPs were also optimized for size and release kinetics (8), and we demonstrated dramatically superior effectiveness compared to orally administered irinotecan in our model system.

Taken together, our preclinical studies suggest that our SN38-TS NP formulation is an attractive new therapeutic approach for NB and other solid tumors. Our results show that this formulation is safe, as well as significantly more effective than oral irinotecan at

targeting NB tumors and controlling tumor regrowth. This formulation could be used to treat any tumor currently treated with irinotecan, and possibly tumors previously thought resistant to this drug, due to the dramatically increased drug delivery. Furthermore, this approach could potentially be applied to other therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NPs	nanoparticles
NB	neuroblastoma
PLA-PEG	poly(lactide)-poly(ethylene glycol)
SN38-TS	SN-38 tocopherol succinate
SRB	Sulphorhodamine B

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Figure 1.

Effect of Irinotecan, SN38-TS NP, SN38 FD, and TS FD on cell growth by Sulphorhodamine B Analysis. Cells were exposed to 1 nM, 3nM (data not shown), 5 nM (data not shown), and 10 nM of the respective compounds in the presence of BDNF. Plates were harvested at 24, 48, and 72 hr post-drug treatment. Cell viability was assayed using SRB dye.

Figure 2.

Biodistribution of SN38-TS NPs in mice at different time points post IV injection. Side and supine views reveal that thoracic fluorescence is primarily due to lymphatic and hepatic NP accumulation. Animals were imaged using the IVIS Spectrum Pre-clinical In Vivo Imaging system.

Figure 3.

Treatment of xenografts with SN38-TS NPs for 4 weeks prolongs tumor regrowth. (A) Tumor volume of xenografts after treatment with irinotecan (5x/week/4 weeks, 10mg/kg) or SN38-TS NP (1x/2weeks/4 weeks; 1x/week/4 weeks; 2x/week/4 weeks, 10mg/kg). Data are shown as means. (B) Survival curves of tumor-bearing animals. indicates ↓ last day of treatment. Animals were followed for tumor regrowth and survival until the tumors reached 3cm³ in volume.

Figure 4.

Treatment of xenografts with SN38-TS NPs for 8 weeks prolongs tumor regrowth. (A) Tumor volume of xenografts after treatment with irinotecan (5x/week/8 weeks, 10mg/kg) or SN38-TS NP (2x/week/4 weeks; 1x/week/8 weeks; 2x/week/8 weeks, 10mg/kg). Data are shown as means. (B) Survival curves of tumor-bearing animals. Log-rank tests revealed $p < 0.0001$ for SN38-TS NP 1x/week/8 weeks group versus irinotecan 5x/week/8 weeks, $p = 0.0007$ for SN38-TS NP 2x/week/4 weeks versus irinotecan 5x/week/8 weeks, $p = 0.0753$ for SN38-TS NP 2x/week/4 weeks versus SN38-TS NP 1x/week/8 weeks, $p < 0.0001$ for SN38-TS NP 2x/week/8 weeks versus irinotecan 5x/week/8 weeks, $p = 0.0142$ for SN38-TS NP 2x/week/8 weeks versus SN38-TS NP 1x/week/8 weeks, $p = 0.0001$ for SN38-TS NP 2x/week/8 weeks versus SN38-TS NP 2x/week/4 weeks. ↓ Indicates last day of treatment. Animals were followed for tumor regrowth and survival until the tumors reached 3cm^3 in volume. * indicates a treatment period of 4 weeks, ** indicates a treatment period of 8 weeks.

Figure 5. SN38-TS NP treatment of large NB tumors. A. survival curve of the tumor-bearing animals. ↓ Indicates last day of treatment. Animals were followed for tumor regrowth and survival until the tumors reached 3cm³ in volume. B. treatment of large tumors with SN38-TS NP promotes maturation of xenografts into a ganglioneuroblastoma phenotype. Tumors were followed for regrowth patterns and harvested when they reached 1 cm³. Increased Tyrosine Hydroxylase staining is seen in the SN38-TS NP-treated tumors.

Table 1

Levels of Irinotecan and/or SN38 in Blood and Tumor Tissue

	Blood	Blood	Tumor	Tumor
	Irinotecan ng/ml $\pm$ SD	SN38 ng/ml $\pm$ SD	Irinotecan ng/g tissue $\pm$ SD	SN38 Ng/g tissue $\pm$ SD
Irinotecan 4 hr	5.13 $\pm$ 3	8.01 $\pm$ 1.7	16.44 $\pm$ 4.7	10.71
Irinotecan 24 hr	<1	<1	<10	<10
Irinotecan 72 hr	<1	<1	<10	<10
SN38-TS hr 4	—	3,937.1 $\pm$ 748.5	—	1974.6 $\pm$ 465.2
SN38-TS hr 24	—	99 $\pm$ 25.7	—	1482.4 $\pm$ 354.6
SN38-TS hr 72	—	29.3 $\pm$ 18.6	—	558.3 $\pm$ 190.7

LLOQ for SN-38 and Irinotecan was 1.0 ng/mL (blood) and 10 ng/g of tissue.

HLOQ for SN-38 and Irinotecan was 1000 ng/mL (blood) and 10000 ng/g of tissue

Tissue distribution of irinotecan and SN38-TS NP. Animals were given a single dose of either irinotecan PO (10mg/kg) or SN-38 T/S NP IV (10mg/kg) and sacrificed at given time points post-treatment. Drug concentrations were analyzed by LC/MS/MS as described in methods. Values are shown $\pm$ the standard deviation (SD).