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The interaction of dendrimer-doxorubicin conjugates with a model pulmonary epithelium and their cosolvent-free, pseudo-solution formulations in pressurized metered-dose inhalers

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ABSTRACT

Oral inhalation (OI) of nano-chemotherapeutics holds great potentials in the treatment of lung cancers as it enables direct targeting of drugs to lung tissues, spatial and temporal control of drug release, and decrease in drug-associated systemic and local lung toxicity. Therefore, the design of chemistry of the nanocarriers and their OI formulations for chemotherapeutics delivery to the peripheral lungs and extrapulmonary tissues of relevance such as lymph nodes, may thus afford new opportunities for treating such relevant diseases. In this work we investigated the effect of polyethylene glycol 1000 Da (PEG1000) density and doxorubicin (DOX) payload on the interaction of poly(amidoamine) dendrimer (PAMAM) with an in vitro pulmonary epithelium model (Calu-3). DOX, which was conjugated to the PAMAM through a pH-labile bond, showed a strong time-dependent cell kill against Calu-3 cells due to sustained DOX release. The conjugation of DOX to PEGylated PAMAM dendrimers significantly enhances DOX transport across pulmonary epithelium compared to free drug, with the rate of transport increasing as PEGylation degree increases. Transient interaction of PEGylated dendrimers with cellular junctions of the polarized epithelium as probed by a reduction in transepithelial electrical resistance, faster mucus diffusion, along with reduced cellular internalization compared to the non-PEGylated counterpart promotes transport across the epithelial barrier. A cosolvent free method was developed to formulate PEGylated PAMAM-DOX conjugates in pressurized metered-dose inhalers. The resulting aerosol formulations show a very high final particle fractions (> 82%). We further demonstrate that aerodynamic particle size distribution of the nanoconjugates can be tweaked with the addition of a biodegradable lactide-based copolymer, which may help tune lung deposition of PAMAM-DOX conjugates to a specific pulmonary area. The combined results suggest that conjugation to PAMAM dendrimers and their surface modification with PEG1000 can be utilized to modulate the transport of DOX across pulmonary epithelium, and also to easily formulate the conjugates in propellant-based inhalers for pulmonary administration of anticancer therapeutics.

1. Introduction

Lung cancers are the leading cause of cancer death for both men and women worldwide (Siegel et al., 2013). Chemotherapy is widely used as a tool to solely or combinatorially treat lung cancers (Lee et al., 2016; Wood et al., 2014). However, one of major challenges that reduce the effectiveness of chemotherapies in the treatment of lung cancer is the low therapeutic concentration (< 5% of administered dose) in lung tumors upon systemic administration (Cheng et al., 2009; Zhu et al., 2010), as is the case for doxorubicin (DOX). While DOX is one of the leading chemotherapeutics in the fight against cancers in general, its low concentrations in the lung tissue, fast plasma clearance, and severe toxicity limit its applicability in the treatment of lung cancer.

The lungs may also serve a portal for drug delivery to

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extrapulmonary tissues and systemic circulation (Koushik et al., 2004; Mohammad et al., 2013). One can thus envision the use of the pulmonary route as a standalone therapy for localized lung tumors, as an adjuvant therapy supplemented to i.v.-based chemotherapy for lung metastases (Otterson et al., 2007), or target other tissues which are hard to reach upon systemic administration. For example, it has been shown that the nanocarriers administered to the lungs can potentially accumulate in the various lymph nodes to a much greater extent than upon i.v. route (Choi et al., 2010; Lehnert et al., 1986; Mohammad et al., 2013; Zhong et al., 2016b). Such efficient targeting of chemotherapeutics to lymph nodes may be a promising strategy to inhibit cancer metastasis through or to lymph nodes and treat primary/secondary lymphomas. The pulmonary delivery of chemotherapeutics thus holds great clinical significance and relevance in the treatment of lung cancer. However, respiratory epithelium represents a first-line barrier for chemotherapeutics topically administered to lungs. The intercellular tight junctional protein complexes serve as checkpoints to select and modulate molecules that transport across epithelial layers (Kondoh and Yagi, 2007). Additionally, mucus layers covered on respiratory epithelium significantly entrap inhaled particulates. Such defense systems may significantly reduce effectiveness of chemotherapeutics administered to respiratory epithelium. Therefore, the increase in the transport of drugs across the pulmonary epithelium will enhance drug availability to lung tissues.

Dendrimers are the nanocarriers of particular interest to chemotherapeutics as their multivalent surface groups can be utilized not only for carrying the drug payload, but also for the conjugation of ligands to modulate the interactions with cells/tissues (Cheng et al., 2008; Ganda et al., 2017) including the pulmonary epithelium (Heyder et al., 2017), as well as targeting subcellular organelles of relevance to cancers (Albertazzi et al., 2010; Bielski et al., 2015). Prior studies have demonstrated PEGylated dendrimers increase the transepithelial transport of carried molecules and the transport rate varies with several factors including dendrimer generations, surface groups, surface charges, and attached ligands (Bharatwaj et al., 2015; Jevprasesphant et al., 2004; Jevprasesphant et al., 2003; Kitchens et al., 2006; Lin et al., 2010; Sweet et al., 2009). The conjugation of DOX to dendrimers through pH-sensitive bonds has shown to reduce DOX-associated lung tissue toxicity which has been regarded as a major drawback of inhalable anticancer drugs (Kaminskas et al., 2014; Zhong et al., 2016a).

The ability to combine the controlled and targeted release of anticancer therapeutics conjugated to dendrimer nanocarriers (DNCs) and oral inhalation (OI) formulations for local delivery of drugs to the lung tissue has, therefore, the potential to develop innovative strategies for the treatment of lung cancer (Bai et al., 2007; Bielski et al., 2017). pMDIs are OI devices of great relevance due to their portability, ease of use and low cost (Myrdal et al., 2014). Furthermore, drug deposition of pMDI formulations is mainly driven by propellant vaporization instead of inspiratory forces (Labiris and Dolovich, 2003), thus providing for an opportunity to achieve improved lung deposition even in the patients with compromised lung function. However, the low solubility of most drugs of interest in FDA-approved hydrofluoroalkane (HFA) propellants for pMDIs is a major challenge in the development of such formulations (Zhu et al., 2015). Another challenge to be addressed in local delivery to lungs is the ability to target different regions of the pulmonary epithelium, such an ability is inspired by in lung cancers as such malignances, may occur in the upper airways (Carvalho et al., 2011). In this work, we use a strategy in which a biodegradable polymeric excipient is employed to modulate aerosol size and thus target different lung region.

In prior studies, we have reported the synthesis and in vitro/in vivo antitumor activity of pH-sensitive dendrimer-DOX conjugates with the temporal/spatial control of drug release, and have also developed a superior pseudo-solution formulation of the conjugates in pMDI with a trace of cosolvent (Zhong et al., 2016a; Zhong and da Rocha, 2016). One of the goals of this work was to assess the effects of PEGylation degree and DOX payload on the interaction of these pH-sensitive dendrimer-DOX conjugates with an in vitro model of the pulmonary epithelium - Calu-3 monolayer. The cellular internalization, transepithelial transport and cell kill were assessed. The effects of the mucus layer and the epithelial barrier on transport were decoupled by assessing the impact of PEGylation and DOX payload on the transport across synthetic mucus. Another goal of the work was to develop a cosolvent free formulation of the conjugates in pMDIs that can tweak the aerosol deposition towards particular lung regions. A melting strategy was used to prepare co-solvent free formulations. Biodegradable polymeric excipients (*co*-polymers of polylactide-polyethylene glycol) were added to the formulations to tune lung depositions of aerosol in accordance with tumor sites on respiratory tract.

2. Materials

Generation 3, amine-terminated poly(amindo amine) (PAMAM) dendrimer (G3NH2, 32 -NH_2 surface groups, theoretical molecular weight = 6909) was purchased from Dendritech, Inc. (Miland, MI, USA). Doxorubicin hydrochloride salt (DOX) was purchased from LC Laboratories (Woburn, MA, USA). Methoxy polyethylene glycol succinimidyl ester, 1000 Da (PEG1000-SE) was purchased from NANOCS, Inc. (New York, NY, USA). *cis*-Acontyl anhydride, sodium hydroxide (NaOH), stannous octoate (95%), 4% para-formaldehyde phosphate buffer saline and mucin from porcine stomach (type III, bound sialic acid 0.5–1.5%) were purchased from Sigma-Aldrich (St Louis, MO, USA). D, L-lactide was a gift from Purac Biomaterials (Amsterdam, Netherland). Dulbecco's Modified Eagle's Medium (DMEM) and penicillin (10,000 U/ml)-streptomycin (10,000 μg/ml) were purchased from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA, USA). Deuterated dimethylsulfoxide ($DMSO\_d_6$) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Ultrapure deionized water (DI H_2O) was obtained from a Barnstead NANOpure DIamond System from Thermo Fisher Scientific (Waltham, MA, USA). Calu-3, a human lung adenocarcinoma epithelial cell line, was purchased from American Cell Collection (Manassas, VA, USA). Amicon® Ultra 15 centrifugal filter device (MWCO = 3000 Da) was purchased from EMD Millipore (Billerica, MA, USA). Costar Transwell® Permeable Support (pore size: 0.3 μm; surface area = 0.33 cm^2) was purchased from Corning Incorporated (Corning, NY, USA). Thin layer chromatography (TLC) Silica gel 60 F_{254} plastic sheet was purchased from Merck KGaA (Darmstadt, Germany). All reagents were used as received unless otherwise stated.

3. Methods

3.1. Cell culture

Calu-3 cells (passages 10 to 15), an adenocarcinoma human airway epithelial cell line, were plated in Corning™ cell culture treated 75 cm^2 flasks (canted neck and vented cap) at a density of 10^4 cells/ml, and cultured in DMEM supplemented with 20% FBS (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were grown in thermally controlled Thermo Scientific™ CO_2 incubator (Thermo Fisher Scientific) with constant purging of 5% CO_2 at 37 °C. The medium was exchanged every two days, and the cells were split when they reached ca. 80% confluence.

3.2. Ability of acid-labile PEGylated dendrimer-DOX conjugates (G3NH2-mPEG1000-nDOX, m = 0, 9, 21, and n = 3, 7) to kill human lung adenocarcinoma cells (Calu-3)

The synthesis and characterization of pH-sensitive G3NH2-mPEG1000-nDOX conjugates has been previously discussed in detail (Zhong and da Rocha, 2016) and important physicochemical properties