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Focal therapy of neuroblastoma using silk films to deliver kinase and chemotherapeutic agents *in vivo*

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ABSTRACT

Current methods for treatment of high-risk neuroblastoma patients include surgical intervention, in addition to systemic chemotherapy. However, only limited therapeutic tools are available to pediatric surgeons involved in neuroblastoma care, so the development of intraoperative treatment modalities is highly desirable. This study presents a silk film library generated for focal therapy of neuroblastoma; these films were loaded with either the chemotherapeutic agent doxorubicin or the targeted drug crizotinib. Drug release kinetics from the silk films were fine-tuned by changing the amount and physical crosslinking of silk; doxorubicin loaded films were further refined by applying a gold nanocoating. Doxorubicin-loaded, physically crosslinked silk films showed the best *in vitro* activity and superior *in vivo* activity in orthotopic neuroblastoma studies when compared to the doxorubicin-equivalent dose administered intravenously. Silk films were also suitable for delivery of the targeted drug crizotinib, as crizotinib-loaded silk films showed an extended release profile and an improved response both *in vitro* and *in vivo* when compared to freely diffusible crizotinib. These findings, when combined with prior *in vivo* data on silk, support a viable future for silk-based anticancer drug delivery systems.

1. Introduction

Neuroblastoma is the most common extracranial tumor in childhood, with a variable disease progression ranging from complete spontaneous remission to life-threatening progression despite intensive combination therapy [1]. High-risk patients are identified using markers such as age, stage, histology, N-myc proto-oncogene status, and DNA ploidy. Although cure rates for early stage neuroblastoma are excellent, the 5-year event-free survival rate for high-risk patients remains only 40% [2].

Current treatment options for high-risk neuroblastoma include intensive systemic neoadjuvant chemotherapy and immune modulation, followed by surgical resection [1]. Nevertheless, even after high dose chemotherapy, an abdominal tumor will still frequently extend along vital vasculatures. Pediatric surgeons are often called upon to skeletonize vessels such as the superior mesenteric artery, the celiac artery, renal vessels, or the inferior vena cava in order to excise these tumors. This is not only a treacherous and time-consuming endeavor, but the morbidity of the procedure is high and can induce vessel thrombosis, stricture, or even organ loss [3]. Furthermore, the side effects of high dose chemotherapeutic agent, including ototoxicity, myelosuppression, and nephrotoxicity, can have lasting consequences in the pediatric population [1].

Neuroblastoma cells often develop drug resistance; therefore, the development of other strategies (e.g., tumor differentiation with retinoids or immunotherapy with disialoganglioside GD2 antibodies combined with cytokines) is encouraging [1]. Kinase inhibitors that target anaplastic lymphoma kinase (ALK) have been developed for other cancers and are currently being evaluated for use in neuroblastoma patients through the Pediatric Preclinical Testing Program of the National Cancer Institute [4]. ALK is associated with a major neuroblastoma predisposition that is reflected by somatic mutations or gene amplifications in 15% of patients

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[2,4]. Because ALK expression is largely restricted to neurons, with a strong up-regulation in neuroblastoma [5,6], ALK inhibitors such as crizotinib are poised to have a significant impact on neuroblastoma therapy [2,4].

Follow-up studies have demonstrated a probability of local progression of 50% in unresected patients versus 10% in stage 4 patients undergoing gross total resection, with an overall survival of 50% and 11%, respectively [7]. Thus, local–regional control of the disease appears to be a key driver for improved clinical outcome. Because high-risk neuroblastoma patients commonly undergo surgical intervention as part of their treatment, focal drug therapy at the time of surgery might be a beneficial approach for the local treatment of unresectable tumors, thereby further improving clinical outcome.

A leaky blood vasculature and reduced lymphatic drainage are hallmarks of established solid tumors [8]. Tumor pathology has been exploited for the rational design of many intravenously administered anticancer nanomedicines (e.g., nanoparticles, micelles, liposomes) [9]; two studies have recently developed crizotinib-based nanomedicines [10,11]. However, poor tumor perfusion and high interstitial pressure can limit nanomedicine accumulation [12]. Therefore focal therapy directly applying the chemotherapeutic agent over the tumor bed appears to be a better approach to treat neuroblastoma patients undergoing surgical intervention.

Focal therapy has remained largely unexplored in the neuroblastoma setting; however, clinical examples exist for other tumors, including brachytherapy of early stage breast cancer [13] and the treatment of high-grade malignant glioma and recurrent glioblastoma multiforme post-resection using controlled-release polymeric wafers to deliver carmustine (Gliadel wafers) [14,15]. For neuroblastoma, features of an ideal drug delivery system for focal therapy would include: (i) a flexible delivery platform for different drugs; (ii) tunable drug release kinetics; (iii) biocompatibility; (iv) biodegradability; (v) conformity to the tumor/tumor bed; and (vi) unidirectional drug release toward the tumor/tumor bed.

Silk has been used in human medicine for hundreds of years as a suture material and is approved as a surgical mesh (Allergan Inc., Irvine, CA, USA). Its unique mechanical properties [16,17], biocompatibility, and the versatility of its various formats [16] make silk attractive for various other biomedical applications, including drug delivery [18]. For example, we have been able to generate self-assembling hydrogels [19] for breast cancer focal therapy, as well as uniform silk nanoparticles that can be used for the pH-mediated lysosomotropic delivery of a model drug, doxorubicin [20]. Silk films have excellent surface conformity [21,22] and are therefore particularly well suited for the direct application to tumors/tumor bed; the wettability of silk also promotes tight adhesion to tissues [23]. In contrast, Gliadel wafers are monolithic, solid disks with no conformity to tumor margins; a physical characteristic and design feature commonly encountered with many synthetic polymeric carrier systems [24].

The goal of the present study was to develop silk films for focal drug therapy using the clinically relevant anticancer drugs, doxorubicin and crizotinib, and to assess the drug loading, drug release, and biological responses of these films, both *in vitro* and *in vivo*.

2. Materials and methods

Human neuroblastoma cell lines SK-N-AS (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM non-essential amino acids. KELLY cells (Sigma–Aldrich, St Louis, MO, USA) were maintained in RPMI 1640 supplemented with 10%v/v fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. All cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C and were trypsin-passaged at 80% confluence.

2.1. Manufacture of drug-loaded silk films

Silk was isolated from Japanese *Bombyx mori* cocoons, as previously described [25]. Briefly, cocoons were boiled in 20 mM sodium carbonate for 30 min, washed with deionized water, and the degummed fibers were air-dried. The silk fibers were then dissolved in 9.3 M lithium bromide at 60 °C for 4 h and the solution was dialyzed (3.4 kDa MWCO, Piece, Rockford, IL, USA) against deionized water for 60 h. Silk films were manufactured using 1, 2, or 4%w/v silk solutions by casting the silk on an 11 mm × 17 mm polydimethylsiloxane (PDMS) mold and allowing the solution to air-dry overnight. The resulting water-soluble films were cut into 7 mm × 11 mm rectangles. A 20-min steam autoclaving cycle was used to induce physical crosslinks, resulting in a stabilized silk film (i.e., water insoluble). The stabilized silk films were loaded with 50 μg doxorubicin (LC Laboratories, Woburn, MA, USA) by soaking them in a doxorubicin solution and verifying loading by measuring doxorubicin-associated fluorescence of the solution (excitation 480 nm, emission 590 nm). Similarly, stabilized silk films were loaded with crizotinib by soaking them in 450 μg crizotinib for 48 h. Drug loading was quantified by mass spectroscopy (detailed below) by determining the difference in amounts of crizotinib in the soaking solution pre- and post-loading. Water-soluble silk films with a nominal doxorubicin loading of 50 μg for each 7 mm × 11 mm film were generated by dissolving the required amount of drug in ddH_2O and adding it to the silk. This doxorubicin silk solution was cast onto PDMS, allowed to air-dry overnight, and then cut to the desired dimensions. Soluble crizotinib films were generated in an analogous fashion, but with a nominal loading of 10 μg of drug per film. Silk films with a gold backing were generated by sputter coating 7 mm × 11 mm films with 100 nm of gold, stabilizing the films by autoclaving, and finally loading with 50 μg doxorubicin using the soaking method described above.

The swelling characteristics of stabilized silk films were determined by measuring the films in their dry and wet states after a 48 h incubation in phosphate buffered saline (PBS). Similarly, the thicknesses of the films were measured with calipers before and after incubation in PBS.

2.2. Measurement of drug release from silk films

In vitro release kinetics were determined by incubating films with 1 ml of PBS under static conditions at room temperature; at each time point, the entire volume of PBS was removed and replaced with fresh PBS to ensure sink conditions. Doxorubicin release was determined by measuring doxorubicin-associated fluorescence (excitation 480 nm, emission 590 nm). The impact of the gold backing on directional doxorubicin release was determined by casting 2 wt.% agarose in PBS into a petri dish and allowing it to cool. Just before the gel had set, silk films were added to the gel and stabilized with a custom-made polystyrene frame to facilitate the correct placement of the film. After 24 h at room temperature, a Xenogen IVIS 200 imaging system, controlled by the Living Image Software 4.2 (Caliper Life Sciences, Hopkinton, CA, USA), was used to measure doxorubicin-associated fluorescence. For *in vivo* studies, the residual amount of doxorubicin remaining in the film was determined by measuring light absorbance at 482 nm. Retrieved films were dissolved using 100 μl 9.3 M lithium bromide at 60 °C for 30–60 min. The resulting solution was diluted with