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Manipulation of variables in local controlled release vincristine treatment in neuroblastoma

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Abstract

Introduction—Local drug delivery minimizes systemic toxicity while delivering high-dose chemotherapy for neuroblastoma patients. We hypothesized that varying burst and maintenance dosing of implanted silk platforms would improve survival.

Methods—Platforms were loaded with vincristine 25µg, 50µg, 100µg, 200µg varying burst (released 1–4 days post-implantation) and maintenance (over next 20 days) dosing. Orthotopic tumors were created in mice using human neuroblastoma KELLY cells. Silk platforms were implanted into tumors when volume>300mm³. Tumor volume was monitored weekly with ultrasound. Experimental endpoints were tumor volume>1000mm³ or weight loss>25%.

Results—Drug release ranged from burst dosing 18.2–80.9µg, maintenance 5.0–111.6µg, and cumulative 23.3–177.4µg. Animals treated with 200µg platform died 9–13 days post-implantation, corresponding to 128.1–141.2µg released (toxic dose). Animals received 30.2±3.4µg day-one survived longer than those that received 10.1±1.1µg(p=0.03), suggesting <10.1µg day-one was insufficient. Tumors treated with 100µg or 50µg silk platform took longer to reach 1000mm³ compared to those treated with control, 44.8±9.5 days(p<0.001) and 26.7±6.7 days(p<0.05), respectively, versus 7.0±1.7 days. Overall survival correlated with higher burst(r=0.446, p=0.004) and maintenance dosing(r=0.353, p=0.02), *Animal survival days*=30.314+0.626×(*dose on day-one*)–0.020×(*tumor volume at day-ten*)(p<0.05).

Conclusion—Platform formulations can be manipulated to vary burst and maintenance dosing, summarized by an equation consisting of these variables.

Keywords

neuroblastoma; controlled release; vincristine

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1. INTRODUCTION

Neuroblastoma is the most common extra-cranial solid tumor of childhood, and it accounts for 15% of all pediatric cancer related deaths [1]. Patients diagnosed with this high-risk disease are treated with intense systemic chemotherapy, including doxorubicin, vincristine, cisplatin, etoposide, cyclophosphamide, and topotecan [1]. The delivery of chemotherapy through an intravenous route has been the mainstay of neuroblastoma treatment, and toxicity from the systemic chemotherapy has resulted in significant patient morbidity such as cardiotoxicity, myelosuppression, renal toxicity, and adverse long term problems including secondary malignancy [2]. As novel drug reservoir systems become available, the adoption of improved drug delivery methods that can maximize tumor kill and minimize systemic side effects is a top-priority, especially for the pediatric population [3–5]. Previously, we have utilized sustained release silk platforms to treat orthotopic neuroblastoma mouse xenografts by implanting the platform into the center of the tumor [6]. This treatment strategy has resulted in significant decrease in tumor growth and reduction in systemic toxicity compared to delivering the same drug dose intravenously.

In this current study, we aimed to further define the variables that can contribute to effective tumor control. We utilized a previously described orthotopic xenograft model [6] and engineered various silk platforms with different drug release profiles. We hypothesized that by varying the amount of drug delivered shortly after silk platform implantation (burst dose) or released over the ensuing days (maintenance dose), we could achieve even a greater decrease in tumor growth. We further postulated that a relationship exists between these variables and interrogating our experimental system by varying the burst dose and maintenance dose can clarify this relationship.

2. METHODS

2.1 Silk fibroin extract

Silk fibroin from *Bombyx mori* silkworm cocoons was extracted as previously described [7]. Briefly, cocoons were cut into approximate 1 cm² pieces and boiled in 0.02 M NaCO₃ for 30 minutes to extract the sericin protein. The silk fibroins fibers were dried overnight followed by dissolution in 9.3 M LiBr for 3 hrs at 60°C to 20% (w/v). The dissolved silk fibroin was dialyzed (3.4 kDa MWCO dialysis cassettes, Thermo Fisher Scientific, Waltham, MA) against ultrapure water at room temperature for two days with seven water changes resulting in an approximately 6.5% (w/v) aqueous silk fibroin solution. The silk fibroin solution was stored at 4°C for later use.

2.2 Vincristine-loaded silk fibroin foams

Vincristine-loaded silk fibroin (vincristine sulfate salt, LC Laboratories, Woburn, MA) foams were fabricated as previously described [6]. Briefly, 100 µL of 6% (w/v) silk fibroin solution was transferred to a 96-well plate and lyophilized to form lyophilized silk fibroin plugs. The lyophilized silk fibroin plugs were transferred to glass petri dishes and autoclaved at 121°C for 20 minutes to transform the protein secondary structure from a predominately random coil to a β -sheet structure rendering the materials insoluble and sterile. The silk

fibroin foams were handled aseptically from this point forward. A solution of 25 µg/mL or 50 µg/mL of vincristine in water was prepared. One milliliter of the vincristine solution was added to silk fibroin foams in sterile, 1.5 mL Eppendorf tubes. The vincristine was allowed to adsorb to the silk fibroin foams as previously reported [6, 8].

2.3 Vincristine-loaded dip-coated reservoirs

Vincristine-loaded dip-coated reservoirs were fabricated following a previously reported method with modifications [9]. Briefly, silk fibroin was diluted to 2% (w/v), sterile filtered and re-concentrated to 8% (w/v) under aseptic conditions via centrifugal filtration (Amicon Ultra-15, 3 kDa NMWL; EMD Millipore Billerica, MA). Centrifugal filters were sterilized with 70% ethanol exposure for 30 minutes followed by four washes with sterile water. Sterile solutions containing 6% (w/v) silk fibroin and 0.5 mg/mL, 1 mg/mL and 2 mg/mL vincristine were made, aliquoted at 100 µL per well in 96-well plates and lyophilized to obtain sterile, vincristine-loaded silk fibroin plugs. The silk fibroin plugs were pressed into 3 mm diameter wafer drug reservoirs and water-vapor annealed at room temperature for greater than 12 hrs to induced the β -sheet confirmation rendering the materials insoluble. A sterile silk fibroin solution of 7% (w/v) or 14% (w/v) was used to dip-coat the vincristine drug reservoirs with either 4 or 6 coats. The deposited silk layers were allowed to completely dry before depositing the subsequent layer. Once all of the layers were deposited, the vincristine-loaded dip-coated reservoirs underwent a final water-vapor annealing step.

2.4 Vincristine release characterization

Vincristine-loaded drug delivery systems were placed into 1 mL of phosphate buffered saline (PBS, pH 7.4 Life Technologies, Grand Island, NY) at 37°C for release quantification. At each time point evaluated, the PBS was completely removed and replaced with fresh PBS. The drug concentration was determined via Ultraviolet/visible (UV/Vis) light spectroscopy (SpectraMax M2 spectrophotometer; Molecular Devices, Sunnyvale, CA). A standard curve for vincristine using an absorbance wavelength of 298 nm was generated to determine the vincristine concentration within the release medium.

2.5 Cell culture

Human neuroblastoma KELLY cells (Sigma-Aldrich, St Louis, MO) were maintained in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. All cells were maintained in a 5% CO₂ atmosphere at 37°C and trypsin-passaged at 80% confluence.

2.6 Mouse orthotopic neuroblastoma model

All mouse procedures were performed in accordance with University of Illinois' recommendations for the care and use of animals and were maintained and handled under protocols approved by the Institutional Animal Care and Use Committee. The establishment of orthotopic neuroblastoma xenograft in a seven-week old female NCr nude mouse (Harlan, Indianapolis, IN, USA) was previously described [6]. Briefly, the mouse was anesthetized with inhalational isoflurane. A transverse incision was made on the left flank to locate the left adrenal gland, and 2 µL of phosphate buffered saline (PBS) containing one million

KELLY cells were injected into the adrenal gland. The fascia and skin were closed in separate layers. Tumor formation was followed by non-invasive ultrasound measurements, and the animals euthanized when the tumor volume exceeded 1000 mm³.

2.7 Implantation of silk fibroin drug delivery system

This procedure was previously described [6]. Briefly, once the tumor volume reached >300 mm³ as determined via high frequency ultrasound imaging, animals underwent treatment. Through a left lateral transverse incision the tumor was identified and dissected free from surrounding structures. Using cautery, the tumor capsule was incised, and the silk fibroin drug delivery system was inserted into the tumor. The fascia and the skin were then closed with sutures. A total of three animals were implanted with the control silk platform, nine with Vin25 – F, nine with Vin50 – F, three with Vin50 – W6, and five with Vin100 – W6 (see Table 1 for labeling of the silk fibroin drug delivery system).

2.8 High frequency ultrasound

After anesthetizing with inhalational isoflurane, the mouse was secured in a prone position, and a VisualSonics Vevo 2100 sonographic probe (Toronto, Ontario, Canada) was placed over the left flank to locate the left adrenal gland and the tumor. Serial cross-sectional images (0.076 mm between images) were taken, and 3-D reconstruction tool (Vevo Software v1.6.0, Toronto, Ontario, Canada) was used to calculate the tumor volume. All tumors were measured every five to seven days after the implantation of the silk fibroin drug delivery system.

2.9 Statistical analysis

The burst dosing, amount of drug released in the first 24 hours or 4 days after implantation, as well as the maintenance dosing, amount of drug released until day 21, were determined for various silk platforms. Using regression modeling on SPSS software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.), a predictive model of overall survival days based upon different treatment variables was created.

3. RESULTS

The release of vincristine from the silk fibroin drug delivery systems was measured *in vitro* (Figure 1A and 1B). The labeling scheme for each group is presented in Table 1A. The results from the foam format were presented previously [6]. In summary, for the silk foams loaded with 25 µg of vincristine, approximately 73% of the drug was released initially and 20% over the next 21 days. For the silk foam loaded with 50 µg of vincristine, about 73% of the drug was released initially, and 30% over the next 21 days (Table 1B).

For the dip-coated reservoirs fabricated with six layers of 7% (w/v) and 50 µg vincristine, 63% released initially, 41% over 21 days, and nearly complete drug release was observed after three weeks (Table 1B). The mass release scaled nearly linearly with the initial drug loading similar to the vincristine-loaded silk fibroin foams [6]: loading with 100 µg of vincristine resulted in similar release kinetics: 66% initially and 30% over 21 days. Nearly linear release kinetics were obtained with the 14% w/v silk fibroin dip coated reservoirs (R²

equal to 0.999 and 0.989 for Vin200 – W4 and Vin200 – W6, respectively). Additionally, nearly complete drug release was observed after seven weeks (Figure 1A and 1B).

When the tumor-bearing animals were treated with Vin200 – W4 or Vin200 – W6, the animals exhibited signs of toxicity such as weight loss (>20%) and decreased activity starting approximately 9 to 13 days after wafer implantation. The animals were euthanized when these signs were detected. At the 9–13 day time point, a total of $128.1 \pm 10.3 \mu\text{g}$ and $141.2 \pm 6.5 \mu\text{g}$ of vincristine was released from Vin200 – W4 and Vin200 – W6 respectively (Figure 1A), representing the toxic cumulative dose. None of the animals treated with the other drug-loaded silk platform resulted in significant toxicity. Excluding those showing treatment-related toxicity, the overall survival days of the tumor-bearing animals after treatment correlated with higher initial burst dosing ($r = 0.446$, $p = 0.004$) and higher maintenance dosing ($r = 0.353$, $p = 0.02$).

When the tumor-bearing animals were treated with Vin50 – W6, the time taken for tumor to reach 1000 mm^3 was slowed significantly compared to those treated with control wafer (26.7 ± 6.7 days versus 7 ± 1.7 days) ($p < 0.05$) (Figure 2A). This difference compared to the control wafer was even more pronounced when the tumor-bearing animals were treated with Vin100 – W6 (44.8 ± 9.5 days versus 7 ± 1.7 days) ($p < 0.001$). Furthermore, animals that received $30.2 \pm 3.4 \mu\text{g}$ on day 1 (Vin100 – W6 group) survived longer than those that received $10.1 \pm 1.1 \mu\text{g}$ on day 1 (Vin25 – F group) ($p = 0.03$). This suggested that drug dose less than $10.1 \mu\text{g}$ on day 1 was insufficient to effect change in tumor growth.

Analyzing the entire set of data and drawing statistical correlation, an equation linking the variables could be formulated to predict the animal survival days after vincristine-loaded silk platform implantation ($p < 0.05$):

$$\text{Days for animal survival} = 30.314 + (0.626 \times \text{drug dose on day 1}) - (0.020 \times \text{tumor volume at day 10})$$

4. DISCUSSION

By varying the formulation of the silk platform, we have engineered a vehicle that can deliver different amounts of vincristine initially (burst) and subsequently (sustained) over time after implanting into the neuroblastoma tumor. These outcomes highlight the versatility of the silk platform and also allows us to determine the minimum amount of vincristine required to effect tumor growth and the maximum amount tolerated by the animal.

Furthermore, our tumor size data between these lower and upper drug limits provided an opportunity to define the relationship between the drug dose, tumor size, and days of animal survival. The demonstration that such a mathematical relationship can be calculated suggests that it is possible to predict tumor response after treatment by measuring certain defined variables such as tumor volume after a few days. And the equation can also be used to improve the delivery profile of the drug to achieve maximal tumor kill with tolerable drug toxicity.

The maintenance dose of vincristine ultimately fell out of significance when all the data were aggregated, and it was not represented in the equation. The rate of drug released over

time followed a similar pattern for each group, thus the maintenance dose variable had to be excluded. Future experiments in manipulating the drug release patterns by varying the number of silk layers can further refine the role of the maintenance dose in affecting tumor growth.

Previously, Coburn et al.[8], has shown that vincristine binds to silk fibroin films via electrostatic interactions, allowing for controlled release over three weeks. The foam-base system used in this work employs this same principle for vincristine binding and release. However, using electrostatic interaction and subsequent desorption in aqueous, salt solution (i.e. phosphate buffered saline) has two limitations, namely limited duration of release and toxicity. The later limitation renders vincristine loadings higher than 50 µg too toxic for animal studies. In this work, a reservoir-based drug delivery system was utilized to increase drug loading while slowing drug release. Utilizing previous methods developed by Pritchard et al.[9], dip-coating of drug-loaded wafers was used to tune the drug release rate. A number of variables, including thickness, which was varied by the number of silk fibroin layers, silk fibroin concentration, and crystallinity which varied by post-processing technique, can be utilized to tune the drug release rate and burst. Most notably in this work, the number of layers and silk fibroin concentration altered the vincristine release rate and initial burst. Additionally, the vincristine-loaded dip-coated reservoirs allowed for loading and release of up to 200 µg vincristine, amounts that were previously found to induce fatal toxicity when using desorption as the primary release mechanism.

The mechanism of release from silk-coated drug reservoirs is similar to other reservoir-based drug delivery systems, including the clinically used drug delivery system Norplant® and Implanon™, where the drug must diffuse through the polymer membrane coating out of the drug-loaded reservoir [9]. These clinically used drug delivery systems are made from non-degradable polymers including silicone and poly[ethylene-co(vinyl acetate)], requiring a second procedure for removal [10, 11]. Silk fibroin degrades via enzymatic degradation to peptides and amino acids which would eliminate the need for a removal procedure [12]. Silk fibroin materials have been implanted into multiple animal models and these studies showed minimal immune response and degradation over time [13–15]. Other biodegradable polymer systems that could be used are polyesters [16]. However, due to the organic solvent requirements for polymer dissolution and acidic degradation products these polymers are less favorable than all aqueous processed, neutral degradation product materials such as silk fibroin.

We have previously reported the approach of implanting a drug-loaded sustained release platform within the center of the tumor to achieve tumor control [6]. This current study furthers that application and provides additional data on the “delivery kinetics.” Future experiments focusing on how the drug delivered within the tumor diffuses across the tumor mass and how this diffusion process can be optimized will be key to applying this treatment approach in the clinical realm.

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A.

B.

Figure 1.

In vitro cumulative vincristine release from the silk fibroin drug delivery systems. Results for (A) the entire study period and (B) through day 7 for a more precise view of the early time point data. Vin25 – F and Vin50 – F reprinted with permission from [6].

Figure 2.

In vivo response to vincristine release from the silk fibroin drug delivery systems. (A) Tumor size, (B) animal weight, and (C) Kaplan-Meier survival curves after implantation of different silk fibroin delivery systems.

Table 1

Labeling and fabrication scheme for each group.

Label	Fabrication Technique	Silk fibroin percentage (w/v) for coatings	Number of silk fibroin layers	Drug mass (µg)
Vin25 – F	Lyophilized foam	N/A	N/A	25
Vin50 – F	Lyophilized foam	N/A	N/A	50
Vin50 – W6	Drug reservoir	7	6	50
Vin100 – W6	Drug reservoir	7	6	100
Vin200 – W4	Drug reservoir	14	4	200
Vin200 – W6	Drug reservoir	14	6	200

Table 2

Summary of vincristine release from silk fibroin drug delivery systems.

	Burst Release (µg) Day 1	Burst Release (µg) Day 4 or 5	Maintenance Dose (µg)	Total Release (µg)
Vin25-F	10.1 ± 1.1	18.2 ± 1.0	5.0 ± 2.0	23.3 ± 2.0
Vin50-F	18.8 ± 2.6	36.6 ± 1.7	15.1 ± 4.1	51.7 ± 3.7
Vin50-W6	14.2 ± 0.4	31.5 ± 0.4	20.4 ± 4.2	51.9 ± 1.7
Vin100-W6	30.2 ± 3.4	66.1 ± 3.4	30.2 ± 0.9	96.3 ± 4.0
Vin200-W4	14.9 ± 0.6	63.6 ± 5.7	111.6 ± 4.5	175.3 ± 10.2
Vin200-W6	7.1 ± 1.0	80.9 ± 4.3	96.5 ± 6.1	177.4 ± 10.1