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Research paper

Dendronized nanoconjugates of lysine and folate for treatment of cancer

Keerti Jain, Umesh Gupta, Narendra K. Jain *

Department of Pharmaceutical Sciences, Dr. H.S. Gour Central University, Sagar, India

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ABSTRACT

Poly-L-lysine (PLL) dendrimers are currently being investigated as antiangiogenic agent for therapy of cancer. In this study, we report folate conjugated poly-L-lysine dendrimers (FPLL) as an efficient carrier for model anticancer drug, doxorubicin hydrochloride (Dox); for pH sensitive drug release, selective targeting to cancer cells, anticancer activity and antiangiogenic activity. This nanoconjugate of Dox showed initial rapid *in vitro* release followed by gradual slow release, and the drug release was found to be pH sensitive with greater release at acidic pH. In the CAM assay and tubule formation assay with HUVEC, Dox-FPLL formulation showed the significant antiangiogenic activity confirming that activity of PLL was not compromised by the presence of Dox and folic acid. The *ex vivo* investigations with human breast cancer cell lines MCF-7 showed enhanced cytotoxicity of Dox-FPLL with significantly enhanced intracellular uptake ($p < 0.001$). The *in vivo* therapeutic potential of nanoconjugate was determined in MCF-7 breast cancer xenograft model in tumor-bearing mice. Dox-FPLL increased the concentration of Dox in tumor by 121.5-fold after 24 h in comparison with free Dox formulation. The folate conjugated dendrimeric Dox showed superior anti-tumor activity in tumor xenograft model with significantly prolonged survival determined by Kaplan Meier survival analysis ($p < 0.001$).

1. Introduction

Tumor growth as well as metastasis strongly depends on the formation of neovasculature *via* angiogenesis hence inhibition of neovascularization is an important strategy for the treatment of cancer. The combination of chemotherapy with inhibition of angiogenesis can result in potentially improved outcome, since the conventional chemotherapy based treatment for cancer is greatly hampered by serious adverse effects with emergence of resistance and results in reduced therapeutic benefit [1,2]. A nanocarrier that can simultaneously deliver anticancer drug with inhibition of angiogenesis may emerge as a promising therapeutic approach for cancer treatment [3–5].

Dendrimers present a well defined three-dimensional polymeric scaffold, which is synthesized in a step-wise controlled manner, achieved by attaching branching units to a radiating focal point known as core. Some dendrimer molecules including poly-L-lysine (PLL) dendrimers have shown intrinsic antiangiogenic activity in previous reports. A thorough investigation on antiangiogenic activity of PLL dendrimers showed reduced vascularization with significant reduction in apoptosis/necrosis of tumor tissue but a moderate regression effect on growth of tumor. This therapeutic effect of PLL dendrimers was equivalent to commercially available antiangiogenic agent, Avastin (anti-VEGF antibody bevacizumab) without significant toxic effect on non-cancerous tissue [6,7]. Folate receptor is highly expressed on a number of malignant

Abbreviations: AFM, atomic force microscopy; ANOVA, analysis of variance; AUC, area under the curve; AUMC, area under the first moment curve; CDH, central drug house; CAM, chorioallantoic membrane assay; CL_t, total clearance; DCC, *n*,*n*-dicyclohexyl carbodiimide; Di-t-Boc, di-tertiary butyl pyrocarbonate; di-t-Boc-lysine, di-tertiary butyl pyrocarbonate protected lysine; DLS, dynamic light scattering; DMEM, Dulbecco's Modified Eagle's Medium; Dox, doxorubicin hydrochloride; Dox-FPLL, doxorubicin loaded folate conjugated poly-L-lysine dendrimers; Dox-PLL, doxorubicin loaded poly-L-lysine dendrimers; EDA, ethylenediamine; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; EPR, enhanced permeation and retention; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FPLL, folate conjugated poly-L-lysine dendrimers; FTIR, fourier transform infrared spectroscopy; HoBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; K_{el}, elimination rate constant; MCF-7, Michigan Cancer Foundation-7; MTT, methylthiazole tetrazolium assay; MRT, mean residence time; NCCS, National Center for Cell Sciences; NMR, nuclear magnetic resonance spectroscopy; PBS, phosphate buffer saline; PDI, polydispersity index; PLL, poly-L-lysine dendrimers; SEC, size exclusion chromatography; $t_{1/2}$, biological half life; TEA, triethyl amine; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; VEGF, vascular endothelial growth factor.

* Corresponding author. Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr. H.S. Gour Central University, Sagar, M.P. 470003, India. Tel./fax: +91 7582 264712.

E-mail addresses: keertijain02@gmail.com (K. Jain), jnarendr@yahoo.co.in (N.K. Jain).

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tumor tissues including breast and lung cancers. Hence, folate conjugated nanocarriers are extensively being explored for site specific delivery of anticancer agents to cancer cells in order to reduce toxicity of anticancer drugs with increased efficacy [8,9]. The present investigation is based on the hypothesis that PLL dendrimers conjugate with a targeting agent, folic acid, might be a promising strategy to further improve the anticancer activity.

In this study we are exploring doxorubicin (Dox) loaded and folic acid conjugated PLL (FPLL) dendrimers (dendronized nanoconjugates of lysine and folic acid) for antiangiogenic activity, anticancer activity and controlled delivery of anticancer drug in *ex vivo* studies with human breast cancer cell lines MCF-7 and *in vivo* studies with breast cancer xenograft model in tumor-bearing mice.

2. Material and methods

2.1. Materials

The drug Dox was a benevolent gift received from M/s Fresenius Kabi Oncology Ltd. (Ghaziabad, India). Di-tertiary butyl pyrocarbonate (di-t-Boc), di-t-Boc protected lysine (di-t-Boc-lysine) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from HiMedia Pvt. Ltd., Mumbai (India). Ethylenediamine (EDA), trifluoroacetic acid (TFA), 1-hydroxybenzotriazole (HoBt), and n,n-dicyclohexyl carbodiimide (DCC) were purchased from Spectrochem, India. Folic acid was procured from CDH, India. Nylon membrane filter (0.22 μm and 0.45 μm) was obtained from Pall Gelman Sciences, USA. All other chemicals were of analytical reagent grade and used without further modification.

2.2. Synthesis and characterization of FPLL dendrimers

PLL dendrimers were synthesized using divergent growth method *via* repetition of coupling and deprotection step using the reported procedure, with slight modifications [10–12] (Fig. 1). Folic acid conjugation to the amino groups of PLL dendrimers was conferred after protection of amino group of folic acid with di-t-Boc. After di-t-Boc protection the γ-COOH group of folic acid was activated using EDC by following a reported procedure with slight modifications [13–15] (Fig. 2). The synthesis of PLL and FPLL dendrimers was assessed by FTIR, ^{1}H NMR spectroscopy, size exclusion chromatography (SEC), dynamic light scattering (DLS), transmission electron microscopy (TEM), and atomic force microscopy (AFM).

2.3. Drug loading and in vitro drug release studies

Dox was loaded into the dendrimers using triethylamine (TEA) following the reported procedure, with slight modifications [8,10]. The *in vitro* drug release profile of Dox was determined at two different pHs, in PBS pH 6.4 and PBS pH 7.4 to simulate the pH of interstitial spaces and endosomes in tumor and pH of blood plasma, respectively. The amount of Dox released was determined by HPLC on a C18 column with a flow rate of 0.2 mL/min and detected at a wavelength of 481 nm [16,17].

2.4. Antiangiogenic activity

In vivo chick embryo chorioallantoic membrane (CAM) assay and inhibition of tubule formation by human umbilical vein endothelial cells (HUVEC) were used to determine the antiangiogenic activity. The CAM assay and tubule formation assay were carried out according to the reported procedures, with slight modifications [7,18].

2.5. In vitro cytotoxicity assay and cancer cell growth inhibition

Cytotoxicity of developed formulations was determined against MCF-7 human breast cancer cell lines purchased from National Center for Cell Sciences (NCCS), Pune, India. MCF-7 cells were seeded in 96-well microculture plates at 1×10^4 cells/well in Dulbecco's Modified Eagle's Medium (DMEM; HiMedia, Mumbai, India) supplemented with 10% heat inactivated fetal bovine serum (FBS; HiMedia, Mumbai, India) and incubated for 24 h at 37 ± 0.5 °C in 5% CO_2 incubator. Formulations (PLL, FPLL, Dox-PLL, Dox-FPLL and Dox) were diluted to various concentrations in culture medium and incubated with MCF-7 cells for 24 h, 48 h and 72 h, respectively. Then percent cell growth inhibition was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] assay standard protocol [2,5].

2.6. Cellular uptake studies

Cellular uptake of developed dendrimeric carrier was determined following the reported procedure, with slight modifications [2,9]. Briefly, MCF-7 cells were incubated with dendrimeric formulations (Dox, Dox-PLL and Dox-FPLL) at 37 °C with 5% CO_2 for 1, 2 and 4 h. Then the medium was removed and cells were washed three times with PBS. The fluorescence due to uptake of formulation was visualized qualitatively (Inverted microscope; Leica, Germany) and analyzed quantitatively with FACSCalibur Flow Cytometer (Beckton, Dickinson Systems, FACS canto™, USA). Further, cells were pre-incubated with excess of free folic acid (10 μM) for 2 h before formulation treatment to assess the effect of conjugated folic acid on the uptake of dendrimeric formulation.

2.7. In vivo studies

The anticancer activity and targeting efficiency of developed formulations were confirmed by performing *in vivo* studies in Balb/C mice. *In vivo* studies comprised of three parts *i.e.* pharmacokinetic studies to elucidate the release behavior and pharmacokinetics of formulation, biodistribution studies to confirm targeting potential, and tumor inhibition studies to verify anticancer activity of dendrimeric formulations of Dox. The *in vivo* studies were performed according to the protocol duly approved by the Institutional Animal Ethical committee, Dr. H.S. Gour University, Sagar, M.P., India [letter number Animal Eths. Comm. 09/DB/177(1)].

2.7.1. Pharmacokinetic studies

For pharmacokinetic studies mice were divided into three groups each group having 6 mice. First group was given intravenously the free drug (Dox) solution (in 0.9% sodium chloride) at a dose of 5.0 mg/kg body weight. Second and third groups were treated intravenously with Dox-PLL and Dox-FPLL formulation at a dose equivalent to free drug (5.0 mg/kg body weight), respectively. Samples were collected at different time intervals by drawing blood from retro-orbital plexus of eye and amount of Dox was estimated by HPLC after loading this clear supernatant in HPLC system (Shimadzu, C18, Japan). Amount of Dox was determined according to previously reported method, with slight modifications [19,20].

2.7.2. Anti-tumor activity and survival analysis

For inducing tumor, mice were inoculated subcutaneously with 1×10^6 MCF-7 cancer cells in 100 μL PBS on the right shoulder of animals. Mice were monitored each day for tumor growth after injecting cell lines by palpating area with index finger and thumb. Mice were divided into four groups; first three groups were treated with Dox-PLL, Dox-FPLL formulation and plain drug (Dox) solution, respectively at a dose having equivalent amount of Dox (5.0 mg/kg