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The effect of size and polymer architecture of doxorubicin–poly(ethylene) glycol conjugate nanocarriers on breast duct retention, potency and toxicity

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ABSTRACT

Although systemic administration of chemotherapeutic agents is routinely used for treating invasive breast cancer, the only therapeutic options for ductal carcinoma in situ (DCIS) are surgery and radiation. Treating DCIS by delivering drugs locally to the affected milk duct offers significant advantages over systemic administration, including reduced systemic and breast toxicities, as well as a greatly reduced need for surgery and radiation. In this study, mammary gland retention and toxicity of intraductally administered poly(ethylene) glycol-doxorubicin (PEG-DOX) polymeric conjugate nanocarriers of varying molecular sizes and architectures were investigated. Nanocarriers were formed by conjugating one or more copies of doxorubicin to PEG polymers, of varying molecular weights (5, 10, 20, and 40 kDa) and architectures (linear, four-arm and eight-arm). Cytotoxicity against MCF7 cells, a human breast cancer cell line, was assessed, and IC_{50} values were calculated. The nanocarriers were intraductally administered into the mammary glands of female retired breeder Sprague-Dawley rats. Whole body images were captured using in vivo optical imaging, and changes in ductal structure as well local inflammation were monitored. Fluorescence intensities were monitored, over time, to evaluate nanocarrier mammary gland retention half-lives ($t_{1/2}$). The IC_{50} values of PEG-DOX nanocarriers against MCF7 cells were 40 kDa PEG-$(DOX)_4$ (1.23 μM) < 5 kDa PEG-DOX (1.76 μM) < 40 kDa PEG-$(DOX)_8$ (3.49 μM) < 10 kDa PEG-DOX (3.86 μM) < 20 kDa PEG-DOX (8.96 μM) < 40 kDa PEG-DOX (18.11 μM), whereas the IC_{50} of free DOX was only 0.14 μM. The $t_{1/2}$ of linear 5, 20, and 40 kDa nanocarriers were 2.2 ± 0.3, 3.6 ± 0.6, and 13.1 ± 3.4 h, whereas the retention $t_{1/2}$ of 4- and 8-arm 40 kDa nanocarriers were 14.9 ± 5.6 h and 11.9 ± 2.9 h, respectively. The retention $t_{1/2}$ of free doxorubicin was 2.0 ± 0.4 h, which was significantly shorter than that of the linear and branched 40 kDa PEG-DOX nanocarriers. Increased molecular weight and decreased branching both demonstrated a strong correlation to enhanced mammary gland retention. Intraductally administered free doxorubicin resulted in ductal damage, severe inflammation and generation of atypical cell neoplasms, whereas PEG-DOX nanocarriers induced only minor and transient inflammation (i.e., damaged epithelial cells and detached cellular debris). The 40 kDa 4-arm PEG-DOX nanocarrier demonstrated the longest ductal retention half-life, the lowest IC_{50} (i.e., most potent), and minimal ductal damage and inflammation. The current results suggest that PEG-DOX nanocarriers with prolonged ductal retention may present the best option for intraductal treatment of DCIS, due to their low local toxicity and potential for sustained therapeutic effect.

1. Introduction

Doxorubicin (DOX), a cytotoxic anthracycline antibiotic, is one of the most effective anticancer drugs for treating breast cancer, childhood solid tumors, soft tissue sarcomas, and aggressive lymphomas (Minotti et al., 2004; Octavia et al., 2012). Doxorubicin exerts its anticancer

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effects by stabilizing the topoisomerase II complex after it breaks the DNA chain, thus preventing the DNA double helix from being resealed and thereby stopping the replication process (Minotti et al., 2004). Doxorubicin also intercalates between DNA base pairs to prevent macromolecular biosynthesis (Fornari et al., 1994; Momparler et al., 1976). An additional plausible mechanism of action is the generation of reactive oxygen species (Minotti et al., 2004). Although doxorubicin shows high cytotoxicity against malignant cells, its clinical utility is limited by dose-dependent toxicities, such as myelosuppression and cardiotoxicity (Prados et al., 2012). Therefore, developing drug delivery systems (DDSs) that reduce doxorubicin toxicity (especially cardiotoxicity) has been a long term goal in the field.

Systemically administered PEGylated liposomal doxorubicin was shown to decrease cardiotoxicity in patients; however, it introduced some untoward effects, such as mucositis and skin toxicity (Ansari et al., 2017). Delivering doxorubicin directly into the mammary ducts provides an alternative approach to systemic treatment, with the potential advantages of reduced systemic exposure and fewer side effects. In animal models of breast cancer, dose matched intraductally administered PEGylated liposomal doxorubicin was more effective than intravenously injected PEGylated liposomal doxorubicin, with the added benefits of low systemic exposure and only transient local inflammation (Murata et al., 2006; Stearns et al., 2011). Some clinical trials have already proven the feasibility of the intraductal approach to breast cancer therapy, as PEGylated liposomal doxorubicin has been safely administered without causing serious adverse events (Love et al., 2013; Zhang et al., 2014). However, local side effects, such as mild erythema and swelling of the breast, were observed over 72 h. Given the widespread use of doxorubicin in the clinic today, these results suggest that new doxorubicin drug delivery systems are needed. Additionally, there is a need for improving ductal retention and reducing both the dose and toxicity of doxorubicin in the mammary ducts and surrounding tissue, to enhance local treatment of early stage breast cancer.

PEGylation is a process in which poly(ethylene glycol) (PEG) is covalently attached to therapeutic agents (Abuchowski et al., 1977a; Abuchowski et al., 1977b). It was first described in 1977 at Rutgers University, by the modification of bovine serum albumin (Abuchowski et al., 1977b) and liver catalase (Abuchowski et al., 1977a). PEG has been approved by the FDA for oral, intravenous and dermal pharmaceutical applications in humans, due to its non-toxic, non-immunogenic, non-antigenic and amphiphilic properties (Brocchini et al., 2008). Along with reduced immunogenicity and antigenicity, the application of PEGylation helps to increase water solubility, prolong plasma half-life, and reduce drug toxicity (Harris and Chess, 2003; Veronese and Pasut, 2005). While classic PEGylation involves attaching a single PEG to a single drug molecule, it is possible to conjugate more than one drug molecule to a single PEG. We previously found that PEGs are excellent nanometer-sized pharmaceutical carriers, exerting enhanced permeability and retention in tumors (Singh et al., 2012b). We further demonstrated the prolonged mammary retention of PEGs in rats, suggesting their usefulness as potential drug carriers for intraductal treatment of breast cancer (Singh et al., 2012a).

In the current report, mammary gland retention and toxicity of PEG-DOX nanocarriers were investigated. PEGs of varying molecular weights (5, 10, 20, 40 kDa) and structures (linear, 4- and 8-arm) were conjugated to doxorubicin, followed by a series of physicochemical characterizations. Cytoxicity was investigated using the MCF7 cell line (a breast cancer cell line), and ductal retention and local toxicity were studied in Sprague-Dawley rats.

2. Materials and methods

2.1. Materials and instruments

Linear and branched (4- and 8-arm) PEG N-hydroxyl succinimide (NHS) esters (MW = 5, 10, 20, 40 kDa) were obtained from NOF America (Whitefield, NY). Doxorubicin hydrochloride was obtained from Changsha Huajia Chemicals (Hunan, China). *N*, *N*-Dimethylformamide (DMF) and Diisopropylethyl amine (DIEA) were obtained from Sigma-Aldrich (St. Louis, MO), whereas Sephadex G50 and G25 (media) were obtained from Thermo Fisher Scientific (Suwanee, GA). All cell culture reagents were received from Invitrogen (Carlsbad, CA), and Aerrane (isoflurane) inhalant anesthetic was purchased from Baxter Healthcare Corporation (Deerfield, IL). The nanocarriers (2 mg/ml) were analyzed by a high performance liquid chromatography (HPLC) system, equipped with ultraviolet (UV) and fluorescence detection, and using a Symmetry 300™ C_{18} column (5.0 µm, 4.6 mm × 50 mm column) (Waters, Milford, MA). The hydrodynamic radii of the polymers were measured by dynamic light scattering on a Malvern Zetasizer Nano ZS. The animal body images were obtained non-invasively, using a Xenogen IVIS® 100 imaging system (Caliper Life Sciences, Hopkinton, MA).

2.2. Synthesis and characterization of PEG-DOX nanocarriers

PEG-NHS (5, 10, 20, 40 kDa linear, and 40 kDa 4- and 8-arm) polymers (200 mg) were dissolved in 10 ml of DMF, containing 20 µl of DIEA. Doxorubicin hydrochloride (2.5 equivalent for each functional group of PEG) was then added to the polymer solution. The reaction mixture was stirred at room temperature, in the dark, for 8 h. Each PEG-DOX nanocarrier reaction mixture was purified on either a Sephadex G25 column (for the conjugates of 5 kDa PEG), or Sephadex G50 columns (for the conjugates of 10, 20 or 40 kDa PEGs), using deionized water as the eluent. The high molecular weight fractions were collected and lyophilized to obtain pure PEG-DOX nanocarriers. The PEG-DOX nanocarriers were then characterized by HPLC, and UV and fluorescence spectrometry.

2.3. Hydrodynamic radii measurements

The hydrodynamic radii of the PEG-DOX nanocarriers were measured at room temperature, using dynamic light scattering. Prior to each measurement, the PEG-DOX nanocarriers were dissolved into deionized water to make a clear solution, then passed through a 0.22 µm membrane filter. All studies were done in quintuplicate, and the data are presented as mean ± SD.

2.4. Cell culture

MCF7, a human breast cancer cell line, was obtained from the American Type Culture Collection (Manassas, VA), and maintained according to the repository's instructions. Briefly, MCF7 was maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Fisher Scientific, Fairlawn, NJ), 1% penicillin/streptomycin and 0.01 mg/ml insulin, at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) in air.

2.5. In vitro cytotoxicity studies

Cytotoxicity was determined using the MTT assay, as previously reported (Ibrahim et al., 2014). MCF7 cells were seeded into 96-well plates, at a density of 4000 cells per well. After incubating for 24 h, the cells were treated with either PEG-DOX nanocarriers or free doxorubicin. Following an additional 48 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, St. Louis, USA) solution (5 mg/ml) was added to each well. The medium was removed 2 h later, and DMSO was added to solubilize the formazan crystals. The absorbance, at 570 nm, was measured using a plate reader. IC_{50} values were also determined.