CHAPTER 5

Prolonged convection-enhanced delivery in the pons with SN-38 loaded nanoparticles and irinotecan

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In preparation
ABSTRACT

Introduction and aim
Inadequate drug delivery due to an intact blood brain barrier (BBB) could explain part of the resistance to conventionally administered chemotherapeutic agents in diffuse intrinsic pontine glioma (DIPG), and consequently its poor prognosis. Convection-enhanced delivery (CED) of chemotherapeutics is an alternative mode of local intracranial delivery and irinotecan, and its biologically active compound SN-38, are considered potentially effective agents. In this study we aim to perform prolonged CED over a period of 24 hours in the pontine area of rats using irinotecan and newly designed SN-38 loaded Poly Lactic Acid (PLA) nanoparticles (NPs).

Methods
Efficacy of irinotecan and SN-38 against patient-derived HSJD-DIPG-007 cells was determined in vitro. Stability and release of SN-38 from the NPs was tested with a dialysis chamber, and release of SN-38NPs and irinotecan from an osmotic Alzet pump was tested in vitro. Prolonged CED (200 μL in 24 h using an Alzet Osmotic pump) was performed in naive rats after the correct targeting coordinates were determined. Maximum tolerated dose (MTD) was determined by neurological follow-up using Rota-rod testing, and rats with HSJD-DIPG-007 tumors were treated with the MTD of SN-38NPs, irinotecan, or vehicle (NaCl 0.9%).

Results
IC50 (50% of growth inhibition) of SN-38 against HSJD-DIPG-007 cells was in the nanomolar range, and IC50 of irinotecan was 1,000 fold higher. Rota-rod scores in rats with HSJD-DIPG-007 tumors and control rats reached a significant difference before difference in body weight could be objectified. Stability of SN-38 and release from nanoparticles was adequate for the aimed experiment and prolonged CED with SN-38 and irinotecan in the pontine area was tolerated at 1.2 μg (corresponding with ~8 µg/kg) and 0.08 mg (corresponding with ~5 mg/kg), respectively. These dosages did not show clear efficacy to treat diffusely growing HSJD-DIPG-007 tumors.

Discussion
This study shows that prolonged CED in the rat pons using potent chemotherapeutic agents is feasible and safe, it validates stability of newly-designed PLA nanoparticles containing SN-38 in our used vehicle, and it shows Rota-rod testing to be an effective tool for studying pathology in the pons and cerebellum of rats. Furthermore, the lack of efficacy in treating HSJD-DIPG-007 tumors highlights the hurdles that need to be taken before translation of CED to the clinic to treat DIPG patients is feasible, and thereby provides new starting points for further research.
INTRODUCTION

Diffuse intrinsic pontine glioma (DIPG) is a primary brain tumor arising in the pons that mostly occurs in children. Children diagnosed with DIPG have a median survival of 9 month and a 2-year survival of only 10% or even less. Because no conventional treatment modalities are able to cure DIPG, alternative treatment strategies should be considered. Convection-enhanced delivery (CED) is a local delivery technique that infuses the drug of choice directly into the tumor under a continuous pressure, over a period of hours to days. Local drug concentrations achieved by CED can be up to 10,000-fold higher as compared to intravenous drug administration, while minimizing systemic exposure. The clinical application of CED is currently being investigated in several clinical trials in adults and children, using multiple agents, among which is irinotecan (NCT02022644). Local delivery of irinotecan has shown to be quite successful in treating a number of preclinical xenograft models and primary brain tumors in dogs, with very little toxicity. However, it is unknown whether the infusion of irinotecan in the brainstem (a tissue with highly important functions for life) is safe as compared to previously tested central nervous system (CNS) locations. Also, pharmacokinetics of irinotecan is complex and the exact mechanism through which irinotecan can be effective after local delivery is unknown. When administered systemically, it is metabolized in the liver by carboxylesterases (CES) mediated hydrolysis to its active form SN-38, which is thought to be 1,000 times more potent than the non-hydrolyzed form. Theoretically, irinotecan will not, or only partially, be metabolized to SN-38 in the brain, leading to low therapeutic efficacy after local delivery. Therefore, performing CED with SN-38 would be much preferred. However, intrinsic chemical properties (low solubility) of SN-38 make it unsuitable for clinical use in its free form. To allow adequate solvability and subsequent clinical use, SN-38 has been changed chemically (as is the case of FDA approved used agents irinotecan and topotecan) or packaged in nanoparticles or nanofibers to enable administration in patients. A novel polymer-based nanoparticle containing SN-38 was recently developed at our laboratory. Nanoparticles are very suitable for delivery via CED because they lead to wide tissue distribution, lower peak concentrations and longer bioavailability.

However, previous work has shown the pons to be particularly sensitive to chemotherapeutic agents, which may limit treatment efficacy. One way to overcome this issue is to perform repeated or continuous CED with lower drug concentrations. Because of potential toxicity using both prolonged infusion and a cytotoxic agent, more
sophisticated and objective neurological follow up techniques are required than have previously been used in preclinical CED experiments. In this study we investigated the use of polymer based SN-38 nanoparticles and irinotecan for prolonged local delivery in the pons via CED, and assessed the feasibility and reproducibility of neurological follow up using Rota-rod function testing. We have determined maximum tolerated dose (MTD), toxicity and distribution in the brain, in order to assess translatable of these agents to clinical trials in DIPG patients. The maximum tolerated dose was tested for efficacy in an orthotopic diffusely growing primary DIPG model.

**METHODS**

**SN-38 polymer nanoparticles**
Poly Lactic Acid (PLA) nanoparticles containing 10% SN-38 were produced by an emulsion-solvent evaporation method, freeze-dried in water with 10% 2-Hydroxypropyl)-
cyclodextrin (HPBCD) (Sigma- Aldrich) as cryopreservant, and extensively characterized by Monterrubio et al. SN-38NPs, 300 nm in size, were reconstituted in sodium chloride (NaCl) 0.9% to the required concentrations. To remove the residual HBPCD to allow for in vivo administration, 3 mL of SN-38NPs were injected in a dialysis cassette (Slide-A-Lyzer™ Dialysis Cassettes, 20K MWCO, 3 mL, Thermo Scientific, 66003) and incubated for 20 minutes in 1 L sterile NaCl 0.9% on a shaking platform at room temperature.

**HSJD-DIPG-007 cell model**
The primary HSJD-DIPG-007 cell line was established from DIPG tumor material obtained after autopsy from a 6-year-old patient and is confirmed to have a H3F3A (K27M) and ACVR1 (R206H) mutation. Cells were cultured in tumor stem cell medium as previously described. Cells were routinely tested for mycoplasm infection and validated by quality controls including short tandem repeat (STR) analysis.

**Animals**
3 or 4-week-old nude rats (Hsd:RH-Foxn1mnu) were purchased from Envigo, Barcelona, Spain, or Charles River (Crl:NIH-Foxn1mnu) and housed under specific pathogen free (SPF) conditions with food and water freely available. All animal experiments were carried out with permission of the institutional animal ethics committee. During this study clinical scores were assigned to rats as ranging from 0 to 4. (0: no clinical symptoms, 1: mild clinical symptoms including subtle decrease in activity and grooming, mild paresis
and lateralization 2: clear clinical symptoms such as mild-moderate inactivity, clear lateralisation or focal neurological symptoms, mild loss of balance, 3: severe neurological symptoms such as severe inactivity, total loss of balance, paralysis and inability to move around the cage freely, 4: death). Humane endpoints were defined as > 20% weight loss or severe neurological symptoms or inactivity.

**Immunohistochemistry**
4 µm cut formaldehyde fixed-paraffin embedded (FFPE) sections were deparaffinized and rehydrated and stained with H&E according to a standardized protocol. To study the extension of the human infiltrative tumor in the rat brain, human nuclei were stained using human specific primary antibodies (Chemicon, Millipore - MAB4383, 1:250) and detected by using the Novolink Polymer Detection Systems Novocastra kit (Leica Biosystems).

**In vitro antiproliferative activity of SN-38, SN-38NP and irinotecan on DIPG cells**
At day 0 cells were mechanically dissociated, counted using a Neubauer chamber and plated with a density of 3,000 cells per well in a 96 well plate. At day 1, cells were incubated with a SN-38 (Seqchem, stock solubilized in DMSO, as previously described), SN-38 nanoparticles and irinotecan (Hospira, Lake City, IL, USA) diluted in medium with concentrations ranging from 0.5 pM to 5 µM (SN-38 and SN-38NP) or 0.05 nM – 500 µM (irinotecan). After 72 hours of incubation, cell viability was determined using an MTS assay (Promega). IC50 values were calculated using Graph Pad Prism (version 5.0b).

**Release and stability of SN-38 PLGA polymer nanoparticles in sodium chloride 0.9%**
To study SN-38 release from SN-38NPs, an adequate amount of lyophilized nanoparticle formulation was weighed and diluted to achieve 0.2 mg SN-38/mL in sterile NaCl 0.9% containing 10% HPBCD to avoid drug adhesion to plastic surfaces. A previously hydrated dialysis cassette (Slide-A-Lyzer™ Dialysis Cassettes, 20K MWCO, 3 mL, Thermo Scientific, 66003) was filled with 2 mL SN-38 nanoparticles and placed in a closed bag containing 10 mL of dialysis fluid (NaCl 0.9 with 10% HPBCD). Only SN-38 released from the nanoparticles passed through the dialysis membrane until reaching equilibrium with the dialysis fluid. Dialysis cassettes were incubated at 37°C on a shaking platform. Samples of the dialysis fluid (n=3) were collected and pH measured at set time points. The dialysis buffer was changed after sample collection. A schematic representation can be seen in figure 2A.
SN-38 lactone and carboxylate forms were quantified in the dialysis fluid using high-performance liquid chromatography (HPLC) as previously described. To assure stability of the lactone fraction after collection, the sample was captured directly in ice-cold methanol (proportion of sample:methanol was 20:80) and stored at -80°C. SN-38 present in nanoparticles was determined by collecting samples from the dialysis chamber at the beginning and the end of the experiment. SN-38 nanoparticles were hydrolyzed in NaOH 0.1M and filtered using a 0.45 nM filter. The filtered solution was further diluted in phosphate buffered saline (PBS) containing 10% HPBCD and SN-38 carboxylate was measured using HPLC as previously described.

**Release of SN-38 polymer nanoparticles and irinotecan from an Alzet osmotic pump**

To study the release profile and the stability of SN-38 released from Alzet osmotic pumps under simulated in vivo conditions, two pumps (model 2ML1) were filled according to the manufacturer’s instructions with 2 mL SN-38NPs (0.2 mg/mL in NaCl 0.9% with 10% HPBCD, pH 5.6) or irinotecan (4 mg/mL). 5 cm vinyl tubing (0.69 mm, Plastics1, Roanoke, USA) was attached to the side arm and the pumps were placed in two separate 50 mL Falcon tubes containing previously warmed PBS (37°C). The tubing was guided through a small opening in the tube’s cap and placed in a small Eppendorf tube covered with parafilm to prevent evaporation. The tubes were incubated at (37°C) on a shaking platform for 7 days and volume collected in the Eppendorf tubes and weighed and sampled every 24 hours to determine volume of outflow from the pump and total SN-38 content.

**Inoculation of orthotopic HSJD-DIPG-007 tumors and study of engraftment and tumor burden**

HSJD-DIPG-007 cells were mechanically dissociated, counted using a Neubauer chamber and diluted in medium to a concentration of 10^5 cells/μL. Cells were injected into the ventricle (coordinates from lambda: -1, +1, -7.5, angle 10°). To study tumor engraftment and tumor burden, rats were sacrificed after 18, 22 and 28 days and at endpoint and perfused with PBS and formaldehyde 4% using previously validated methods modified from Gage et al. Brains were sliced using an Adult Rat Brain Slicer Matrix with 1.0 mm sagittal section slice intervals (Zivic Instruments, Pittsburg, PA, USA, BSRAA001-2) and tumor burden was studied on slides from paraffin embedded sections.
Follow up by Rota-rod neurological function test
To detect neurological damage in the rats due to DIPG disease or to toxicity of convection-enhanced infusion of treatments, rats were trained on the Rota-rod (Panlab, Harvard Apparatus, LE8300) during two weeks prior to treatment. To train, rats were placed on the Rota-rod for a minimum of 5 minutes. After 5 minutes the speed of the rod was increased to 10 rotations per minute (RPM) and this speed was maintained 5 minutes. If rats failed to complete 5 minutes on the Rota-rod, they were trained again the next day. Training sessions took place at least two times per week during two weeks. If rats could not learn Rota-rod in two weeks, they were excluded from the study. During the follow-up phase, rats were tested bi-weekly on the Rota-rod. They were placed on the stationary Rota-rod for one minute. After one minute, the speed was gradually increased to 10 RPM. Rats had to complete three trials of 3 minutes. Total time on the Rota-rod during three sessions was documented (Rota-rod score) and the trial was considered failed if they are unable to complete at least one 3-minute trial.

Determining targeting coordinates for CED in 7 week old nude rats
To determine the adequate coordinates to implant catheters infusing the brainstem of 7-week-old nude rats, 3 rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg) and perfused with PBS and formaldehyde 4% using previously validated methods modified from Gage et al. After perfusion rats were placed in a stereotactic frame and the skin on the scalp was removed. A burr hole was made at previously set coordinates and an angled (26G) Hamilton syringe filled with trypan blue was inserted into the brain. Successive injections with 0.5 μl of trypan blue were performed using different coordinates to determine correct infusion location and absence of leakage to the ventricle. Brains were sliced using an Adult Rat Brain Slicer Matrix with 1.0 mm sagittal section slice intervals (Zivic Instruments, Pittsburg, PA, USA, BSRAA001-2) and analyzed. Targeting coordinates were evaluated based on location of the catheter tip (aimed location: central pons) and presence or absence of trypan blue in the ventricle. A maximum of three coordinates were tested in one rat brain.

Prolonged CED using an osmotic subcutaneous pumps
To implant permanent infusion catheters in the brainstem, 7 week-old weighing 150 – 200 grams rats were anesthetized with ketamine (80 mg/kg) / xylazine (10 mg/kg) and placed on a warmth pad in a stereotactic frame. The skin on the scalp was removed showing both lambda and bregma. A burr hole was made at +2, -1 mm from lambda.
Three additional burr holes were made at 1-2 mm from the first hole. These holes were used to fit the screws for attaching the catheter. The screws (2.40mm Plastics1, Roanoke, USA 0-80 x 3/32) were placed sticking out slightly above the skull base. An L-shaped infusion catheter, (Plastics1, 330OP/DW/Spc, infusion depth 9.5 mm) with a dummy wire and a dust cap on the side arm, was placed in an angled mounting holder (Plastics1, MH-325-G/3.1) in the stereotactic frame with an angle of 20°, and lowered 9.5 mm below the skull base. Dental cement (Ketac Cem Easymix, 3M, USA) was applied around the catheter pedestal and the screws to ensure a secure attachment. The catheter was kept in place for 5 – 10 minutes to allow hardening of the cement. The upper part of the pedestal was cut and the skin was closed with sutures. Rats were placed in individual cages and monitored closely until fully recovered. Three to four days after placement of catheter, rats were anesthetized with isoflurane (1-2 L O2 100% + 2.5% isoflurane). The skin on the scalp was reopened and after confirming the catheter to be adequately anchored to the skull, the dust cap and dummy wire were removed from the side arm. A previously filled Alzet pump (2001D, Alzet Osmotic Pumps, USA) attached to tubing was placed subcutaneously and the tubing was attached to the side arm. The skin was sutured and the rats were placed in individual cages and again monitored closely. If any neurological symptoms arose after these procedures (placement of catheter or pump), rats would be excluded from the study.

Targeting and distribution of nanoparticles in the brainstem
To study the pattern of distribution of SN-38NPs infused in the brainstem, catheters were placed at day 1 as previously described. Alzet pumps (2001D) were filled with SN-38NPs containing a 1% proportion of fluorescein isothiocyanate (FITC)-conjugated PLA and attached to the catheters at day 4. After 36 h rats were sacrificed by perfusion and brains were sliced using the Rat Brain Slicer Matrix. Fluorescence in brains was imaged using a camera (Hamamatsu Photonics Deutschland) coupled to Hokawo imaging software. (Schematic overview in supplemental figure 1A.)

Determining MTD of SN-38 and irinotecan in naïve rats
5-week-old nude rats were trained for two weeks to perform the Rota-rod test. An infusion catheter and Alzet pump was placed at the age of 6 weeks as previously described. Infusion took place in 24 hours following the implantation of the pump and follow up consisted of daily weight measurements, determination of neurological symptoms (clinical score 0-4) and tri-weekly Rota-rod function tests. Alzet pumps were removed
after 72 hours and rats were sacrificed if humane endpoints were reached. At day 14 rats were sacrificed by perfusion and brains were analysed for the presence of histological toxicity. Maximum tolerated dose (MTD) was defined as: no rat at a certain dose reaching humane endpoint or clinical score > 2 during follow up. Concentrations and dose tested are shown in table 1, a schematic overview can be seen in supplemental figure 1B.

**Efficacy of prolonged CED with SN-38NPs and irinotecan to treat HSJD-DIPG-007 in vivo**

HSJD-DIPG-007 cells were injected into the brainstem in 4-week-old rats (n=18). 18 days after injection of cells, the catheter was implanted and 3-4 days later an Alzet pump (2001D) filled with irinotecan (0.4 mg/mL), SN-38NP (6 μg/mL, dialysed 30 minutes) or NaCl 0.9% was placed subcutaneously and attached to the catheter. One rat of each group was sacrificed 7 days after the CED treatment to evaluate local activity. Follow up of the remaining rats consisted of daily observations and weight measurements. Rats were sacrificed by perfusion after humane endpoints were met. Differences in survival were analysed by Kaplan Meier curves and significance was calculated using log rank testing using Graph Pad Prism software (version 5.0b) and p < 0.05 (2 sided) will be considered significant. A schematic overview of the experiment can be seen in supplemental figure 1C.

**RESULTS**

**Efficacy of SN-38, irinotecan and SN-38NP on HSJD-DIPG-007 primary DIPG cells**

First, toxicity of SN-38 and irinotecan against HSJD-DIPG-007 cells was tested in vitro. HSJD-DIPG cells proved to be very sensitive to treatment with SN-38 both in free form and when supplied to the cells in nanoparticles. IC50 values were 3 nM (free SN-38) and 38 nM (SN-38 NP). Irinotecan showed a much higher IC50 value of 2 μM (figure 1 A-C).

**Release and stability of SN-38 polymer nanoparticles in NaCl 0.9%**

Release from SN-38NPs and stability of SN-38 in the vehicle used was tested in an in vitro setup. A schematic representation of this setup can be seen in figure 2A-C. The pH directly after reconstitution of nanoparticles was 6.1, decreased to a minimum of 5.2 and rose to 5.6 at day 7. The pH of irinotecan diluted in NaCl 0.9% was 3.6 at the start of the experiment (figure 2D). Such low pH ensures that SN-38 remains predominantly in the lactone (active) form until released in the targeted tissue. Dialysis showed release
of SN-38 from polymer nanoparticles incubated at 37°C to be gradual up to 50% after seven days (figure 2E). Incubation of the 2ML Alzet pump in PBS, shaking at 37°C lead to a constant release of fluid from the pump at a rate of 7.7 μL/h for SN-38NP and 7.9 μL/h for irinotecan (figure 2F). The concentration of SN-38 present in fluid released from the Alzet pump filled with SN-38NPs gradually decreased from initial 100%, to 72% at 24 hours, reaching 10% of the initial concentration at day 7 of incubation at 37 °C. SN-38 in a SN-38NPs NaCl 0.9% solution stored at 4°C was much more stable and only decreased to 80% during the 7 day period (figure 2G).

**Targeting coordinates of the pons in 7 week-old nude rats**

Three separate targeting experiments were performed to determine the optimal coordinates for placement and length of the CED catheter. First the coordinates targeting the ventricle were confirmed by sacrificing an animal directly after injection of HSJD-DIPG-007 cells into the ventricle (supplemental figure 2, first row). In the first and second targeting experiment supplemental figure 2, second and third row), trypan blue could be detected in the right location in the pons in two out of six injections, but using these coordinates, trypan blue was also leaking into the ventricle, indicating possible leakage through the catheter path during CED. Increasing the angle of injection from 10 to 20° and increasing the injection depth from 8.5 to 9.5 mm circumvented the ventricle and targeted the ventral pons as planned (supplemental figure 2, last row). The final coordinates used to place the infusion catheter were: -1, *2, -9.5 under a 20° angle (from lambda). Infusion of SN-38 nanoparticles containing FITC confirmed location of infusion and absence of leakage to the ventricle (figure 3).
Figure 2 | Release and stability of SN-38NPs. (A) Setup of SN-38NP loaded dialysis chamber. (B) In vitro setup to test release from Alzet pump. (C) Table with timing of sampling from dialysis fluid (DF), dialysis chamber (DC), pH and Alzet pump (AP). (D) pH measured in dialysis fluid over time. (E) Release of SN-38 from nanoparticles over time measured by HPLC; total SN-38 content (black line), SN-38 content in dialysis chamber (striped red line with triangles), SN-38-lactone content in dialysis fluid (green dotted line with down facing triangles), SN-38-carboxylate content in dialysis fluid (orange striped line with black dots). (F) Volume released from Azlet pump: SN-38 (black line) and irinotecan (striped purple line). (G) Total SN-38 content measured by HPLC. SN-38 released from Alzet pump (striped green line) or total SN-38 kept at 4°C (dotted blue line).
Follow up of rats with HSJD-DIPG-007 tumors using Rota-rod

Rats with growing HSJD-DIPG-007 tumors were analyzed using a Rota-rod function test to identify pathology in the pons and cerebellum. Rota-Rod training starting at 4-7 weeks of age was successful within 2 weeks in 100% of the rats trained during this study (n=24). Average Rota-rod score in naïve animals was 8.55/9 (SD 0.57) throughout the study. Rats injected with HSJD-DIPG-007 tumors had an average score of 8.4/9 (SD 0.70) one week after injection of cells. In the first 4 weeks after injection of HSJD-DIPG-007 the average score was 7.9/9 (SD 0.93) and in 4-6th week after injection average score was 7.5/9 (SD 1.76). In the last week before all animals reached the humane endpoints, average Rota-rod score was 0.93/9 (SD 0.80) (figure 4A). The weight of the rats with or without tumor were comparable up until reaching human endpoints (figure 4B). There was a significant difference between reaching human endpoint (median 61 days) and Rota-rod failure (median 54 days, p < 0.05) (figure 4C). 54 days after injections, Rota-Rod score differed significantly between tumor baring rats and control rats (p < 0.01, figure 4D). At this stage in the disease, no significant difference could be detected in average normalized weight between the two groups (p < 0.01, figure 4A and E) indicating Rota-rod function tests to be a more sensitive method than weight loss for detecting pathology in the pons and cerebellum. At endpoint weight differed significantly between naïve and tumor bearing rats (figure 4F).

Timing of treatment in HSJD-DIPG-007

To determine correct timing of treatment rats injected with HSJD-DIPG-007 tumor cells were sacrificed a different time points. Human HSJD-DIPG-007 cells were detected using immunohistochemical (IHC) staining with human specific anti-nuclei antibody.
Figure 4 | (A) Rota rod score over time starting from injection of tumor cells (total min on the Rota-rod in 3 individual 3 minute trials). Black dots: rats with HSJD-DIPG-007 tumor, blue squares rats without tumor. (B) Rat weight normalized by weight at time of injection of tumor cells of tumor baring rats (black dots) or naïve rats (blue squares). (C) Kaplan-Meier curve of failure of rota-rod test (not being able to finish one 3 min trial, purple striped line) and survival (black line) of rats injected with HSJD-DIPG-007 tumors. (D) Normalized weight at day 54 after injection of HSJD-DIPG-007 cells. No significant difference between rats with tumor (black dots) or without tumor (blue squares). (E) Rota rod score at day 54 after injection of HSJD-DIPG-007 tumor cells. Significant difference between rats with tumor (black dots) and rats without tumor (blue squares) ** P< 0.01. (F) Weight at endpoint of rats with tumor, significant difference between rats with tumor (black dots) and rats without tumor (blue squares) * P< 0.05
Human HSJD-DIPG-007 cells were detected in the ventricle directly after injection (figure 5A) and showed clear engraftment and spread throughout the ventral pons and cerebellum at day 18 after injection (figure 5B,C). There was remarkable diffuse growth pattern, consistent with human DIPG (5D,E). Based on the pattern of spread, optimal timing (large enough tumors to be clinically relevant but still confined to pontine and cerebellar area) of treatment would be between 18 and 22 days.

**Clinical toxicity of SN-38NP and irinotecan**

Rats showed severe clinical and histological toxicity after administration of high dose irinotecan (4 mg/mL) and SN-38 (0.2 mg/mL), but toxicity was similar in animals treated with the initial vehicle (NaCl 0.9% with 10% HBPCD) (supplemental figure 3, table 1). Additional experiments (supplemental 3A-E) confirmed the toxicity of HBPCD, even at low concentrations (0.2%), and toxicity of high dose irinotecan without HBPCD (figure 6A,B). Clinical and histological toxicity was especially pronounced in animals treated...
Figure 6 | (A) Weight and (B) clinical score (0-4) of rats treated with HPBCD 0.2% (yellow dotted and striped line with black dots), irinotecan 1.3 mg/mL (red line with black and white squares), irinotecan 0.4 mg/mL (dotted purple line with squares), and SN-38 0.02 mg/mL + 0.2% HPBCD (black line with down facing triangle). (H) Histology of rat brain after treatment with SN-38 0.02 mg/mL in 0.2% HPBCD. (D) Weight and (E) Rota-rod score (total minutes on the Rota-rod in 3 individual 3 minute trials) of rats treated with CED with NaCl 0.9% vehicle (blue line and blue dots), irinotecan 0.4 mg/mL (purple line with closed squares), SN-38NPs 6 µg/mL (black dotted line with black triangle) and control (green line with down facing triangle). Histology of rat brain treated with (F) vehicle NaCl0.9% (G) irinotecan 0.4 mg/mL and (H) SN-38NPs 6 µg/mL. Right panel shows black squares at higher magnification.
with SN-38NP in vehicle containing HBPCD (figure 6C). HBPCD is required to adequately
freeze dry and reconstitute SN-38NPs, but after the first experiments, HBPCD was
removed from the reconstituted solution by dialysis to avoid toxicity.

Subsequent dose de-escalation determined the MTD of irinotecan to be 0.08 mg/200 µL (0.4 mg/mL; i.e., approximately 5 mg/kg in a 24 h infusion) (vehicle NaCl 0.9%) and of
SN-38NP to be 1.2 µg/200 µL (6 µg/mL; i.e., approximately 8 µg/kg in 24 hours) (vehicle
NaCl 0.9%, dialyzed) (figure 6 D, E). Rats treated at the MTD of irinotecan or SN-38NP
or with vehicle (NaCl 0.9%) showed mild to moderate transient clinical symptoms and
functional deficits after infusion. All animals, irrespective of treatment group experienced
transient weight loss after infusion (figure 6D) and Rota-Rod score declined in all treated
animals 24 hours after the end of infusion, but recovered between 4 to 6 days after the
infusion (figure 6E).

**Table 1 | Summary of clinical and histological findings in rats after CED with irinotecan, SN-38NPs and vehicle (HPBCD and NaCl 0.9%).**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>N</th>
<th>Vehicle</th>
<th>Clinical outcome</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Irinotecan</strong></td>
<td>4 mg/ml</td>
<td>3</td>
<td>NaCl 0.9%</td>
<td>Severe toxicity directly after infusion</td>
<td>Severe loss of tissue and necrosis</td>
</tr>
<tr>
<td></td>
<td>1.3 mg/ml</td>
<td>1</td>
<td>NaCl 0.9%</td>
<td>Severe toxicity, 2 days after infusion</td>
<td>Necrosis, inflammation and edema</td>
</tr>
<tr>
<td></td>
<td>0.4 mg/ml</td>
<td>2</td>
<td>NaCl 0.9%</td>
<td>Moderate transient toxicity</td>
<td>Inflammation and reactive changes</td>
</tr>
<tr>
<td><strong>SN-38NP</strong></td>
<td>0.2 mg/ml</td>
<td>3</td>
<td>HPBCD 10%</td>
<td>Severe toxicity directly after infusion</td>
<td>Severe inflammation and necrosis</td>
</tr>
<tr>
<td></td>
<td>0.02 mg/ml</td>
<td>1</td>
<td>HPBCD 0.2%</td>
<td>Severe toxicity after 6 days</td>
<td>Severe necrosis, loss of tissue</td>
</tr>
<tr>
<td></td>
<td>6 µg/ml</td>
<td>2</td>
<td>NaCl 0.9%</td>
<td>Moderate transient toxicity</td>
<td>Influx of inflammatory cells and reactive changes</td>
</tr>
<tr>
<td><strong>Vehicle</strong></td>
<td>HPBCD 10%</td>
<td>3</td>
<td>-</td>
<td>Severe toxicity directly after infusion</td>
<td>Severe inflammation, loss of tissue and edema</td>
</tr>
<tr>
<td></td>
<td>HPBCD 0.2%</td>
<td>1</td>
<td>-</td>
<td>Moderate-severe toxicity 2 days after infusion</td>
<td>Loss of tissue, inflammation and necrosis</td>
</tr>
<tr>
<td></td>
<td>NaCl 0.9%</td>
<td>2</td>
<td>-</td>
<td>Moderate transient toxicity</td>
<td>Some mild/moderate reactive changes</td>
</tr>
</tbody>
</table>

**Histological toxicity of SN-38NP and irinotecan**

Histological toxicity corresponded well to clinical toxicity. Severe toxicity including loss of tissue, influx of inflammatory cells and bleeding was observed in rats treated with high dose and intermediate dose irinotecan (4 mg/mL and 1.33 mg/mL). SN-38NP
(0.2 mg/mL and 0.02 mg/mL) and vehicle with HBPCD 10% and 0.2%. Animals treated with high dose were sacrificed 24-48 h after infusion and tissue responds was primarily characterized by acute inflammation (figure D and E). In animals sacrificed at later time points (SN-38 0.02 mg/mL), severe necrosis, bleeding and loss of tissue can be seen (figure 6C). Animals treated at safe dose (irinotecan 0.4 mg/mL, SN-38NP 6 μg/mL and NaCl 0.9%) showed mild (NaCl 0.9%, SN-38 6 μg/mL) to moderate (irinotecan 0.4 mg/mL) reactive changes with influx of inflammatory cells (figure 6 F,G,H). Two animals with HSJD-DIPG-007 tumors treated at clinical endpoint with high dose irinotecan (4 mg/mL) and SN-38 (0.2 mg/mL) showed extensive necrosis, inflammatory responds and loss of tissue of HSJD-DIPG-007 tumor cells. In these animals the CED catheter and Alzet infusion pumps were placed in the same session, and histological analysis shows a poor distribution of drug with extensive backflow (supplementary figure 3G).

**Survival of tumor-bearing rats treated with SN-38 and irinotecan delivered via CED**

Treatment with CED of rats with HSJD-DIPG-007 tumors did not improve survival using any of the compounds used. Rats treated with SN-38NPs at maximum tolerated dose led to a (statistically not significant) shorter survival time (median survival 41 days), possibly due to long-term local toxicity that was not detected during the MTD assays, or to the presence of tumor that led to increased local inflammation due to the drug effect on tumor cells. Median survival of irinotecan treated rats was 61 days, as compared to 56 days for vehicle-treated controls (P = 0.1966) (Figure 7).

Figure 7 | Survival of rats with HSJD-DIPG-007 tumors treated with CED with vehicle (blue line), Irinotecan (purple dotted line) or SN-38NPs (black striped line). Differences in survival are not statistically significant.
DISCUSSION

In this study we determined the feasibility of performing prolonged – high volume CED in the rat brainstem with newly designed SN-38 nanoparticles and its commercial prodrug irinotecan. We first showed SN-38, and to a lesser extend irinotecan to be effective against DIPG cells \textit{in vitro} and confirmed \textit{Rota-rod} to be a reproducible and sensitive method to study the neurological effects of infiltrative tumors and acute toxicity in the pons and cerebellum as previously described by Lui et al.\textsuperscript{53} We confirmed the stability, sustained release and activity of SN-38 nanoparticles using an \textit{in vitro} CED setup. We showed that Rota-rod follow up allowed to determine maximum tolerated dose for both SN-38NP and irinotecan for local pontine delivery. We observed both clinical and histological toxicity after administration of irinotecan doses that have previously been shown to be safe after local administration to the striatum\textsuperscript{169,192,202}. Upon reduction of such dose, a theoretically effective maximum tolerated dose could be administered with only transient clinical symptoms. These results are in accordance with previous preclinical reports of prolonged convection-enhanced delivery in the rat brainstem with fluorescent dye and small molecule kinase inhibitors\textsuperscript{203,204}. No efficacy studies of prolonged CED in the pons have yet been published and no previous report has described prolonged CED to the pons using cytostatic agents. This single dose prolonged CED with a cytostatic agent was however not sufficient to treat diffusely growing orthotropic DIPG tumors \textit{in vivo} effectively.

Because high toxicity of locally delivered chemotherapeutic agents in the pons was observed in previous studies\textsuperscript{223}, a quantitative, sensitive and reproducible method was required to study neurological toxicity of CED. Rota-rod function tests can be used to study balance, coordination, physical condition and motor-planning in rodents\textsuperscript{205}. In the current study we correlated Rota-rod performance to pathology in the brainstem and cerebellum caused by CED or tumor growth. Rota-rod testing proved more sensitive compared to weight measurements and is less subjective and less prone to inter-observer variability compared to clinical observation alone. Especially when studying toxicity after local delivery or performing follow up of tumors that cannot be monitored by \textit{in vivo} bioluminescence imaging or MRI, using Rota-rod can substantially increase internal validity of the results of preclinical rat-DIPG studies. Most importantly, the test could help detect the antitumor activity of future treatments against DIPG.
Because it is not known whether irinotecan is converted to its active metabolite SN-38 upon local delivery in the CNS, and given that the infusion of free SN-38 is not possible due to the extremely low solubility of this drug\textsuperscript{196} we proposed the use of a new SN-38 releasing polymer formulation. Results from our experiments suggest SN-38NPs can be used in a prolonged CED experiment lasting 24 hours, but the vehicle and formulation should be further optimized to enable longer infusion times. We measured SN-38 content in vehicle containing HPBCD. However, since this showed to be toxic in the rat brain, HPBCD was cleared from the formulation before further administration \textit{in vivo}. This underlines the low tolerance of the pons to not only chemotherapeutic agents, but also to chemicals widely used in drug formulation. Previous studies have shown that SN-38 adheres to plastic surfaces and precipitates unless 10\% HPBCD is included in the release buffer\textsuperscript{200}. Thus, we are currently uncertain how this could have influenced activity and concentration of SN-38 in the final formulation. We also do not know how SN-38NPs will behave after local delivery \textit{in vivo}. Microdialysis experiments could be of aid to study exact interstitial concentration of free SN-38 carboxylate and lactone after CED\textsuperscript{196,200}.

Neither CED with SN-38NPs or irinotecan led to a survival benefit in rats with HSJD-DIPG-007 tumors and irinotecan at high dose showed similar toxicity compared to SN-38NPs. Because only low conversion of irinotecan to SN-38 is expected in the brain due to absent levels of CES, toxicity is likely caused by irinotecan itself. Some preclinical reports suggest tumor cells themselves are able to convert irinotecan to SN-38 causing higher concentrations of SN-38 at the tumor site leading to a favorable toxicity/efficacy profile\textsuperscript{206}. Summarizing both our \textit{in vitro} and our \textit{in vivo} results we consider it unlikely that DIPG tumor cells convert irinotecan to SN-38, but \textit{in vivo} microdialysis experiments might be of aid to study this in more detail.

DIPG is still an incurable disease and CED could play a role in a multi-modality treatment regimen. This preclinical study underlines some of the hurdles that need to be taken to translate CED into a successful treatment for DIPG patients. First we observe toxicity of chemotherapeutic agents to be high, when directly administered to the pontine area. This has been observed earlier (223) when administering doxorubicin to the brainstem. It is possible that inability to infuse high doses due to intrinsic sensibility of resident brain cells causes sub-therapeutic levels of therapeutic agents in the tumor after CED with subsequent treatment failure as result. Another reason for treatment failure can
be lack of adequate distribution after CED. Even though nanoparticles containing FITC showed a good distribution throughout the brainstem using our methods, therapeutic concentrations at the edge of the infusion ring might have been sub-optimal. Smaller (<100 nm) sized nanoparticles could potentially lead to a better distribution. Furthermore, we observed that implantation of the CED catheter and Alzet pump in one session, and subsequent direct commencing of infusion after placement of the CED catheter yielded a very poor area of distribution after CED, with extensive backflow in 2 rats. In the rest of our study, infusion was started with an interval of 3-4 days after placement of the CED catheter. Although numbers are small, this suggest tissue recovery and remodeling after catheter placement reduces backflow and this could have important implications for clinical CED and should therefore be studied in more detail. Efflux from the brain or from tumor cells by ABC-transporters could also have prevented adequate exposure to the drugs, since SN-38 and irinotecan are both substrates to drug efflux pumps. A fourth reason for treatment failure could be the highly diffuse and infiltrative character of the HSJD-DIPG-007 xenograft tumor. This tumor might have spread beyond the infusion area before treatment commenced. Analysis of the brain of animals treated with CED will follow to get more insight into the growth pattern of the tumor after treatment with CED, to conform or exclude this hypothesis.

DIPG is a highly infiltrative tumor, with tumors cells extending far beyond the primary tumor as visible on MRI. This will be one of the most difficult aspects when using local drug delivery as at treatment modality. Although this study provides important leads for further studies to improve (clinical) CED, it also underlines the need for a multi-modality treatment regimen to treat this devastating tumor.

Acknowledgments

Work funded by ISCIII-FEDER (CP13/00189), Fondo Alicia Pueyo, AECC Scientific Foundation, Fundacion Joan Ribas Araquistain, and European Union Seventh Framework Programme (FP7/2007-2013) under Marie Curie International Reintegration Grant (PIRG-08-GA-2010-276998). The international collaboration necessary to complete this project was made possible by a grant from the “Ter Meulen Fonds”, supported by the KNAW (National Academy for Arts and Sciences, The Netherlands). DIPG research at the VUmc has been made possible by the invaluable support of the Semmy Foundation, Stichting Egbers and of Stichting Kika (Children-Cancer-free).
Supplemental figure 1 | Schematic overview of (A) distribution experiment (B) toxicity experiment and (C) efficacy study.

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Supplemental figure 2 | Targeting coordinates for placement of CED catheter. Leakage to the ventricle can be seen in the second and third brain (*)
Supplemental figure 3 | (A) Clinical score (0-4) and (B) normalized weight of rats treated with CED with HPBCD 10% (orange with black dots), irinotecan (IRN) 4 mg/mL in 10% HPBCD (purple with black squares), SN-38 0.2 mg/mL in 10% HPBCD (back striped line with triangle), or control animal with dummy wire (DW) in place (green line with down facing triangle). Histology of (C) control brain (D) 10% HPBCD and (E) SN-38 0.2 mg/mL in 10% HPBCD. (F) Weight of two rats with HSJD-DIPG-007 tumors at endpoint treated with high dose irinotecan (4 mg/mL) and SN-38NP (0.2 mg/mL). (G) Histology of rat brain with HSJD-DIPG-0007 tumor at endpoint treated with irinotecan 4 mg/mL. Placement of CED catheter and subcutaneous Alzet pump took place in one session. Left panel shows anti-human nuclei staining, right panel shows H&E staining.
Prolonged CED with SN38 and irinotecan
Bevacizumab targeting diffuse intrinsic pontine glioma: results of $^{89}$Zr-bevacizumab PET-imaging in brain tumor models


* These authors contributed equally

Molecular Cancer Therapeutics, 2016 (9)15: 2166-2174
ABSTRACT

The role of the vascular endothelial growth factor (VEGF)-inhibitor bevacizumab in the treatment of diffuse intrinsic pontine glioma (DIPG) is unclear. We aim to study the biodistribution and uptake of zirconium-89 (\(^{89}\text{Zr}\))-labeled bevacizumab in DIPG mouse models.

Human E98FM, U251-FM glioma cells and HSJD-DIPG-007-FLUC primary DIPG cells were injected into the subcutis, pons, or striatum of nude mice. Tumor growth was monitored by bioluminescence imaging (BLI) and visualized by Magnetic Resonance Imaging (MRI). Seventy-two to 96 hours after \(^{89}\text{Zr}\)-bevacizumab injections, mice were imaged by Positron Emitting Tomography (PET) and biodistribution was analyzed \(ex\) \(vivo\).

High VEGF expression in human DIPG was confirmed in a publically available mRNA database, but no significant \(^{89}\text{Zr}\)-bevacizumab uptake could be detected in xenografts located in the pons and striatum at an early or late stage of the disease. The E98FM, and to a lesser extent the U251-FM and HSJD-DIPG-007 subcutaneous tumors, showed high accumulation of \(^{89}\text{Zr}\)-bevacizumab. VEGF expression could not be demonstrated in the intracranial tumors by in situ hybridization (ISH), but was clearly present in the perinecrotic regions of subcutaneous E98FM tumors.

The poor uptake of \(^{89}\text{Zr}\)-bevacizumab in xenografts located in the brain suggests that VEGF targeting with bevacizumab has limited efficacy for diffuse infiltrative parts of glial brain tumors in mice. Translating these results to the clinic would imply that treatment with bevacizumab in DIPG patients is only justified after targeting of VEGF has been demonstrated by \(^{89}\text{Zr}\)-bevacizumab immuno-PET. We aim to confirm this observation in a clinical PET study with DIPG patients.