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Doxorubicin-conjugated polypeptide nanoparticles inhibit metastasis in two murine models of carcinoma

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ABSTRACT

Drug delivery vehicles are often assessed for their ability to control primary tumor growth, but the outcome of cancer treatment depends on controlling or inhibiting metastasis. Therefore, we studied the efficacy of our genetically encoded polypeptide nanoparticle for doxorubicin delivery (CP-Dox) in the syngeneic metastatic murine models 4T1 and Lewis lung carcinoma. We found that our nanoparticle formulation increased the half-life, maximum tolerated dose, and tumor accumulation of doxorubicin. When drug treatment was combined with primary tumor resection, greater than 60% of the mice were cured in both the 4T1 and Lewis lung carcinoma models compared to 20% treated with free drug. Mechanistic studies suggest that metastasis inhibition and survival increase were achieved by preventing the dissemination of viable tumor cells from the primary tumor.

1. Introduction

Engineered drug delivery vehicles for cancer treatment seek to improve the clinical efficacy of chemotherapeutics by increasing the amount of drug deposited in the tumor while decreasing its accumulation in healthy tissues [1–4]. This is necessary because chemotherapeutics are commonly comprised of small hydrophobic molecules that have short plasma half-lives leading to poor bioavailability after systemic administration [5]. Sequestration of drugs into the hydrophobic core of a soluble nanocarrier has been shown to enhance the solubility and bioavailability of the drug, improve its biodistribution by preventing rapid renal clearance due to low molecular weight, and stabilize the active form of the drug within the plasma environment [6–8]. Furthermore, nanocarriers ranging between 20 and 100 nm are ideally suited to both extravasate through the leaky vasculature characteristic of rapid and uncontrolled tumor growth and accumulate within the extracellular matrix due to impaired lymphatic drainage, two pathophysiological features of tumors collectively referred to as the enhanced permeability and retention (EPR) effect [9,10].

These attractive features of drug-loaded nanoparticles led us to develop a class of recombinant chimeric polypeptide (CP) nanoparticles for the delivery of chemotherapeutics to solid tumors [1,11]. CPs are comprised of two components: (1) a hydrophilic elastin-like polypeptide (ELP) domain consisting of repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is any amino acid except Pro, and (2) a C-terminal $C(GGC)_7$ peptide segment that provides eight unique cysteine residues that can be used as sites for drug attachment. Conjugation of 4–6 copies of the chemotherapeutic doxorubicin (Dox) to the C-terminal drug attachment domain through an acid-labile linker results in the spontaneous self-assembly of ~40 nm diameter spherical micelles within which the drug is sequestered. We have previously demonstrated the efficacy of these CP-Dox nanoparticles in the C26 murine colon carcinoma model where ~90% of a tumor-bearing cohort was cured following a single injection [1]. While the efficacy of CP-Dox against primary tumors is encouraging, the greatest clinical need is for drugs that interfere with the metastatic cascade, as metastasis accounts for the vast majority of cancer deaths [12].

Therefore, we examine herein the overall and metastasis-free survival rate for mice bearing two syngeneic metastatic tumors, mammary 4T1 carcinoma (4T1) and Lewis lung carcinoma (LLC) engineered to

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express firefly luciferase to enable *in vivo* tracking of metastasis. Furthermore, we used a clinically relevant treatment model in which the mice were treated with a combination of chemotherapy and surgical removal of the primary tumor, enabling us to directly correlate mortality with metastatic disease.

2. Methods

2.1. Cell culture

4T1-luciferase murine mammary carcinoma cells were provided by Prof. Mark Dewhirst at Duke University Medical Center (cells certified pathogen free on 6/26/13 by IMPACT Profile III). Lewis lung carcinoma LL/2-Luc-M38 (LLC) cells were purchased from Caliper Life Sciences (certified pathogen free on 1/21/2011), after which the cells were passaged for less than 5 generations before use in animal experiments. Both cell lines were grown in DMEM supplemented with 10% FBS and cultured at 37 °C in a humidified 5% CO_2 environment.

2.2. Cytotoxicity assays

4T1 and LLC cells were seeded (10^4 cells per well) in a 96-well plate and grown for 24 h, after which they were exposed to CP-Dox or free Dox (0–100 μM equivalents) for 24 h. Cell viability was determined based on their ability to reduce tetrazolium dye (MTT assay; Promega, Madison, WI). Viability was normalized to untreated controls, and the concentration required to achieve 50% inhibition of signal (IC_{50}) was calculated.

2.3. CP-Dox synthesis

2.3.1. Synthesis and expression of chimeric polypeptides

The gene encoding the CP was synthesized from custom oligomers purchased from IDT Inc. by recursive directional ligation, as described previously [1]. The gene was cloned into a pET25b + expression vector (Novagen, Madison, WI) and transformed into BL21 (DE3) *Escherichia coli* cells (EdgeBio, Gaithersburg, MD). Transformed cells were used to inoculate 50 mL flasks supplemented with 100 μg/mL ampicillin and grown overnight at 37 °C and 190 rpm. Each 50 mL flask was used to inoculate six 1 L cultures of Terrific Broth (MOBIO, Carlsbad, CA) supplemented with ampicillin (100 μg/mL), which were grown overnight in a shaker incubator at 37 °C and 190 rpm. Protein expression was induced 5 h following inoculation by the addition of IPTG to a final concentration of 0.5 mM. Purification of the CP was carried out by inverse transition cycling (ITC), as described previously [13].

2.3.2. Conjugation of Dox to the CP

Dox was conjugated to the CP as described previously [1]. Briefly, Dox was activated by conjugation to n-ß-maleimidopropionic acid hydrazide (BMPH, Pierce Biotechnology, Rockford, IL) *via* an acid-labile hydrazone bond by stirring for 16 h in methanol supplemented with 0.1% (v/v) TFA. Separately, the purified CP was dialyzed overnight in deionized water and then reduced for 30 min in 20 mM tris carboxyethyl phosphine hydrochloride, pH 7.4 (TCEP, Pierce Biotechnology, Rockford, IL). The CP phase transition was triggered by the addition of 2.8 M NaCl, and the CP was concentrated by centrifugation (14,000 rpm for 10 min at 30 °C), after which the CP pellet was re-solubilized in 100 mM phosphate buffer (pH 7, without saline). The activated Dox-BMPH conjugate dissolved in methanol was then added dropwise to the phosphate buffer and CP solution. The final ratio of methanol to PB was 2:1. After 3 h, TCEP was added to a final concentration of 30 mM to ensure the availability of free cysteine residues for maleimide bond formation. The reaction was then left to stir overnight. The reaction solution was centrifuged using 10K MWCO Amicon centrifugal ultrafilters (Millipore, Billerica, MA) and washed with a 30% acetonitrile and 70% PBS solution for multiple cycles at 2000 rpm for 45 min to solubilize and remove unconjugated Dox-BMPH until the sample was >98% pure by gel-filtration HPLC. Finally the buffer was exchanged with PBS with additional centrifugal ultrafiltration, and endotoxin was removed by passing the CP-Dox solution through a bed of Detoxi-gel™ resin (Pierce Biotechnology, Rockford, IL). The solution was sterilized by filtration (0.2 μm pore size, VWR, Radnor, PA) and concentrated by another centrifugal ultrafiltration step (Amicon 10K MWCO, 2000 rpm, 60 min). The topographical atomic force microscopic (AFM) images were collected in tapping mode using silicon nitride cantilevers (DNP-S, Bruker, 0.35 N/m nominal spring constant; 65 kHz nominal resonant frequency) with Multimode (Bruker, Santa Barbara, USA) in liquid. AFM images were obtained at a resolution of 512 × 512 pixels using 1 Hz scan rates.

2.4. Animal studies

All animal experiments were performed in accordance with protocols approved by the Duke Institutional Animal Care and Use Committee (IACUC). BALB/c mice (Charles River, 6–10 weeks old) were inoculated with 8×10^5 4T1-luciferase cells in the 4th mammary fat pad. Albino BL6 mice (Charles River, 6–10 weeks old) were shaved and inoculated subcutaneously on the flank with 1×10^6 LLC-luc cells. Mice were treated on day 8 (post-inoculation) with free Dox or CP-Dox at the maximum tolerated dose (5 mg/kg and 20 mg/kg, respectively). Mice were sacrificed if they appeared moribund or lost more than 15% of their baseline body weight, or if the tumor volumes exceeded 2000 mm^3.

2.5. Pharmacokinetics and biodistribution

Mice were inoculated with 4T1 and treated with free Dox (5 mg/kg) or CP-Dox (20 mg/kg) on day 8 as described above. At 2, 24, 28, and 72 h after treatment, mice were sacrificed and blood and tissue samples (tumor, liver, lung, heart, spleen, kidney and paw) were obtained, processed, and analyzed for doxorubicin content by fluorescence as described previously [1]. Briefly, samples were homogenized and treated with acidified isopropanol to extract the doxorubicin, and the fluorescence of the doxorubicin in the supernatant was quantified using 485 nm for excitation and 590 nm for emission. Background fluorescence was subtracted according to calibration curves made for each organ. Drug concentration in tissues was calculated as percent of the total injected dose per gram of tissue, using calibration curves made from serial dilutions of known standards.

2.6. Primary tumor regression study (4T1)

Mice were treated on day 8 and day 15 post-inoculation with free Dox or CP-Dox at their maximum tolerated dose (MTD, 5 mg/kg and 20 mg/kg, respectively), or an equivalent volume of PBS [1]. Tumor dimensions (length and width) were measured every other day, and tumor volumes were calculated using the formula Volume (mm^3) = (length ∗ $width^2$) / 2. Groups contained 5–8 mice.

2.7. Metastasis inhibition studies

Mice were inoculated with either 4T1-luc or LLC-luc, as described above (10–12 mice per treatment group). On day 8 post-inoculation, mice were treated with free Dox (5 mg/kg) or CP-Dox (20 mg/kg). On day 15, mice were anesthetized and tumors were surgically resected. On day 22, mice were again treated with free Dox or CP-Dox at the MTD. Mice were monitored for metastasis and primary tumor recurrence 2×/week using the IVIS Xenogen bioluminescent imaging system (Caliper LS, Hopkinton, MA). Mice were sacrificed after observing a detectable metastatic signal in two consecutive imaging sessions, or if the mice became moribund (>15% weight loss or the presence of hyperventilation). The presence of metastases that were detected *via* bioluminescence was later confirmed by post-mortem dissection.