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Development of a liposome formulation for improved biodistribution and tumor accumulation of pentamidine for oncology applications

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ABSTRACT

Pentamidine isethionate, widely used for the treatment of parasitic infections, has shown strong anticancer activity in cancer cells and models of melanoma and lung cancer. Systemic administration of pentamidine is associated with serious toxicities, particularly renal, affecting as many as 95% of patients (O'Brien et al., 1997). This work presents the development of a liposome pentamidine formulation for greater tumor accumulation and lower drug exposure to vulnerable tissues. Liposomes formulated with saturated/unsaturated phospholipids of different chain lengths, varying cholesterol content, and surface PEG were explored to understand the effects of such variations on drug release, encapsulation efficiency, stability and *in vivo* performance. Saturated phospholipids with longer chain lengths, higher cholesterol content and PEG resulted in greater stability. The optimal formulation obtained showed significantly lower clearance rate (3.6 ± 1.2 mL/h/Kg) and higher AUC_{0-inf} (348 ± 31 μmol/L × h) *in vivo* when compared to free drug (414 ± 138 mL/h/Kg and 2.58 ± 0.74 μmol/L × h, respectively). In tumor-bearing mice, liposomal delivery decreased kidney drug levels by up to 5-fold at 6 and 24 h post-administration. Tumor drug exposure was up to 12.7-fold greater with liposomal administration compared to free drug. Overall, the liposomal pentamidine formulation developed has significant potential for the treatment of solid tumors.

1. Introduction

Pentamidine, a dicationic diarylfuran used clinically as pentamidine isethionate (Nguewa et al., 2005; Qiu et al., 2012), is a therapeutic historically employed for the treatment of leishmaniasis caused by the parasite *Leishmania donovani* (Singh and Dey, 2007; Lopes et al., 2014), as well as other parasitic diseases including trypanosomiasis, pneumocytosis and babesiosis (Pathak et al., 2002). Although the precise mechanism of its antiparasitic action remains poorly understood (Pathak et al., 2002; Nguewa et al., 2005), drug action is in part due to inhibition of topoisomerase II (Singh and Dey, 2007). Pentamidine has also been found to bind to AT-rich DNA regions, but this has not been shown to influence its antimicrobial action (Choudhury and Leibowitz, 2003; Jung et al., 2011).

Besides its role in combating parasitic infections, the anticancer effects of pentamidine have emerged relatively recently (Zerbini et al., 2014). It has shown promising antiproliferative activity *in vitro* in renal cell carcinoma, melanoma, ovarian, lung, breast, and prostate cancer cell lines, and *in vivo* in tumor models of melanoma and lung carcinoma (Pathak et al., 2002; Chow et al., 2004; Jung et al., 2011; Zerbini et al., 2014). The mechanism of action of pentamidine against cancer cells appears to be multifactorial, and its effects on cancer-relevant targets are continuously being elucidated. Pentamidine inhibits endo-exonuclease, an enzyme overexpressed in various cancers that is involved in DNA double-stranded break repair (Qiu et al., 2012; Zerbini et al., 2014), and phosphatase of regenerating liver kinases (PRLs), which are

Abbreviations: PEG, polyethylene glycol; DNA, deoxyribonucleic acid; TRAIL, TNF-related apoptosis-inducing ligand; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; MS, mass spectrometry; UV, ultraviolet; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitylphosphatidylcholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; EPC, egg phosphatidylcholine; HBS, HEPES buffered saline; SCID, severe combined immunodeficiency; chol, cholesterol; %ID/g, percent injected dose per gram; MTD, maximum tolerable dose; MPS, mononuclear phagocyte system; PLGA, poly (lactic-co-glycolic acid); PC, phosphatidylcholine; EPR, enhanced permeability and retention.

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oncogenic and abundantly found in various cancer types (Pathak et al., 2002). Recent evidence reveals that pentamidine can hinder the expression of hypoxia-inducible factor 1 alpha (HIF-1α), a transcription factor involved in response to hypoxia crucial for cancer cell survival, by interfering with protein translation in prostate and breast cancer cell lines (Jung et al., 2011). The drug's demonstrated inhibitory action on topoisomerase II in parasites is likely to contribute to its antitumor effects, as topoisomerase II inhibitors prevent DNA strand separation during mitosis and lead to single and double-stranded DNA breaks that accumulate and eventually result in cell death (Imreh et al., 2011).

Pentamidine also shows promise in potentiating chemotherapy when used in combination. For instance, it sensitizes myelogenous leukemia cells to TRAIL-mediated apoptosis, a mode of apoptotic induction exclusive to cancer cells (Qiu et al., 2012). It also yields chromosome segregation defects, contributing to synergism when used with microtubule-targeting agents in tumor models (Lee et al., 2007). Further, its anti-endo-exonuclease activity may sensitize cells to the DNA-damaging action of radiotherapy, and in fact shows synergism with DNA-damaging agents *in vivo* (Chow et al., 2004). Exploratory studies have recently assessed pentamidine's anti-cancer potential in non-small cell lung cancer (NCT01844791) and metastatic colorectal cancer (NCT01378143) in combination with conventional chemotherapeutics.

Although successful in the treatment of parasitic infections, systemic administration of pentamidine has posed serious toxicity concerns, particularly renal (Smith et al., 2010). In a range of studies involving intravenous pentamidine administration, nephrotoxicity occurred in as many as 95% of patients (O'Brien et al., 1997). The present work focuses on the development of an intravenous pentamidine formulation, with the aim of achieving decreased drug levels within tissues vulnerable to toxicity, and increased accumulation at tumor sites, both of which are issues faced with currently available parenteral formulations. To this end, we chose to formulate pentamidine in liposomal form. This drug has a high water solubility of >100 mM (Kitamura et al., 1995) and a Log *P* of 2.66 (Bento et al., 2014). Due to its amphiphilic nature, pentamidine encapsulation into the aqueous core of liposomes is feasible. Liposomes have been extensively explored for therapy and diagnosis of cancer due to their enhanced accumulation at tumor sites, which spares healthy tissues from drug-related toxicities while increasing drug exposure to target sites (Jia et al., 2013). Following intravenous administration, superior liposomal deposition into tumors relies on the enhanced permeability and retention (EPR) effect, where heightened permeability of tumor vasculature and deficient lymphatic clearance allow for extravasation and consequently increased accumulation of such nano-formulations in tumor tissues compared to healthy tissues (Matsumura and Maeda, 1986; Ait-Oudhia et al., 2014).

Various liposome formulations were developed with differing pentamidine release kinetics, achieved by employing phospholipids with the same polar head group but different hydrocarbon chains, with shorter chain lengths theoretically providing faster release *in vivo*, and exploring the amount of cholesterol (chol) in the liposome membrane to examine their impact on stability. Further, the presence or absence of polyethylene glycol (PEG) was studied. Five superior formulations were selected for *in vitro* evaluation of encapsulation and release of pentamidine in media, in an effort to predict release behavior in the blood compartment. Pharmacokinetics and biodistribution studies of the five formulations were done in non-tumor-bearing mice. One optimal formulation was chosen for tumor accumulation and biodistribution studies in mouse models of colorectal, lung, ovarian and breast cancers.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitylphosphatidylcholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] ammonium salt (DSPE-mPEG$_{2000}$) were purchased from Corden Pharma Switzerland LLC (Liestal, Switzerland), egg phosphatidylcholine (EPC) from Avanti Polar Lipids (Alabaster, USA), [^{3}H] pentamidine isethionate from Moravek (California, USA), Solvable® tissue solubilizer from PerkinElmer (Waltham, USA), USP NaCl 0.9% sterile and hionic-fluor from the medical store at the University of Toronto. Pentamidine isethionate (referred to as "pentamidine" going forward) was provided by Oncozyme Pharma (Montreal, Canada). HPLC-grade 100% methanol was purchased from Caledon Laboratories Ltd. (Georgetown, Canada). Chol and all other chemicals unless otherwise specified were purchased from Sigma–Aldrich (Oakville, Canada). Fetal bovine serum (FBS) was obtained from Sigma–Aldrich (Oakville, Canada). McCoy's 5A and RPMI 1640 media, as well as penicillin-streptomycin solution were purchased from Life Technologies (Burlington, Canada). DMEM/Ham F12 medium (1:1) was obtained from Gibco Life Technologies (Grand Island, USA).

2.2. HPLC Analysis

Analysis of non-radioactive pentamidine was performed by HPLC (Agilent Technologies 1200 series) with UV detection (Waters 2487 Dual λ Absorbance Detector) at a wavelength of 265 nm. Briefly, a mobile phase gradient was employed using a mixture of 100% methanol and ammonium acetate buffer (3% NH_4Ac w/w, pH 7.2). The ammonium acetate buffer was prepared by dissolving 60 g of ammonium acetate in 2 L of deionized water with adjustment of the pH to 7.2 using ammonium hydroxide (6 N aqueous solution). The buffer was subsequently filtered with a 0.45 μm membrane filter (Pall Corporation, Ann Arbor, USA) prior to use for HPLC. A 65/35 (v/v) mixture of methanol and ammonium acetate buffer, respectively, was used as a diluent for the preparation of samples and standards. Accordingly, liposomal samples of pentamidine were diluted 1:1000 (before dialysis) or 1:100 (after dialysis), vortexed thoroughly and subsequently injected as a 20 μL volume onto an XTerra® MS C18 reversed-phase column (5 μm particle size, 4.6 mm × 250 mm dimensions; Waters Ltd., Mississauga, Canada). The pentamidine exhibited a retention time of 5.7 min at a mobile phase flow rate of 0.8 mL/min. Drug quantification was performed by injecting a set of standards of known drug concentration and generating a calibration curve with each run. All standards and samples were prepared in triplicate.

2.3. Preparation of liposome formulations

The liposome formulations were composed of DSPC, DPPC, DMPC or EPC, chol and mPEG$_{2000}$-DSPE (PEG) at different molar ratios (Fig. 1). A formulation composed of DSPC: chol: PEG in the molar ratio of 55:45:5 was used in the liposome formulation that was carried forth to *in vivo* tumor uptake studies, referred to here as "45% chol." Molar ratios for the other formulations were as follows: 60:35:5 in the "35% chol" formulation, 70:25:5 in the "25% chol" formulation, and 55:45:0 in the "PEG-free" formulation.

For liposome preparation, the phospholipid, chol and PEGylated lipid were dissolved in chloroform, evaporated under N_2 with slight agitation, dried under vacuum overnight and warmed to 72 °C. Pentamidine was dissolved in HBS buffer (pH 7.4) and pre-