Targeted Doxorubicin-Loaded Bacterially Derived Nano-Cells for the Treatment of Neuroblastoma

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Abstract

Advanced stage neuroblastoma is an aggressive disease with limited treatment options for patients with drug-resistant tumors. Targeted delivery of chemotherapy for pediatric cancers offers promise to improve treatment efficacy and reduce toxicity associated with systemic chemotherapy. The EnGeneIC Dream Vector (EDVTM) is a nanocell, which can package chemotherapeutic drugs and target tumors via attachment of bispecific proteins to the surface of the nanocell. Phase I trials in adults with refractory tumors have shown an acceptable safety profile. Herein we investigated the activity of EGFR-targeted and doxorubicin-loaded EDVTM (EGFREDVTM) for the treatment of neuroblastoma. Two independent neuroblastoma cell lines with variable expression of EGFR protein [SK-N-BE(2), high; SH-SY-5Y, low] were used. EGFREDVTM induced apoptosis in these cells compared to control, doxorubicin, or non-doxorubicin loaded EDVTM. In three-dimensional tumor spheroids, imaging and fluorescence lifetime microscopy revealed that EGFREDVTM had a marked enhancement of doxorubicin penetration compared to doxorubicin alone, and improved penetration compared to non-EGFR-targeted EDVTM with enhanced spheroid penetration leading to increased apoptosis. In two independent orthotopic human neuroblastoma xenograft models, short-term studies (28 days) of tumor-bearing mice led to a significant decrease in tumor size in EGFREDVTM-treated animals compared to control, doxorubicin, or non-EGFR EDVTM. There was increased TUNEL staining of tumors at day 28 compared to control, doxorubicin, or non-EGFR EDVTM. Moreover, overall survival was increased in neuroblastoma mice treated with EGFREDVTM (P < 0.007) compared to control. Drug-loaded bispecific-antibody targeted EDVTM offer a highly promising approach for the treatment of aggressive pediatric malignancies such as neuroblastoma.

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Introduction

Neuroblastoma is a childhood tumor that arises from the neural crest cells of the sympathetic nervous system and accounts for 6% to 10% of deaths from childhood cancer (1). Children with neuroblastoma frequently present with advanced stage disease. Intensive therapeutic regimens for children with advanced neuroblastoma include high-dose chemotherapy and bone marrow transplantation. Chemotherapeutic agents are given systemically such as liposomal carriers or nanoparticle-based delivery vehicles. Another approach to engineered material delivery is to use naturally-derived nano-sized structures from biological systems as therapeutic response, biodistribution, and reduce toxicity to normal tissues in the body (3). Convergence of engineering, chemistry, and biology has led to the development of engineered materials such as liposomal carriers or nanoparticle-based delivery vehicles. Another approach to engineered material delivery is to use naturally-derived nano-sized structures from biological systems including exosomes, vesicles, and bacterial membranes (4, 5). EnGeneIC Ltd. (Sydney, Australia) has developed EDVTM-nanocells (EDVTM), bacterially-derived delivery system consisting of nonviable nanocells that are 400 ± 20 nm in diameter by reactivating polar sites of cell division in bacteria (6). The EDVTM can be loaded with chemotherapeutic agents, siRNA or miRNA. Furthermore, bispecific antibodies can be attached to the surface of the EDVs allowing the EDVs to target-specific tumor cell antigens (6). The EDVs are well tolerated with earlier safety and toxicity studies in both pigs and dogs displaying only a mild, transient inflammatory response to repeated dosing.
with targeted EDVTM loaded with doxorubicin or other toxic payloads (6, 7).

In a first-in-human study, epidermal growth factor receptor (EGFR) targeted EDVTM nanocells loaded with paclitaxel were tested for safety in patients with solid tumors (8). Twenty-two of 28 enrolled patients completed the trial with six patients being withdrawn due to progressive disease with the most common treatment-related side effects being rigors and pyrexia (8). A recent phase 1 trial of EGFR targeted EDVTM nanocells loaded with doxorubicin in adults with recurrent EGFR-expressing glioblastoma was also well tolerated, with no dose limiting toxicities or withdrawals from the study due to adverse effects (9). To date, the EDVTM nanocells have not been tested for efficacy against solid pediatric tumors.

In this study we investigated the ability of EGFR targeted EDVTM to target and deliver a chemotherapy drug to neuroblastoma cells and induce cell death in vitro. Moreover, we investigated the ability of the chemotherapy drug-loaded EGFR-EDVTM to reduce tumor growth and prolong survival in an orthotopic mouse xenograft model of neuroblastoma.

Methods and Materials

EnGeneIC Dream Vector (EDVTM)

EDVTM were produced and purified from a Salmonella enterica serovar Typhimurium (S. Typhimurium) minCDE-strain as previously described (6). Doxorubicin (Teva Pharmaceuticals) loading, EGFR targeting, lyophilization, and dose preparation have also been previously described (6). Briefly, lyophilized vials were first rehydrated with a trehalose solution, followed by dilution with injectable saline to the desired concentration for loading, EGFR targeting, lyophilization, and dose preparation. Dose preparation with Dox, EGFREDVTM, EDVTM, or free Dox was added at an equivalent concentration of 1 µmol/L and incubated with spheroids for 1, 4, or 24 hours before imaging. Two types of confocal microscopy—Zeiss LSM 780 and Leica TCS SP5—were utilized to acquire images, both equipped with an environmental chamber which controls the atmospheric conditions, humidity, and temperature for live-cell imaging. Spheroids were imaged using the 10 × 0.45 NA and 20 × 0.8 NA air objectives. Analysis of spheroid penetration was done in Image J. Images acquired 100 µm from the bottom of the spheroid were converted to surface plots and a 1D fluorescence intensity profile was taken by drawing a across the plot from edge to edge. Three to six spheroids were analyzed per treatment per time point and averaged to produce the penetration depth profile.

Scanning electron microscopy

Spheroids with an average diameter of ~400 to 500 µm after 4 days growth were used for nanoparticle penetration studies via confocal microscopy and FLIM experiments. A total of 1 × 106 EDVTM, EGFREDVTM, or free Dox was added at an equivalent concentration of 1 µmol/L and incubated with spheroids for 4, 1, or 24 hours before imaging. Two types of confocal microscopes—Zeiss LSM 780 and Leica TCS SP5—were utilized to acquire images, both equipped with an environmental chamber which controls the atmospheric conditions, humidity, and temperature for live-cell imaging. Spheroids were imaged using the 10 × 0.45 NA and 20 × 0.8 NA air objectives. Analysis of spheroid penetration was done in Image J. Images acquired 100 µm from the bottom of the spheroid were converted to surface plots and a 1D fluorescence intensity profile was taken by drawing a across the plot from edge to edge. Three to six spheroids were analyzed per treatment per time point and averaged to produce the penetration depth profile.

Spheroid formation and viability assay

Neuroblastoma tumor spheroids with an average diameter of 500 to 700 µm were generated in 96-well round-bottom plates (Sigma-Aldrich) as previously described by our laboratory (14). After 4 days growth, spheroids were treated with EDVTM, Dox or EGFREDVTM, EDVTM, or Doxorubicin (Pfizer; 0.2 µmol/L) (Dox) for 72 hours. Spheroids were then manually disrupted and whole cell lysates prepared. PARP cleavage was measured by Western blotting to assess viability.

Annexin V staining and flow

SH-SY-5Y and SK-N-BE(2) tumor spheroids with an average diameter of ~400 to 600 µm after 4 days growth were used for assessment of early and late apoptosis. Spheroids were treated with Dox, EGFREDVTM, EDVTM, or free EDVTM at 0.2 µmol/L doxorubicin. At 24 hours posttreatment, spheroids were disaggregated with trypsin and stained with the Annexin V-FITC Apoptosis Detection Kit (Sigma). Flow cytometric analysis was performed on a Gallios flow cytometer (Beckman Coulter) and analyzed using the FlowJo software package (FlowJo, LLC, and Illumina, Inc.).

Western blotting

Western blot analyses on whole cell lysates were performed using the following antibodies as described previously (12, 13): cleaved PARP (Asp214) (D64E10) rabbit monoclonal antibody (Cell Signaling Technology), EGF receptor (D38B1) rabbit monoclonal antibody (Cell Signaling Technology), and GAPDH (clone 6C5; Abcam).

EDV binding

The ability of SH-SY-5Y and SK-N-BE(2) to bind effectively to EGFR targeted EDVTM was assessed via flow cytometric analysis on a Gallios flow cytometer (Beckman Coulter). Briefly, cells were collected with Versene to preserve cell surface receptors. EDVTM and EGFREDVTM were added to the cells at a ratio of 10,000 EDVTM per cell and incubated for 2 hours in suspension with rotation at 4°C. Cells were then washed thoroughly to remove excess unbound EDVTM before fixation in 4% PFA. EDVTM were labeled with an anti-LPS Alexa Fluor 488 antibody (Thermo Fisher) for detection by flow cytometry.

Confocal microscopy

Spheroids with an average diameter of ~400 to 500 µm after 4 days growth were used for nanoparticle penetration studies via confocal microscopy and FLIM experiments. A total of 1 × 106 EDVTM, EGFREDVTM, or free EDVTM was added at an equivalent concentration of 1 µmol/L and incubated with spheroids for 1, 4, or 24 hours before imaging. Two types of confocal microscopes—Zeiss LSM 780 and Leica TCS SP5—were utilized to acquire images, both equipped with an environmental chamber which controls the atmospheric conditions, humidity, and temperature for live-cell imaging. Spheroids were imaged using the 10 × 0.45 NA and 20 × 0.8 NA air objectives. Analysis of spheroid penetration was done in Image J. Images acquired 100 µm from the bottom of the spheroid were converted to surface plots and a 1D fluorescence intensity profile was taken by drawing a across the plot from edge to edge. Three to six spheroids were analyzed per treatment per time point and averaged to produce the penetration depth profile.
repetition rate of 40 MHz. Fluorescence emission was detected 550 nm long-pass filter using a single-photon avalanche diode (SPAD) and PicoHarp300 TCSPC electronics. Fluorescein was used to calibrate the phasor plot to a monoexponential lifetime of 4 nanoseconds. Phasor analysis was performed using simFCS (developed by Enrico Gratton, Laboratory of Fluorescence Dynamics, Irvine, CA).

Orthotopic neuroblastoma model
SH-SY-5Y/TGL or SK-N-BE(2)/TGL cells 
(2 × 10⁶) were injected orthotopically into the adrenal fat pad of 6- to 8-week-old male SCID-Beige mice as described with minor modifications (10, 11). All animal experiments were approved by the Animal Ethics Committee, UNSW Australia (ACEC number 12/68). Briefly, mice were anesthetized using gaseous isoflurane. A small incision was made near the left adrenal gland for retroperitoneal access to the adrenal fat pad, and neuroblastoma cells suspended in 20 μL of Cultrex basement membrane extract (Bio Scientific) was injected. Tumors were left to form for 7 to 14 days post cell implantation with growth monitored once weekly using the Xenogen bioluminescence imaging system. Once a luminescent signal was detectable (minimum total flux 1 × 10⁹ photons/sec for SK-N-BE(2) and 1 × 10⁸ photons/sec for SH-SY-5Y; defined as Day 0), mice were randomized into groups and treatment was commenced the next day. Mice were treated via intravenous tail vein injection twice weekly with one of the following treatments: saline, 2 mg/kg doxorubicin (Dox; Hospira Australia), 2 mg/kg liposomal doxorubicin (Lipo Dox; Sun Pharmaceuticals) or 5 × 10⁷ to 1 × 10⁸ EngeneIC Dream Vector (EDVTM). EDVTM were prepared and formulated as previously described (4) as EDVTM with EGFR targeting antibodies (EGFR/EDVTM). EDVs loaded with doxorubicin (EDVTM_Dox) and EGFR targeted EDVs loaded with doxorubicin (EGFR/EDVTM_Dox). For short-term studies, mice were treated for 4 weeks. At the end of the fourth week (Day 28), mice were humanely killed and primary tumors were harvested and measured using digital calipers and tumor volume calculated as \( \frac{\pi}{6} \times \text{length} \times \text{width} \times \text{height} \). Tumors were fixed in 10% formalin and embedded in paraffin for routine histological analysis (hematoxylin and eosin staining) and/or TUNEL staining. Tumor viability was determined by a light microscopic examination of hematoxylin and eosin stained sections of tumor by a practicing Paediatric Pathologist. The extent of tumor necrosis and hemorrhage was visually estimated, with the remainder of the tumor regarded viable, that is, viable tumor (%) = 100 − (necrosis + hemorrhage). For long-term studies, xenogen signal and tumor size by palpation was used as the endpoint for determining “survival.” When the Xenogen signal was close to or over 1.5 × 10⁶ photons/seconds, tumors were monitored closely by palpation and once tumors palpated to ~1 cm³, mice were humanely killed. Tumors were then harvested, measured via calipers, fixed in formalin, and embedded in paraffin for routine histologic analysis as described above.

Statistical analysis
All statistical analysis was performed using the GraphPad Prism software package. Data are represented as mean ± SD or SEM. Statistical significance between groups was determined by a one way ANOVA, followed by the Tukey’s multiple comparison test. IC₅₀ was determined via regression analysis. Survival curves were analyzed using the Log-rank (Mantel Cox) test. For all tests, \( P \) values were as follows: *, \( P \leq 0.05; **, P \leq 0.01; ***, P \leq 0.001, \text{ and } ****, P \leq 0.0001.

Results
EGFR protein expression in neuroblastoma cells
EDVTM were designed to target the EGFR protein known to be present on the surface of a large number of tumor cells. In order to determine if cell surface EGFR protein levels were sufficiently high to mediate the therapeutic efficacy of the EDVTM, a panel of neuroblastoma cell lines were assessed for EGFR protein levels by Western blot analysis (Fig. 1A). The cell lines examined expressed EGFR protein at variable levels and as such, a high expressing EGFR cell line [SK-N-BE(2)] and a low expressing EGFR cell line (SH-SY-5Y) were selected for all subsequent studies. The ability of the EGFR targeted EDVTM to effectively bind to cell lines with differing levels of EGFR was then assessed (Fig. 1B). Both the low expressing and high expressing cell lines were equally capable of binding EGFR/EDVTM with minimal nonspecific binding of nontargeted EDVTM.

Efficacy of doxorubicin loaded EDVTM in neuroblastoma two-dimensional and three-dimensional models
Initial viability studies examined the efficacy of both targeted and nontargeted doxorubicin (Dox) loaded EDVTM in two-dimensional (2D) monolayer cell cultures of both SK-N-BE(2) and SH-SY-5Y as well as normal MRC-5 fibroblasts (Supplementary Fig. S1A). EDVTM delivering the same Dox concentration as free Dox (Dox) showed similar efficacy against all cell lines in 2D-cultures with Dox-treated monolayers displaying an increase in cleaved PARP levels over EDVTM-treated samples as shown in Western blots from SH-SY-5Y cells (Supplementary Fig. S1B). Because of the kinetics of EDVTM uptake and drug release, efficacy in 2D was in vitro cell culture models possesses little correlation to in vivo efficacy. However, in three-dimensional (3D) neuroblastoma spheroids, which more closely mimic in vivo tumors, EDVTM and EGFR/EDVTM_Dox treated spheroids showed both a larger increase in cleaved PARP and apoptosis than those treated with free Dox at the same concentration in both SK-N-BE(2) and SH-SY-5Y (Fig. 2; Supplementary Fig. S2). Densitometric analysis of cleaved PARP demonstrated a significant increase in cleaved PARP as compared to both nonloaded EGFR/EDVTM and free Doxorubicin in SK-N-BE(2) spheroids and as compared to all other treatments in SH-SY-5Y spheroids (Fig. 2A) and in fact, both EDVTM formulations displayed an increase in cleaved PARP expression as compared to free Dox at twice the concentration in SK-N-BE(2) spheroids (Supplementary Fig. S2). The efficacy data were further supported by Annexin/PI staining on SK-N-BE(2) and SH-SY-5Y tumor spheroids treated with Dox loaded EDVTM and EGFR/EDVTM_Dox treatment in SK-N-BE(2) spheroids resulted in 49.1% total apoptosis (early + late) versus 41.4% for the EDVTM_Dox treated spheroids and 32.7% for the Dox-treated spheroids. In the SH-SY-5Y, EGFR/EDVTM_Dox treatment resulted in 38.3% total apoptosis as compared to 36.6% for the EDVTM_Dox-treated spheroids and 22.8% for the Dox-treated sample. Figure 2B shows flow density plots and quantitation of the SH-SY-5Y cells in which more extensive early apoptosis is occurring in the spheroids treated with the EDV loaded samples as compared to the same amount of free Dox.
Penetration of EDVTM into neuroblastoma tumor spheroids

To further explore the reason for the increase in efficacy observed in 3D-spheroid cultures, drug diffusion studies were conducted via confocal microscopy in order to visualize penetration of EDV’s into SK-N-BE(2) tumor spheroids (Fig. 3A). Consistent with a previous study (14), Dox can only penetrate a few cell diameters into the spheroid (maximum penetration ∼50 μm; Fig. 3A and B). In contrast, within 1 hour exposure to either EGFREDVTM-Dox or EDVTM-Dox, drug penetration had already progressed 50 μm from the edge of the spheroid, with full penetration of a 400 μm spheroid after only 4 hours (Fig. 3A and B). Penetration studies were carried out to 24 hours (Fig. 3B) with no change in penetration profiles after 4 hours. Given the large size of the EDVTM (400 nm), this spheroid penetrating ability of large particles is unexpected. As such, to determine if EDVTM penetrate in an intact state, fluorescence imaging microscopy (FLIM) was employed at 1 and 4 hours post exposure to Dox or EDVTM (Fig. 3C). The fluorescence lifetime of Dox increases when Dox is associated or encapsulated in a nanoparticle. As such, FLIM measurements on 3D-spheroid cultures could be used to determine whether the Dox detected was still particle associated or had been released. In this case, the phasor analysis was used. The phasor plot (15) provides a model free assessment of the fluorescence decays, which overcomes some of the problems of exponential fitting fluorescence decays. In FLIM images, the fluorescence decay is fitted at every pixel. In phasor analysis, data are converted into the frequency domain, with fluorescence decays from each pixel represented as sine and cosine Fourier transforms and plotted in a polar plot (15). All single exponential lifetimes lie on the universal circle while multi-exponential lifetimes are a linear combination of their components (Fig. 3C, left). The phasor plot has been color coded for ease of interpretation, with nuclear associated free Dox colored pink. In the case of free Dox, the majority of detectable Dox is present in the nucleus after 4 hours. In contrast, EGFREDVTM-Dox has released its Dox payload in the cytoplasm (green) with some nuclear localization (pink) only at the edge of the spheroid. The EGFREDVTM-Dox towards the center of the spheroid has the longest lifetime (red) indicating that EDVTM penetrate the spheroids fully intact, with a somewhat delayed release profile towards the center of the spheroid. A similar FLIM penetration and release profile was seen for the nontargeted EDVTM-Dox (Supplementary Fig. S3).

In vivo efficacy of targeted EDVTM in an orthotopic neuroblastoma model with high EGFR protein levels

Having demonstrated the in vitro efficacy of EGFREDVTM-Dox, we investigated the potential of this delivery agent in vivo. Recently we described the establishment and validation of a luciferase-expressing metastatic neuroblastoma orthotopic mouse model (10, 11) and used this model in this study to investigate activity of EGFREDVTM-Dox. SK-N-BE(2)/TGL cells were injected directly into the left adrenal fat pad of male SCID-Beige mice to induce a primary orthotopic neuroblastoma tumor (n = 4–5 mice/group). Mice were treated twice weekly for 4 weeks with either 1 × 10⁵ (containing ∼500 ng Dox) EDVTM-Dox or EGFREDVTM-Dox, 2.5 mg/kg Dox (which corresponds to 50 μg of Dox for a 20 g mouse), or saline and monitored weekly via Xenogen bioluminescence imaging. EDVTM treatment was well tolerated and resulted in minimal weight loss throughout the course of the experiment, whereas mice receiving Dox showed excessive weight loss in week 3, at which point dosage was decreased to 2 mg/kg (Supplementary Fig. S6). A significant reduction in tumor growth and/or viability as determined via bioluminescence was seen in the EGFREDVTM-Dox treated group when compared with all other treatments by day 28 (Fig. 4A and C). Similarly, final tumor measurements of harvested tumors demonstrated a significant decrease in tumor volume of the EGFREDVTM-Dox-treated group as compared to the other treatment groups, whereas both Dox and EDVTM-Dox-treated groups
Figure 2. Effect of \textit{EDVTM\textsubscript{Dox}} on induction of cell death of neuroblastoma cells. A, Representative Western blots showing cleaved PARP expression following treatment of SK-N-BE(2) or SH-SY-5Y tumor spheroids with either control (cells only, no treatment), \textit{EDVTM\textsubscript{Dox}} or \textit{Dox}. GAPDH was included as a protein loading control. B, Apoptosis as determined via Annexin V/PI staining in tumor spheroids of SK-N-BE(2) and SH-SY-5Y treated with \textit{EDVTM\textsubscript{Dox}}, \textit{Dox} or \textit{EDVTM\textsubscript{Dox}} and “cells only” were included as a treatment free sample. Representative flow density plots shown for SH-SY-5Y (n = 3 independent experiments).
exhibited final tumor sizes, which were significantly smaller than control mice (Fig. 4B). Untargeted EDVTM-Dox can be expected to have some effect on tumor growth as it will passively deposit in the tumor via the enhanced permeability and retention effect followed by a small amount of nonspecific uptake related to the close proximity to cells in the tumor microenvironment, whereas EGFR targeting will greatly enhance this uptake. Furthermore, although free Dox can be an effective agent in the treatment of
neuroblastoma, it is important to note that the concentration of Dox delivered via EDV\textsuperscript{TM} is approximately 100-fold less than the free Dox dose. Combined, these results demonstrate the effectiveness of EGFREDVTM Dox in reducing primary tumor growth in orthotopic neuroblastoma tumors with high EGFR levels (Fig. 4A–C).

In vivo efficacy of targeted EDV\textsuperscript{TM} in an orthotopic neuroblastoma model with low EGFR protein levels

Efficacy in a second neuroblastoma in vivo mouse model (SH-SY-5Y/TGL) with low levels of EGFR protein was also assessed to determine if the levels of EGFR protein were critical to the success of EGFR-targeted EDVs. As demonstrated in the EDV flow cytometry experiments (Fig. 1B), high EGFR levels were in fact not necessary for effective targeted EDV binding and merely the presence of even low levels of EGFR was sufficient, and we investigated whether this would be reflected in vivo.

Because SH-SY-5Y tumors exhibit a slower growth profile than SK-N-BE(2) tumors, an initial dose–response study was undertaken to assess the efficacy of \(5 \times 10^7\) EGFREDVTM\textsubscript{Dox} as compared with \(1 \times 10^8\) EGFREDVTM\textsubscript{Dox} in order to determine if a lower dose could produce a similar response. As shown in Supplementary Fig. S4A to S4C, the lower dose demonstrated a similar response in terms of reduced tumor growth with less weight loss as compared to those receiving a higher dose. As such, the short-term efficacy study in SH-SY-5Y tumor bearing mice was conducted at the reduced EDV\textsuperscript{TM} dosage. For the short-term efficacy study, orthotopic SH-SY-5Y tumors were induced in male SCID-Beige mice (\(N = 5–6\) mice/group). Mice were treated twice weekly for 4 weeks with either \(5 \times 10^8\) (containing \(~250\ ng\ Dox\) EDV\textsuperscript{TM} \textsubscript{Dox} or \(E\)GFREDVTM\textsubscript{Dox} 2 mg/kg Dox, or saline and monitored weekly via Xenogen bioluminescence imaging. The concentration of Dox was decreased from 2.5 to 2 mg/kg for this and all subsequent studies due to toxicity observed in the initial study as evidenced by weight loss, fur

Figure 4.

In vivo efficacy of targeted EDV\textsuperscript{TM} in an orthotopic neuroblastoma mouse model with high EGFR protein levels. Mice bearing orthotopic SK-N-BE(2)-Luc neuroblastoma tumors were treated with control (saline), EGFREDVTM\textsubscript{Dox}, EDV\textsuperscript{TM}\textsubscript{Dox} or Dox twice weekly for 4 weeks. Quantitation of bioluminescence emitted from the whole-body of mice from each group was measured. Data represent the mean total flux (photons/second) ±SEM (error bars) for each group \((n = 4–5\text{/group})\). \(\ast\, P < 0.05; \ast\ast, P < 0.01\), taken every 7 days up to 28 days. A, At the end of the 28 days, the tumors were excised and measured using calipers and expressed as size in mm\(^3\). B, Representative pseudocolor images of mice bearing orthotopic SK-N-BE(2)-Luc neuroblastoma tumors at the end of the study at 28 days. C, Colored scale bar represents the degree of bioluminescence emitted, as measured in photons/second/centimeter squared/steradian \((\mu\text{sec/cm}^2/\text{sr})\). Arrows indicate dosing days.
ruffling, lethargy, and hunched posture which developed in most Dox-treated mice by the 3rd week of that study. In addition, for comparison to a lipid-based nanoparticle doxorubicin formulation, an additional group of mice was treated with a clinical liposomal doxorubicin formulation (Lipo-Dox). Lipo-Dox was delivered at a therapeutic concentration (2 mg/kg, corresponding to 40 μg for a 20 g mouse). It should be noted that at this dosage, the concentration of Dox in the Lipo-Dox formulation would be more than 100-fold higher than what is delivered in a 5 × 10⁹ dose of EGFREDVTM (Dox) EDVTM treatment was well tolerated and resulted in less than 5% weight loss throughout the course of the experiment, mice receiving Lipo-Dox showed ~2% reduction in weight, whereas mice receiving Dox showed ~7.5% weight loss (Supplementary Fig. S4B). A significant reduction in tumor growth as determined via bioluminescence was observed in the EGFREDVTM (Dox)-treated group when compared to all other treatments with the exception of the Lipo-Dox by day 28 (Fig. 5A). In addition, tumor sizes at the termination of the experiment demonstrated a significant decrease in tumor volume in the EGFREDVTM (Dox)-treated group compared when to saline and EGFREDVTM-treated tumors (Fig. 5B).

SH-SY-5Y tumors were sectioned and stained with hematoxylin and eosin (H&E, Fig. 5C) or Tunnel (Fig. 5D). Histologic examination was undertaken in the EGFREDVTM (Dox)-treated SH-SY-5Y tumors from the short-term study and demonstrated features consistent with undifferentiated neuroblastoma. The tumor cells contained intermediate-sized hyperchromatic nuclei with nucleoli that were less conspicuous than those observed in SK-N- BE(2) xenografts (Fig. 6). The morphologic appearance of the treatment effect comprised variably sized zones of coagulative necrosis with minimal hemorrhage and pigmented histiocytes were observed in some tumors (Fig. 5C). The greatest degree of treatment response, was seen in the EDVTM (Dox) mice, where 80% to 85% viable tumor remained following treatment. Essentially, no cell death was seen by H&E in either the EGFREDVTM (Dox) or EGFREDVTM, where 98% to 99% of viable tumor remained. A similar response was evident in the Lipo-Dox, saline control, and doxorubicin alone treated mice, which demonstrated 93% to 95% viable tumor following treatment (Table 1). It is important to note that despite the lack of cell death visible in H&E sections, when the apoptotic-specific TUNEL staining was used an increase in the percentage of apoptotic cells was observed. Combining this finding with the significant reduction in tumor size and luminescent signal in the EGFREDVTM (Dox)-treated mice indicates that this treatment exhibited the most effective antitumor response.

As mentioned above, TUNEL staining of primary tumors showed a notable increase in apoptotic cells in EGFREDVTM (Dox) treated tumors in comparison to all other treatments (Fig. 5D, additional fields of view Supplementary Fig. S5). Little to no apoptosis was observed in the control, EGFREDVTM, or Dox-treated tumors, with a small amount visible in EDVTM (Dox)-treated tumors. In contrast, areas of apoptosis indicated by positive TUNEL staining were observed in Lipo-Dox–treated tumors, whereas large areas of the EGFREDVTM (Dox)-treated tumors displayed positive TUNEL staining. Collectively, the reduction in tumor growth combined with the large degree of apoptosis within EGFREDVTM (Dox)-treated tumors demonstrates a high degree of efficacy for EGF-targeted EDVTM even in neuroblastoma tumors expressing low levels of EGFR.

To determine whether the short-term study on tumor growth was reflected in overall survival, survival studies were conducted. SK-N-BE(2)-Luc tumors were established in a new cohort of mice (n = 8–10/group). As mice with neuroblastoma tumors are generally asymptomatic until tumors are extremely large, palpation to 1 cm³ and mouse wellbeing were used to determine "survival" endpoint in this model. Mice were again treated twice weekly with either Saline, 2 mg/kg Dox, or 1 × 10⁹ EDVTM with the following formulations: EDVTM (Dox), EGFREDVTM (Dox), or EGFREDVTM (an EGF targeted EDV with no payload). Changes in mouse weights (Supplementary Fig. S6) during the treatments were comparable to the two short-term in vivo studies. Mice treated with EGFREDVTM (Dox) demonstrated a significant increase in survival time compared to all other treatments (Fig. 6A; Table 2). In fact, on day 32, 7/9 mice in the EGFREDVTM (Dox) group were still alive whereas only 1 mouse in the EDVTM (Dox) group survived until day 32, and no mice were alive in any other group at day 32. Furthermore, 5/9 mice in the EGFREDVTM (Dox) treatment group survived to day 36, and 2/9 survived to day 46. As shown in Fig. 6B, tumors harvested at the survival "endpoint" had an average volume of 1 cm³ for each treatment group indicating no biased in palpation of tumors. On histologic examination of SK-N-BE(2)-Luc xenografts from the long-term survival study demonstrated features consistent with poorly-differentiated neuroblastoma (Fig. 6C). The tumor cells were intermediate to large in size and contained single to multiple prominent cherry-red nucleoli, consistent with the known MYCN amplification status of SK-N-BE(2)-Luc cells. The morphologic appearance of the treatment effect comprised variably sized zones of coagulative necrosis with minimal hemorrhage. Viable tumor was preferentially observed surrounding blood vessels. Hemosiderin-laden macrophages were present in all tumors, indicative of prior hemorrhage. Despite final tumor measurements being similar in all treatment groups, histologic analysis of tumor sections indicated that EGFREDVTM (Dox) showed the greatest response with only 30% viable tumor remaining following treatment. A moderate response was evident in the tumor treated with nontargeted EDVTM (Dox) where 65% viable tumor remained. In contrast, minimal response was seen in either the EGFREDVTM or doxorubicin alone treated tumors, where ≥90% of viable tumor remained (Table 1).

**Discussion**

The majority of patients with neuroblastoma have advanced disease at time of diagnosis. This necessitates the use of aggressive treatment approaches including surgery and high dose chemotherapy (16). Toxicity to normal tissues associated with chemotherapy can be dose limiting and potentially life threatening. Although survivors of neuroblastoma often have life-long health issues due to the toxic effects of the treatment. There is extensive interest in the delivery of chemotherapy to tumor cells while sparing normal nontumor cells in order to decrease toxicity and potentially increase efficacy. However, studies on drug delivery for pediatric cancers have been limited. In this study, we demonstrated for the first time the ability of the EGF-antibody linked drug-loaded bacterially-derived delivery system, EGFREDVTM (Dox) to enter and kill neuroblastoma cells in vitro and in clinically-relevant in vivo models of neuroblastoma.
**Figure 5.**

*In vivo* efficacy of targeted EDVTM in an orthotopic neuroblastoma mouse model with low EGFR protein levels. Mice bearing orthotopic SH-SY5Y-Luc neuroblastoma tumors were treated with control (saline), EGFREDVTM, Liposomal doxorubicin (Lipo Dox), EGFREDVTMDox, EDVTM, or dox twice weekly for 4 weeks. **A,** Quantitation of bioluminescence emitted from the whole-body of mice from each group was measured. Data represent the mean total flux (photons/second) ±SEM (error bars) for each group (n = 5–6/group), *P < 0.05; **P < 0.01 taken every 7 days up to 28 days. B,** At the end of the 28 days, the tumors were excised and measured using calipers and expressed as size in mm³. Arrows indicate dosing days. **C,** Histologic appearance of neuroblastoma SY-5Y cell line xenografts showing (i) Liposomal doxorubicin (Lipo Dox; sample ID 757), (ii) EDVTM, (III) EGFREDVTM, (iv) EGFREDVTM, (v) saline control, and (vi) doxorubicin (DOX; sample ID 822) treated tumors. Note less prominent nucleoli in non-MYCN amplified SY-5Y xenografts (i–iv) compared to BE(2) xenografts (Fig. 6); coagulative necrosis (ii); and an autonomic ganglion incorporated into a tumor (bottom of iv). Magnification: 600×. **D,** TUNEL staining of tumor sections from mice receiving the six different treatments.
Drug delivery strategies for cancer in the clinic have focused on adult malignancies with limited focus on pediatric cancers. Doxorubicin is an effective agent used in the treatment of neuroblastoma, however, it is adverse short- and long-term side effects are well studied (17). The EDV technology provides a way to deliver drugs preferentially to the tumor thereby both enhancing efficacy, and reducing the amount of drug needing to be administered. A recent first-in-human clinical trial in adults showed that EGFREDVTM packaged with paclitaxel were safe and showed some efficacy. In glioblastoma, a first in human trial using EGFREDVTM packaged with doxorubicin also found that the delivery system was well tolerated with no severe adverse effects noted (9). As doxorubicin forms part of the chemotherapeutic cocktail used to treat neuroblastoma, in this study we focused on examining the efficacy of EGFREDVTM_Dox in neuroblastoma. The six neuroblastoma cell lines we screened for EGFR showed variable levels of protein expression with SK-N-BE(2) and SHEP cells having the highest expression and SH-SY-5Y and IMR-32 cells having the lowest expression. Our in vitro and in vivo studies focused on the activity of the EGFREDVTM_Dox in two independent cell lines with high (SK-N-BE(2)) and low (SH-SY-5Y) EGFR expression. The EGFREDVTM_Dox was more effective at inducing cell death than doxorubicin alone as demonstrated by increased PARP cleavage and apoptosis in both the SK-N-BE(2) and SH-SY-5Y cells. Even though the SH-SY-5Y cells have low expression of EGFR there was a clear induction of apoptosis using the EGFREDVTM_Dox. The EDVTM_Dox (lacks the EGFR antibody) and this was also able to induce apoptosis in both cell lines, however there was a trend towards greater efficacy with the EGFREDVTM_Dox.

An effective drug delivery system also relies on the ability of the system to penetrate solid tumors. Using a 3D spheroid model and FLIM analysis we demonstrated that EGFREDVTM_Dox could effectively penetrate 3D tumor spheroids compared to doxorubicin alone (low penetration) or EDVTM_Dox (partial penetration; Fig. 3). Importantly, this resulted in a marked increase in PARP cleavage for 3D spheroids treated with EGFREDVTM_Dox at equivalent doses of doxorubicin. Consistent with our findings in vitro we found that EGFREDVTM_Dox treatment of mice bearing orthotopic human neuroblastoma xenografts resulted in a significant reduction in tumor growth and tumor volume at day 28 compared to control, indicating that the EGFREDVTM_Dox is effective against two independent neuroblastoma tumor models. Our data showed that EGFREDVTM_Dox significantly increases overall survival of mice harboring neuroblastoma tumors. Even though the short-term animal studies (28 days) showed some evidence of activity of EDVTM_Dox (lacks EGFR antibody), the long-term survival experiment clearly shows that EGFREDVTM_Dox induces a significant increase in overall survival. EDV delivery is likely to benefit from the enhanced permeability and retention (EPR) effect (18) and allowing higher amounts of drug-loaded EDVs to enter the tumor site that has a poor clearance rate. In previous studies, the biodistribution of intravenously administered 125I-labeled minicells in nude mice bearing subcutaneous EGFR overexpressing breast tumors demonstrated that at 2 hours post-treatment ~30% of the EGFREDVTM_Dox were localized in the tumor as compared with only ~1% of the free Dox (4, 6). The rapid extravasation of EGFREDVTM_Dox followed by engagement with cell surface EGFR due to the proximity of siRNA to tumor cells will allow targeted delivery via endocytosis and bypass any drug efflux pumps operating in the tumor cells. Therefore, although liposomal doxorubicin and untargeted EDVTM are able to take advantage of the EPR effect in a similar fashion, they rely exclusively on nonspecific tumor cell uptake which is a much less effective mechanism of nanoparticle uptake. Furthermore, nanoparticle extravasation and uptake are greatly affected by a variety of factors including nanoparticle size and surface chemistries as well tumor vascularity and microenvironment characteristics. The results presented here and elsewhere have demonstrated the need to deliver liposomal formulations of doxorubicin and other chemotherapeutics at ~100-fold higher dosages in order to achieve similar efficacy to EGFR-targeted EDVTM carrying the same payload (4, 6), which is likely due to a combination of enhanced tumor cell uptake via active targeting and differences in nanoparticle characteristics between liposomal and EDVTM formulations.

Given the safety profile of the recent first in human trial of EGFREDVTM_Dox in the treatment of glioblastoma in adults (9), there is great promise to advance EDVTM drug delivery to pediatric patients. We have now commenced and have patients enrolled in a Phase I Study of Intravenous EGFR-targeted EDVTM loaded with mitoxantrone in Children with Recurrent/Refractory Solid or CNS Tumors Expressing Epidermal Growth Factor Receptor (Trial registration ID: NCT02607386) that is underway at the Sydney Children’s Hospital, Randwick, and the Children’s Hospital Westmead, Sydney, Australia.

This system is highly adaptable to different type of drug loading, siRNA and miRNA, and can be linked to different receptors expressed on cancer cells making this a potential new treatment approach to increase efficacy while minimizing toxicity for pediatric patients.

### Table 1. Summary of pathology data for treated orthotopic neuroblastoma tumors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Tumor morphology*</th>
<th>Tumor viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH-SY5Y-Luc</td>
<td>Lipoo Dox</td>
<td>UDNB</td>
<td>95</td>
</tr>
<tr>
<td>EDVTM</td>
<td>Lipoo Dox</td>
<td>UDNB</td>
<td>80</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>Lipoo Dox</td>
<td>UDNB</td>
<td>85</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>EGFREDVTM</td>
<td>UDNB</td>
<td>99</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>EGFREDVTM</td>
<td>UDNB</td>
<td>98</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>EGFREDVTM</td>
<td>UDNB</td>
<td>98</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>Dox</td>
<td>UDNB</td>
<td>93</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>Dox</td>
<td>UDNB</td>
<td>93</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>PDDN</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>PDDN</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>PDDN</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Dox</td>
<td>PDDN</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

*Median survival and change in survival, days.

### Table 2. Survival for mice treated with EGFREDVTM_Dox

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival* (range)</th>
<th>Change in survival (Treatments vs. saline) (P value)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>26 (19–29)</td>
<td>0.0007</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>29 (22–31)</td>
<td>0.006</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>26 (22–29)</td>
<td>0.005</td>
</tr>
<tr>
<td>EDVTM</td>
<td>29 (22–32)</td>
<td>0.006</td>
</tr>
<tr>
<td>EGFREDVTM</td>
<td>36 (26–47)</td>
<td>10</td>
</tr>
</tbody>
</table>

*p Values represent EGFREDVTM_Dox vs. other treatments.
Disclosure of Potential Conflicts of Interest

H. Brahmbhatt has ownership interest (including patents) in EnGeneIC. J.A. MacDiarmid has ownership interest (including patents) in EnGeneIC. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Sagnella, M. Kavallaris

Figure 6.

EGFREDVTM_Dox increases survival of neuroblastoma-bearing mice treated with targeted EDVs. Mice bearing orthotopic SK-N-BE(2)-Luc neuroblastoma tumors were treated with control (saline), EGFREDVTM_Dox, EDVTM_Dox or Dox twice weekly until tumors reached 1,000 mm³ in size by palpation (n = 8–10/group). A, Mice were monitored daily by palpation and weekly bioluminescence emitted from the whole-body of mice until mice were sacrificed, and survival curves plotted. B, At sacrifice, the tumors were excised and measured using calipers to ensure tumors size was within an acceptable range of the target 1,000 mm³. C, Histologic appearance of SK-N-BE(2)-Luc cell line xenografts showing (i) EGFREDVTM, (ii) EDVTM_Dox, (iii) doxorubicin (Dox), and (iv, v, vi) EGFREDVTM_Dox-treated tumors. Note presence of cherry red nucleoli in xenografted cells consistent with MYCN amplification of the SK-N-BE(2)-Luc cell line (A); coagulative necrosis where cell outlines are preserved (left-hand side of ii and iv), and rosettes containing neuropil (vi) indicative of a poorly-differentiated neuroblastoma (F). Magnification: 400× or 600×.
Neuroblastoma Drug Delivery

Study supervision: S.M. Sagnella, J.A. MacDiarmid
Other (I am a paediatric pathologist and as such I am responsible for the interpretation of the histopathological data in the manuscript.):
A.J. Gifford

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