Preclinical evaluation of convection-enhanced delivery with liposomal doxorubicin to treat pediatric diffuse intrinsic pontine glioma and thalamic high grade glioma.
ABSTRACT

Introduction and Aim
Pediatric high grade gliomas (pHGG) including diffuse intrinsic pontine gliomas (DIPG) are primary pediatric brain tumors that have a high mortality and morbidity. Because of too poor brain penetrance of therapeutic drugs, systemic chemotherapy regimens have failed to deliver satisfactory results and convection-enhanced delivery (CED) may be an alternative mode of drug delivery. Anthracyclines are potent chemotherapeutics that have been successfully delivered via CED in preclinical supratentorial glioma models. This study aims to assess the potency of anthracyclines against DIPG and pHGG cell lines in vitro, and to evaluate the efficacy of CED with anthracyclines in orthotopic pontine and thalamic tumor models.

Methods:
Sensitivity of primary pHGG cell lines to a range of anthracyclines was tested in vitro. Preclinical CED with free doxorubicin and pegylated liposomal doxorubicin (PLD) was performed in the brainstem and thalamus of naïve nude mice. Maximum tolerated dose (MTD) was determined by observation of clinical symptoms and brains were analyzed after H&E staining. Efficacy of the MTD was tested in adult glioma E98FM-DIPG and E98FM-thalamus models and in the HSJD-DIPG-007-Fluc primary DIPG model.

Results
Both pHGG and DIPG cells were sensitive to anthracyclines in vitro. Doxorubicin was selected for further preclinical evaluation. MTD of CED with free doxorubicin and PLD in the pons was 0.02 mg/ml and dose tolerated in the thalamus was ten times higher (0.2 mg/ml). 0.02 mg/ml free doxorubicin or PLD via CED was ineffective against E98FM-DIPG or HSJD-DIPG-007-Fluc in the brainstem but when applied in the thalamus, 0.2 mg/ml PLD slowed down tumor growth and increased survival in a subset of animals with small tumors.

Conclusions
Local delivery of doxorubicin causes severe toxicity when delivered to the brainstem, even at doxorubicin concentrations that are safe in the thalamus. As a consequence we could not establish a therapeutic window for treating orthotopic brainstem tumors in mice. For tumors in the thalamus, therapeutic concentrations could be reached to slow down tumor growth. These data suggest that anatomical location determines severity of toxicity after local delivery of therapeutic agents, and that caution should be used when translating data from supratentorial CED studies to treat infratentorial tumors.
INTRODUCTION AND AIM

Diffuse intrinsic pontine glioma (DIPG) and thalamic glioma are diffusely infiltrating midline gliomas, often harboring histone H3K27M mutations, that are most frequently observed in childhood. These tumors have a very high mortality and morbidity as effective treatment strategies still do not exist, despite significant progress in understanding the biological processes that play a role in the development and progression of these tumors. Tumor cells are highly resistant to chemo- and radiotherapy and the presence of the blood brain barrier (BBB) prevents drugs from reaching the bulk tumor and infiltrating tumor cells in sufficient concentrations.

None of the clinical trials that have been performed in DIPG and pHGG, using a large number of different chemotherapeutic agents, including cytostatic agents, targeted antibodies, and small molecule inhibitors, has yet shown a clear benefit that can be translated to standard clinical practice. Recently, preclinical research has identified pHGG and DIPG cell lines to be sensitive to a number of “classical” cytostatic agents, especially some anthracycline drugs. Anthracyclines are the cornerstones of many chemotherapeutic treatment regimens in a wide variety of cancer types in both adults and children. They are, despite their assumed value, associated with serious adverse events, including debilitating or even life threatening cardiotoxicity. Almost all anthracyclines have been identified as strong substrates of ATP-binding cassette transporters (ABC transporters) P-gP, MRP1 and BCRP causing limited brain penetration. The limited brain penetrance of anthracyclines in brain tumors with an intact BBB, such as diffuse gliomas, might also explain the lack of efficacy when used in the treatment of pHGG including DIPG.

Convection-enhanced delivery (CED) is a local delivery technique that is being considered a potential drug delivery strategy in DIPG and other brain tumors as it circumferences the BBB. Drug distribution is facilitated by a pressure gradient at the tip of the infusion catheter, resulting in a bulk flow through the interstitial spaces of the brain. However, choosing the right drug to administer via CED, is difficult: it should be effective against pHGG cells, have favorable chemical properties to enable adequate distribution, and give no-, or limited toxicity in healthy brain tissue. Using liposomal formulations of a drug can potentially improve distribution, bioavailability, biological half-life and efficacy after CED as suggested by several preclinical studies.
In this study we aimed to investigate the translational potential of using anthracyclines in the treatment of DIPG and pHGG using CED. Preclinical animal studies have already shown the potential for local delivery of anthracyclines to treat brain tumor models, but no study has investigated the administration of anthracyclines directly to the pons or thalamus.\textsuperscript{169,170} We studied the efficacy of different anthracyclines in vitro, and determined the feasibility of delivering anthracyclines to delicate brain areas such as the brainstem and thalamus. Subsequently we investigated the efficacy of nanoliposomal and free anthracyclines in treating orthotopic high grade tumors in the brainstem and thalamus \textit{in vivo}.

**MATERIALS & METHODS**

**TOPIIA expression in DIPG and pHGG**
Because TOPIIA expression is associated with good clinical response to treatment with anthracyclines in a number of cancer types\textsuperscript{171,172}, TOPIIA mRNA expression in diffuse intrinsic pontine glioma (DIPG) (n=27\textsuperscript{101}) and pediatric high grade glioma (pHGG) (n=53\textsuperscript{29}) was determined in silico, using publicly available datasets, and compared to a dataset of non-malignant brain tissue (n=44\textsuperscript{23}), low grade brainstem glioma (n= 6\textsuperscript{101}) and adult HGG (n=284\textsuperscript{24}). These datasets include tumor material from biopsy (adult and pHGG and DIPG), resection (adult and pHGG) and autopsy (DIPG). All expression analyses were performed using R2, a web-based microarray analysis and visualization platform (http://r2.amc.nl).

**Processing of Tumor Material and Cell Culture**
Single cell cultures were established from biopsy samples derived from pediatric glioblastoma multiforme, anaplastic astrocytoma, anaplastic oligodendroglioma and diffuse intrinsic pontine glioma. Informed consent was obtained according to institutionally-approved protocols. Tumor pieces were collected into DMEM (Dulbecco's Modified Eagles Medium, PAA Laboratories GmbH, Pasching, Austria) and washed twice with PBS to remove blood clots. Samples were sliced into small (3–5 mm$^3$) pieces and either mechanically dissociated by filtering through a cell strainer (BD Falcon Biosciences, Bedford, USA), or dissociated by incubation with Accutase (PAA Laboratories GmbH, Pasching, Austria). Single cells were seeded in DMEM-F12, constituted with stable glutamine, 10% fetal bovine serum (PerBio Science Nederland B.V., Etten-Leur, The Netherlands), 1% penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria), and 0.5% sodium pyruvate. For primary astrocytes 15% fetal bovine serum was used. Cells were grown at 37°C in a 5% CO$_2$ humidified atmosphere. Primary cell cultures
genetically characterized using karyotyping or array-CGH as previously described by Veringa et al.\textsuperscript{48} The primary HSJD-DIPG-007 cell line was established from DIPG tumor material obtained at Hospital Sant Joan de Déu (Barcelona, Spain) after autopsy from a 6-year-old patient and was confirmed to have a H3F3A (K27M) and ACVR1 (R206H) mutation\textsuperscript{61}. HSJD-DIPG-007-Fluc was cultured in serum free tumor stem medium (TSM) as previously described\textsuperscript{175}.

**Drug Treatment**

The primary pediatric glioma- and astrocyte cultures were exposed to different anthracycline drugs (idarubicin, epirubicin, mitoxantrone, doxorubicin and daunorubicin) at concentrations ranging from 0.01-1000nM. Fifteen hundred cells were seeded per well in 96-well tissue culture plates. After 96 hours, cell survival was assessed using the Acumen eX3 laser cytometer (TTO Labtech, UK), using 300 mM of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) as readout. Results were analyzed using Acumen Explorer software, calculating the survival percentage for each compound tested in the assay. Each experiment was performed at least four times.

**Drugs**

Clinical formulations of idarubicin, epirubicin and daunorubicin used for the in vitro drug screens were supplied by the department of pharmacology of the VU University Medical Center. Free- and pegylated liposomal doxorubicin (PLD) was supplied by 2-BBB Medicines BV (Leiden, The Netherlands). PLD was prepared according to the commercial Doxil/Caelyx preparation method, i.e. using active doxorubicin loading against an ammonium sulfate gradient, as previously described\textsuperscript{176}. Mean liposome size was 95 nm and contained 2 mg/ml doxorubicin; more than 90% of which was encapsulated in the liposomes. Liposomes were stored in liposome buffer (9.4% sucrose with histidine, 1.55 mg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands) at 4°C for no longer than three months after production. The vehicle was previously shown to be non-toxic in mice.\textsuperscript{176} For details on the stability of doxorubicin and its liposomal formulation we refer to previously published data by Gaillard and Barenholz\textsuperscript{176,177}

**Animals**

Animal experiments were performed in accordance with the Dutch law on animal experimentation and the protocol was approved by the committee on animal experimentation of the VU University Medical Center. Athymic Nude-Foxn1\textsuperscript{nu} mice (six
weeks old) were purchased from Harlan (Horst, The Netherlands), kept under filter top conditions and received food and water ad libitum.

Orthotopic DIPG mouse models
The E98 adult glioblastoma cell line, was transduced to express firefly luciferase (Fluc) and mCherry (E98FM cells) and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin and streptomycin. These cells were injected subcutaneously in female athymic nude mice (6-8 weeks of age) to expand the number of cells. When the subcutaneous tumor reached a diameter of 1 cm, the tumor was removed and a single cell suspension was prepared by mechanical disruption through 100 µm nylon cell strainer. HSJD-DIPG-007-Fluc cells were injected directly from culture after mechanical dissociation and counting. The cells were washed once with phosphate buffered saline (PBS) and concentrated to 1x10⁵ cells per µl (both E98FM and HSJD-DIPG-007-Fluc). Mice were stereotactically injected with 5x10⁵ cells in a final volume of 5 µL into either the pons (-1.0 mm X, -0.8 mm Y, 4.5 mm Z from the lambda) or thalamus (x: 1.5, y: -2 and z: -3.2 from bregma). Coordinates were based on "The mouse brain in stereotaxic coordinates" by Franklin and Paxinos and previously validated using injections with trypan blue (data not shown).

Convection-enhanced delivery in vivo
The CED procedure was performed as previously described by us using a stepped catheter specifically designed for this purpose (figure 2a). In vivo targeting of the brainstem was determined by infusion of trypan blue via CED (figure 2c) and an MRI was performed after infusion of 15 µL of gadolinium 5 µM (Dotarem, Guerbed figure 2d). MRI was performed on mice anesthetized with isoflurane inhalation anaesthesia (1.5 L O₂/minute and 2.5% isoflurane) using a preclinical PET-MRI system (Nanoscan system, MEDISO, Budapest, Hungary). T1 weighed images were acquired and analyzed using MIPAV software (Medical Image Processing, Analysis, and Visualization, version 7.2.0). To perform in vivo CED animals were injected with buprenorphine 0.05-1 mg/kg and anesthetized with isoflurane 2-3% in 100% oxygen. After placing the animals in a stereotactic frame on a heated platform (37°C) the CED catheter was introduced into the pons (figure 2b). The coordinates used for CED were the same as used for intracranial injections. During 30 minutes, a total of 15 µL of free doxorubicin, PLD, or vehicle was infused in the brain with a flow velocity of 0.5 µL /min. After the procedure, animals were returned to their cages to recover and resumed normal active behavior within 3-12 hours.
Toxicity study
CED toxicity (n=3) with 0.02 mg/ml (35 µM, pons) or 0.2 mg/ml (345µM, pons and thalamus) or 2 mg/ml (3448 µM, pons) doxorubicin or PLD was determined by clinical observations, including weight loss and clinical score. Clinical scores ranged from 0 to 4 and referred to 0: normal active behavior, 1: subtle inactivity or subtle neurological symptoms, 2: mild to moderate inactivity or neurological symptoms, 3: severe neurological symptoms, inactivity, loss of reflexes, inadequate grooming, 4: dead. Half point scores were assigned to mice that were behaving in between two scores. Endpoints due to toxicity were defined as weight loss more than 15%, severe neurological symptoms or severe inactivity. When no clinical endpoints were met, mice were sacrificed six weeks after CED to determine histological toxicity. MTD was selected on clinical features after treatment of three mice (no endpoint reached in all three animals, no clinical score > 2).

CED and IV efficacy studies
Start of treatment was determined by a rise in BLI signal, indicating tumor engraftment and growth. At day seven or eight after intracranial injection of tumor cells for the establishment of orthotopic brain tumors, mice were stratified on the basis of BLI signal intensities into different treatment groups. For the CED studies, animals harboring a pontine (E98FM-DIPG or HSJD-DIPG-007-Fluc) or thalamic (E98FM-Thalamus) tumor were assigned to receive CED with 0.02 mg/ml (35 µM) or 0.2 mg/ml (345µM) of free doxorubicin or PLD, or vehicle (NaCl 0.9%) (n=4/8 per treatment group). For the IV studies, mice were assigned to receive PLD (18 mg/kg, 1x/week, 2x) or vehicle (NaCl 0.9%) injected intravenously in the tail vein (n=3 per treatment group). Dosing was performed at previously described MTD \(^{180}\). Follow-up included daily observations and assignment of clinical scores, weight measurement as well as measurement of BLI signal twice a week (E98FM). Endpoints were defined as weight loss more than 15%, severe neurological symptoms or severe inactivity. Researchers were blinded as to which treatment group the mice belonged to. Mice were sacrificed via pentobarbital overdose. Brains were removed and fixed in 3.7% phosphate buffered saline (PBS) buffered formaldehyde. Differences in survival were analyzed by Kaplan-Meier curves and logrank tests for significance. Non-parametric Kruskal-Wallis test followed by a Dunn’s post-hoc test were used to determine differences in BLI signal. A \(p < 0.05\) was considered statistically significant.
Tissue staining and histological scoring
Haematoxylin and eosin (H&E) staining was performed on 4 μm formalin-fixed, paraffin-embedded tissue sections cut in the coronal plane, using a standard H&E protocol. To determine histological toxicity in non-tumor bearing animals, sections were selected at the site of the needle tract and 100 μm both rostrally and posteriorly. Two researchers (AS, TL) and an independent neuropathologist (PW) performed assessment of tissue damage and inflammation. They were blinded to the experimental procedure that the animals underwent.

RESULTS

Anthracyclines are promising drugs to treat pHGG and DIPG
TOPIIA was significantly overexpressed in pHGG and DIPG as compared to LG-BSG and normal brain (p < 0.01) (figure 1a-b). Cytotoxicity of clinically available anthracyclines against was tested in vitro against pHGG and DIPG primary cells and showed moderate to excellent sensitivity with IC50-values ranging from 1 μM to 10 pM (figure 1c-g). Doxorubicin showed to be particularly active against DIPG cells (figure 1g). Next, potency of doxorubicin was tested against Eg8FM cells, used to establish orthotopic Eg8FM-DIPG and Eg8FM-Thalamus models and normal human astrocytes, showing an excellent therapeutic window (figure 1h). For doxorubicin, used in subsequent in vivo experiments, IC50 values ranged from 1 nM in cell line VU-DIPG-A to 0.8 µM in VUMC-HGG-05. HSJD-DIPG-007-Fluc had an intermediate sensitivity profile (IC-50, 40 nM, supplemental figure 1).

“Clinical” toxicity of doxorubicin is determined by anatomical location
CED with a high dose (2 mg/ml) doxorubicin and PLD gave severe clinical toxicity when delivered to the pons (figure 3a). Severe symptoms occurred later after the CED procedure in the PLD treated animals (six days), compared to animals treated with free-doxorubicin (1-3 days) and difference in survival and weight loss was statistically significant (p<0.05, table 1, supplemental figure 1a,b). Eventually, all animals treated in the brainstem with high dose doxorubicin had to be sacrificed due to unacceptable toxicity (figure 3a). Symptoms consisted of weight loss (> 15%) and neurological deficits including paresis and loss of balance.
Preclinical CED with doxorubicin to treat DIPG

**Figure 1** | Expression of TOP2A in (A) adult GBM, pHGG, DIPG, LG-BSG and normal brain. TOP2A expression in (B) DIPG, LG-BSG and normal brainstem (individual cases plotted) as assessed by mining a publicly available database (R2.amc.nl). Sensitivity in pM (IC50) of pHGG and DIPG cell lines to (C) idarubicin (D) epirubicin (E) daunorubicin (F) mitoxantrone (G) and doxorubicin. Sensitivity of normal human astrocytes and E98FM cells to doxorubicin.

Severe clinical toxicity still occurred with CED to the pons at medium dose (0.2 mg/ml). All animals in the free-doxorubicin group had to be sacrificed. In the PLD group, one animal had to be sacrificed due to severe symptoms and one animal had clear neurological symptoms but remained active, with weight loss within acceptable limits (<15%), these neurological symptoms regressed after approximately three weeks (table 1, figure 2b).
None of the animals treated with low dose doxorubicin or PLD (0.02 mg/ml) to the pons showed significant clinical toxicity, illustrated by absence of clinical symptoms after CED (data not shown). In our experience, weight loss or inadequate weight gain of the animals is a sensitive symptom of toxicity, and all animals treated with 0.02 mg/ml showed normal weight gain after treatment (figure 2c). To find out what the maximum tolerable dose for injection of doxorubicin in the pons was, mice were treated with CED with doses of 0.1 and 0.04 mg/ml as well. These doses still caused intolerable symptoms that were beyond the criteria set for MTD (clinical endpoint reached > 1 animal, clinical score > 2, data not shown).

Toxicity of doxorubicin delivered via CED to the thalamus was significantly less pronounced. Medium dose (0.2 mg/ml) of free doxorubicin and PLD could be delivered to the thalamus without any clinical symptoms or weight loss (figure 2d). Due to the severe toxicity seen after treatment with 2 mg/ml doxorubicin to the pons, this dose level was not further assessed in the thalamus because of ethical considerations.

Histological toxicity of CED is related to dose and formulation of doxorubicin. After the mice were sacrificed, the brains were histologically analyzed. Animals were sacrificed six weeks after treatment unless the mice had to be sacrificed at an earlier time.
point due to unacceptable clinical symptoms (CED to the pons with free doxorubicin high and middle dose and PLD high dose, table 1).

After treatment with high dose PLD (2 mg/ml), in the pons tissues obtained six days after treatment consistently revealed a sharply demarcated lesion with incomplete necrosis, dispersed macrophages, and relative sparing of the microvessels (figure 4a). In animals treated with high dose free doxorubicin (2 mg/ml, 1-3 days after treatment) lesions were much less circumscribed and showed insipient necrosis of intervascular tissue and more widespread spongic changes of the neuropil (figure 4b).

**Figure 3** | Clinical symptoms of mice treated with CED with doxorubicin in the brainstem and thalamus. Clinical score (0-4) of mice treated with (A) high dose (2 mg/ml) or (B) intermediate dose (0.2 mg/ml) of free doxorubicin (red), PLD (purple) or vehicle (blue) in the brainstem. (C) Normalized weight gain of mice treated with vehicle (blue), free doxorubicin 0.02 mg/ml (red) or PLD 0.02 mg/ml (purple) in the brainstem. (D) Normalized weight gain of mice treated with vehicle (blue), free doxorubicin 0.2 mg/ml (red) or PLD 0.2 mg/ml (purple) in the thalamus.
In brains treated with medium dose doxorubicin to the brainstem (0.2 mg/ml), the tissue damage was more variable. In sharp contrast to the functional neurological deficits shown in these animals, treatment with 0.2 mg/ml free doxorubicin, histological abnormalities at 2-3 days after treatment were generally absent (figure 4c). After treatment with 0.2 mg/ml PLD (brains analyzed 3-6 weeks after CED) some focal gliosis and deposition of iron pigment was found.

**Figure 4** | H&E stainings of brain slices from the pontine area of mice treated with (A) PLD 2 mg/ml (B) free doxorubicin 2 mg/ml, (C) PLD 0.2 mg/ml or (D) free doxorubicin 0.02 mg/ml (black squares indicate enlarged areas) or H&E stainings from brain slices from the thalamic area treated with CED to the thalamus with intermediate dose (E&F) free doxorubicin (0.2 mg/ml), (G&H) PLD (0.2 mg/ml) or (I&J) vehicle. Scale bars represent 100 μm.
Six weeks after treatment with low dose free doxorubicin (0.02 mg/ml), the brainstem showed only focal areas with more coarse texture, consistent with astrogliosis (figure 4d), similar to what was found in some of the animals that were treated with low dose PLD or CED with vehicle only.

After six weeks of follow-up, brains treated with 0.2 mg/ml free doxorubicin to the thalamus showed clear toxicity characterized by a variably circumscribed area of tissue decay, pericellular thickening of the walls of microvessels, some iron pigment deposition (partly in macrophages) and some proliferation of (myo)fibroblasts (4e&f). Brains treated with PLD 0.2 mg/ml still showed similar but less pronounced lesions (figure 4h&g). No histological abnormalities were detected in animals treated with vehicle to the thalamus (figure 4i&j).

Table 1 | Range of days after CED that mice had to be sacrificed due to reaching of clinical endpoint or end of follow up

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<th>Location</th>
<th>Dose</th>
<th>Formulation</th>
<th>Sac after CED (days)</th>
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<td>Pons</td>
<td>High</td>
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<td>PLD</td>
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<td>Middle</td>
<td>Doxorubicin</td>
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<td>PLD</td>
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<td>Low</td>
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<td>Thalamus</td>
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E98FM cells form diffuse infiltrative high grade tumors in the pons and thalamus. Adult GBM derived E98FM cells have previously been described to grow as diffuse tumors in the pons with histological and clinical features quite similar to human DIPG 50. In order to study CED in diffuse high grade gliomas in the thalamus, we established thalamic tumors, using E98FM cells. To this end, we injected E98FM cells into previously validated thalamic coordinates and followed tumor growth in vivo using BLI. A subset of mice injected with E98FM to the thalamus and pons were sacrificed at set time points, these brains were analyzed to study size and histology of the tumors (figure 5 a-c, g-i). Others were followed until endpoint (figure 5 m- n). Histological assessment showed
diffuse infiltrative tumors located in the thalamus with tumor size proportionate to bioluminescence signal. Median survival of mice with E98FM-thalamus was 23.5 days (n=8, figure 5m) and 22 days in the pons (n=6, figure 5n).

Treatment of E98FM-DIPG and E98FM-thalamus with free doxorubicin or PLD
Both E98FM-DIPG, HSJD-DIPG-007-Fluc and E98FM-thalamus tumors were treated by CED with free-doxorubicin, PLD, or vehicle at the maximal tolerated dose as determined in the previous toxicity experiments. The CED catheter and schematic treatment schedule are depicted in figure 6a-b. Median survival of E98FM-DIPG and HSJD-DIPG-007-Fluc animals treated with free doxorubicin or PLD at low dose (0.02 mg/ml) did not differ significantly from animals treated with vehicle (figure 7a,g), and bioluminescence signal was not significantly different for any of the groups (figure 7b,h). Of note, one mouse with HSJD-DIPG-007-Fluc treated with PLD survived beyond the 90 days of follow up. In mice with E98FM-thalamus tumors treated with medium- dose (0.2 mg/ml), bioluminescence signal of E98FM-thalamus tumors was significantly lower in mice treated with PLD compared to mice treated with vehicle or free doxorubicin (figure 6d). Survival did not differ significantly for the whole group however, two out of eight animals treated with PLD had a prolonged decrease in BLI signal and prolonged survival (figure...
Treating mice with E98FM-DIPG tumors intravenously with PLD did not significantly influence clinical course (figure 6e) or bioluminescence signal (figure 7f).

**Tumor size influences efficacy in E98FM tumors treated with PLD via CED**

In the efficacy study, using E98FM-thalamus orthotopic tumors, we found a significant difference in bioluminescence signal in the first week after treatment with 0.2 mg/ml PLD, and noticed a survival benefit in a small proportion (2/8) of mice. To determine whether this survival benefit was due to a true treatment dependent decrease in tumor burden, or just a random occurrence we studied these animals in more detail. One noticeable difference in animals that responded to treatment was a relatively low bioluminescence signal at start of treatment. Even though median BLI signal was not significantly different between groups, there was a large variation in signal at start of treatment (figure 7 a,c, g). We studied response of each individual animal in relation to bioluminescence signal at start of treatment. Thereto we stratified mice into low- (< 10^6
photons/sec), medium- (<10^7 photons/sec) or high- (>10^7 photons per second) tumor burden at start of treatment.

By doing so, we identified that mice with Eg8FM-thalamus tumors with low tumor burden showed response (figure 7f) to treatment with PLD 0.2 mg/ml, while no responsive subgroup could be identified in the Eg8FM-DIPG or HSJD-DIPG-007-Fluc tumors treated either with CED (PLD or Dox, figure 7c, supplemental 3) or IV (figure 7i). In these mice, BLI signal rose exponentially without change, similar to vehicle treated animals. This suggests that tumor size at the start of treatment has substantial influence...
on efficacy of CED in the thalamus treated with PLD at maximal tolerated dose but not in tumors in the pons treated with CED at the MTD or intravenously.

**DISCUSSION**

Using in silico and in vitro experiments, we identified anthracyclines to be an interesting class of chemotherapeutics that could potentially be used for the treatment of DIPG and pHGG. TOPIIa, which was previously shown to be correlated with anthracycline efficacy in patients \(^\text{171,172}\), was highly expressed in both DIPG and pHGG compared to normal...
brain and normal brainstem. Furthermore, we show that pHGG and DIPG cells were sensitive to anthracyclines in vitro. The severity of toxicity after local delivery by CED however, limits effective treatment in vivo.

The method of action of anthracyclines is not fully elucidated but is thought to be multifactorial. The best-known effect of anthracycline drugs is inhibition of topoisomerase II (TOPII), which is necessary to avoid supercoiling of DNA in dividing cells. Inhibition of TOPII leads to double stranded breaks and subsequent cell death via apoptosis. Few clinical trials have been published treating children with pHGG, DIPG or other recurrent or progressive brain tumors with anthracyclines. Results have so far been variable. In one study, four out of eight included patients with recurrent or progressive pHGG responded with stable disease for a period of 9 to 48 weeks on a regimen of pegylated liposomal doxorubicin (PLD) and oral topotecan, but toxicity of this systemic treatment was high. In another study, children with recurrent or progressive brain tumors were treated with liposomal daunorubicin, that led to a treatment responds 6 out of 14 children with relatively mild toxicities. Interestingly, our in vitro data show that some DIPG cells (VU-DIPG-A) appeared to be ultra-sensitive for 4 out of 5 anthracyclines tested, with IC50 values well below those needed to treat all other cell lines in this panel and other glioma cell lines reported in literature. Only this particular cell line carries a mutation in histone gene H3F3A at lysine 27 (K27M) which can be found in approximately 60% of DIPG tumors. This mutation alters the organization of chromatin, by inability of EZH2 to trimethylate lysine 27. Absence of Lys 27 trimethylation causes a more open chromatin structure and leads to gross changes in expression profiles of various cell types. It was discovered recently that certain anthracyclines, including doxorubicin and daunorubicin, can cause histone eviction, especially in chromatin regions that have an absence in trimethylation at lysine 27. This mechanism could potentially add to the sensitivity of H3F3A-mutated DIPG cells to anthracyclines. Further experiments to elucidate the role of histone eviction are beyond the scope of this manuscript. Unfortunately the VU-DIPG-A cell line does not engraft after inoculation in the brain of mice, and therefore could not be used to perform the in vivo efficacy experiment. Therefore another H3F3A K27M mutated cell line (HSJD-DIPG-007-Fluc) was used, that was not part of our initial screen. This cell line was intermediately sensitive to doxorubicin in vitro, but no efficacy of low dose doxorubicin via CED could be established using our methods.

Despite the potential, the limited brain penetration of these compounds after systemic
delivery greatly limits their clinical use in the treatment of brain tumors. In this study we investigated the feasibility of using CED for local delivery of doxorubicin in the brainstem and thalamus. To our surprise, doxorubicin showed a MTD that was 100 times lower compared to what was previously described to be safe for local delivery in the rat striatum. In our hands, anatomical location clearly influenced clinical toxicity after CED. Tolerable dose when treating mice with CED of doxorubicin in the thalamus was ten times higher compared to the MTD in the brainstem. Meanwhile, histological analysis of brains after CED, showed similar tissue damage in the infused regions. Why this difference in toxicity occurs is not completely understood. We hypothesize that damage to the brainstem, including the pons is more likely to give functional deficits as compared to damage to structures in the thalamus, a phenomenon that is well known in human neurology. Of note, we only studied indirect distribution of doxorubicin by observing spread of histological toxicity. In theory it is possible that distribution of free doxorubicin or PLD differs between pons and thalamus, causing differences in clinical presentation after CED. In this study, we also did not investigate more subtle defects in performance in mice treated with moderately high dose doxorubicin in the thalamus. By doing so, we might have observed functional deficits that now escaped our attention using basic observations. The expected tolerable dose in the thalamus still differed 10 fold from safe concentrations delivered to the striatum of rats described in literature. Although toxicity after CED appears to be related to concentration and not total dose, this could still be explained by the use of a relatively large volume (15 µl) compared to CED studies in most studies using rats (average 20 µl). Furthermore, additional toxicity could be species or even strain related.

Liposomal formulation of doxorubicin gave rise to clinical symptoms in mice with a (much) longer time interval, but despite this lag time, free and liposomal doxorubicin had a similar MTD. Distribution of PLD was more sphere like, as illustrated by the circumscribed lesions found in the brains of high dose treated animals. High protein affinity of free doxorubicin (nearly 98%) could potentially lead to poor distribution, and PLD used in this study, should have a nearly ideal size (100 nm in diameter) for convection through the brain interstitial spaces. Presented data implies PLD indeed gives a more gradual release of doxorubicin and a wider area of distribution after CED. Both effects may enhance efficacy of CED in patients.

The low dose doxorubicin (MTD pons) was ineffective in treating a diffusely growing orthotopic DIPG model with a single delivery, suggesting no therapeutic window
could be reached and a one time infusion of doxorubicin via CED will most likely have no role in the treatment of DIPG. This result is contrasted by the data from our in vitro experiments, showing cytotoxicity at much lower in vitro IC50 values than delivered to tumors in vivo (up to a theoretical million fold difference) and a substantial difference in cell survival between E98FM and normal human astrocytes (figure 1h). The difference between in vitro found efficacy and lack of efficacy in vivo can be caused by inadequate coverage of the tumor due to inadequate distribution, or fast efflux of doxorubicin from the brain by efflux pumps causing a much lower area under the curve as compared to treatment in vitro. When using the ten fold higher dose (MTD thalamus), PLD was effective in a slowing down tumor growth in the thalamus. These findings suggest data from supratentorial CED studies cannot be translated directly to design trials to treat infratentorial tumors, and stresses the need for selective agents to avoid excessive toxicity to healthy surrounding tissue, especially to treat tumors in delicate areas of the brain. One preclinical study has already shown that infusing more targeted small molecule inhibitors dasatinib, everolimus and perifosine can be performed safely in the rat pons using long-term CED.

Using our methods, we were able to infuse a substantial area of the pons, but even when treating this area, CED was only effective when tumors were still very small. This is particularly problematic considering the small size of the murine compared to the human pons. Translating this knowledge to a clinical setting would imply only treating small, very early stage tumors. Since this is not the clinical reality of DIPG and thalamic HGG, CED will require drugs with a very high therapeutic index or long-term continuous infusions with lower concentration drugs. To achieve the latter, it will be necessary to apply more sophisticated techniques such as using multiple infusion catheters and computer modeling for targeted infusion or brain-penetrating nanoparticles with regulated release. These projects are currently ongoing, and results from clinical studies are eagerly awaited. To progress CED to an effective treatment strategy for pHGG including DIPG, will be a clinical, biological and technical challenge, requiring a comprehensive multidisciplinary approach.

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**Supplemental figure 1** | (A): Kaplan-Meier curve of naïve mice treated with 2 mg/ml free doxorubicin (red), PLD (purple) or vehicle (blue) (B) Normalized weight curves of naïve mice treated with 2 mg/ml free doxorubicin (red), PLD (purple) or vehicle (blue).

**Supplemental figure 2** | In vitro sensitivity of HSJD-DIPG-007-Fluc to doxorubicin

**Supplemental figure 3** | BLI data of HSJD-DIPG-007-Fluc treated with doxorubicin, PLD or vehicle plotted for each mouse individually and showing one long-term survivor treated with PLD.