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Effective melanoma cancer suppression by iontophoretic co-delivery of STAT3 siRNA and imatinib using gold nanoparticles

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ABSTRACT

Co-delivery of chemotherapeutic agents improve anti-tumor efficacy and reduce cancer resistance. Here, we report development of layer-by-layer assembled gold nanoparticles (LbL-AuNP) containing anti-STAT3 siRNA and imatinib mesylate (IM) to treat melanoma. The combination treatment with STAT3 siRNA and IM in B16F10 melanoma cells showed greater suppression of STAT3 protein, decreased cell viability and increased apoptotic events compared with LbL-AuNP containing either STAT3 siRNA or IM. *In vivo* efficacy studies in melanoma tumor bearing mice showed that non-invasive topical iontophoretic administration (0.5 mA/cm^2) of LbL-AuNP was comparable with intratumoral administration. Co-delivery of STAT3 siRNA and IM using LbL-AuNP showed significant ($p < 0.05$) reduction in percentage tumor volume, tumor weight and suppressed STAT3 protein expression compared with either STAT3 siRNA or IM loaded LbL-AuNP. Taken together, LbL-AuNP can be developed as nanocarrier system for co-delivery of siRNA and small molecule drugs for topical iontophoretic delivery.

1. Introduction

Melanoma is one of the leading causes of death among all cancer conditions (Ferlay et al., 2015). This aggressive form of skin cancer is associated with proliferation of pigment producing melanocytes in epidermis (Shain and Bastian, 2016). Melanoma treatment includes surgical resection and chemotherapy (Mavrakis et al., 2015). The commonly administered first line of targeted chemotherapeutic agents include dabrafenib, vemurafenib, and tremetinib in patients expressing BRAFV600E mutation (Russo et al., 2014). Imatinib has been investigated in patients expressing KIT mutation and ipilimumab as immune-modulatory therapeutic agent (Johnson and Sosman, 2015). More recently, a combination of chemotherapeutic agents have been investigated to overcome the resistance of cancer cells and improve the efficacy by targeting multiple anti-cancer mechanisms (Long et al., 2014; Kwong and Davies, 2014). Furthermore, combination therapy allows reduction in the required dose of individual chemotherapeutic agents, thereby reducing the potential adverse drug reactions. Such examples include a combination of vemurafenib and tremetinib to inhibit BRAF and MEK signaling molecules (Kwong and Davies, 2014). However, cancer cells were found to develop resistance to these targeted chemotherapeutic agents (Liu et al., 2013). Signal transduction and activator of transcription factor 3 (STAT3) has been found to be constitutively activated in majority of melanomas and is an important target in control of melanoma progression (Cao et al., 2016). However, there were no clinically approved small molecule inhibitors of STAT3. Gene silencing agents such as small interference RNA (siRNA) can be utilized to effectively suppress the production of STAT3 protein. In addition to STAT3 signaling, there are other signaling factors that contribute to melanoma progression. Among those, c-kit protein

Abbreviations: STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA; LbL-AuNP, layer-by-layer assembled gold nanoparticles; IM, imatinib mesylate; SC, stratum corneum; PAMAM, polyamidoamine; SA, sodium alginate; PVP, poly(N-vinyl pyrrolidone); FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; CS, chitosan; Cy3, cyanine3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RP-HPLC, reversed phase-high performance liquid chromatography; PDI, poly dispersity index; FTIR, Fourier transform infrared; DSC, differential scanning calorimetry; PBS, phosphate buffered saline; PKC, protein kinase C; VEGF, vascular endothelial growth factor.

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(CD117) is a membrane-bound tyrosine kinase receptor overexpressed in melanoma (Smalley et al., 2009). C-kit receptor stimulation activates the micropthalmia-associated transcription factor expression and downregulation of anti-apoptotic protein, Bcl2 (McGill et al., 2002). Here, we utilized imatinib mesylate (IM), a selective c-kit receptor inhibitor to co-deliver with STAT3 siRNA for effective treatment of melanoma. Earlier, we have reported loading of STAT3 siRNA in layer-by-layer assembled gold nanoparticles (LbL-AuNP) (Labala et al., 2016). Similarly, we have also reported development of LbL-AuNP as a nanocarrier for topical delivery of imatinib mesylate (Labala et al., 2015). The LbL-AuNP prepared using sequential adsorption of natural polyelectrolytes, chitosan and sodium alginate resulted in stable nanoparticles with lower cytotoxicity compared with synthetic polyelectrolytes of polyethylene imine and polystyrene sulfonate (Labala et al., 2016, 2015).

Early stages of melanoma is localized within the epidermal layer of skin (Shain and Bastian, 2016; Gray-Schopfer et al., 2007). Application of chemotherapeutic agents topically in the localized region is advantageous for effective management of the cancer. This localized delivery also limits the exposure of healthy internal tissues to cytotoxic chemotherapeutic agents, and thereby improve the quality-of-life of patients. However, the formidable barrier property of skin would preclude permeation of active agents (Prausnitz and Langer, 2008). To overcome this skin barrier property, various chemical and physical enhancement techniques have been developed. The first generation physical permeation enhancement technique, iontophoresis utilizes small electric current ($\leq$0.05 mA/cm^2) to drive the charged drug/carrier systems across the outer-most layer of skin, stratum corneum (SC) (Prausnitz and Langer, 2008; Barry, 2001). Iontophoresis enhances the skin penetration by a combination of electrorepulsion and electroosmosis (Guy et al., 2000). Our group and others' have widely reported on the application of iontophoresis for non-invasive drug transport through skin (Venuganti et al., 2015; Huber et al., 2015). Topical iontophoresis of generation 4 PAMAM dendrimer-antisense oligonucleotide nanocomplex against Bcl2 anti-apoptotic protein reduced the skin tumor volume by 55% compared with control treatment (Venuganti et al., 2015). Earlier, we have also showed that LbL-AuNP reach viable epidermis after application of 0.5 mA/cm^2 anodal iontophoresis in porcine skin model (Labala et al., 2016).

To that end, the objective of the present study was to prepare and characterize LbL-AuNP containing both STAT3 siRNA and IM, and evaluate the efficacy of combination therapy delivered through topical iontophoresis application in controlling the progression of melanoma.

2. Materials and methods

2.1. Materials

Tetrachloroaurate trihydrate ($HAuCl_4$ $3H_2O$), sodium alginate (SA, MW: 80 kDa), poly(N-vinyl pyrrolidone) (PVP K-30, MW: 40 kDa) and imatinib mesylate (IM) were purchased from Sigma Aldrich (Bengaluru, India). Chitosan (CS, MW: 15 kDa, degree-of-deacetylation: 85%) was purchased from Polysciences Inc., USA. STAT3 siRNA (sense sequence: 5′AAAUGAAGGUGGUGGAGAAUU3′; antisense sequence: 5′UUCUCCACCACCUUCAUUUUU3′) was purchased from Dharmacon Inc., USA. Scrambled sequence siRNA, STAT3 monoclonal antibody, β-actin primary antibody and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc., USA. Silencer® Cy3 labeled siRNA was purchased from Life Technologies Inc., USA. All the cell-based assays were performed in B16F10 murine melanoma cells procured from National Center for Cell Science (NCCS), Pune, India. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Himedia Laboratories (Mumbai, India). Milli Q water (Millipore Inc., USA) was used for all the experiments.

2.2. Preparation of the LbL-AuNP for co-delivery of siRNA and IM

Blank LbL-AuNP (AuNP-CS/SA/CS) and siRNA loaded LbL-AuNP (AuNP-CS/siRNA/CS) were prepared as described in our earlier report (Labala et al., 2016). For the preparation of co-delivery system, IM was entrapped in AuNP-CS/siRNA/CS nanoparticles. AuNP-CS/siRNA particles were incubated with chitosan solution (1 mg/ml) containing IM (20 mg/ml) for 1 h under continuous stirring at 1000 rpm. After incubation, IM loaded AuNP-CS/siRNA/CS nanoparticles were centrifuged at 15000 rpm for 35 min to remove free chitosan and IM. To determine the loading efficiency, supernatant was analyzed by a RP-HPLC method (Labala et al., 2015). The loading efficiency of IM was calculated using Eq. (1).

$$\text{Loading efficiency}\,(\%) = \frac{\textit{Concentration of IM in LbL} - \textit{AuNP}}{\textit{Concentration of LbL} - \textit{AuNP}} \times 100 \quad (1)$$

STAT3 siRNA replaced with scrambled siRNA or sodium alginate (SA) in LbL-AuNP were used as control formulations.

2.3. Characterization of the IM loaded LbL-AuNP

The average particle size, polydispersity index (PDI) and zeta-potential of nanoparticles were determined using Zetasizer (Nano ZS, Malvern Instruments Inc., UK) as described earlier (Labala et al., 2016). Sequential adsorption of polymers on to AuNP-CS core was studied using Fourier transform infrared (FTIR) spectrometer (FT/IR-4200, Jasco Inc., USA). Furthermore, FTIR was also used to characterize the encapsulation of IM in LbL-AuNP. For analysis, sample (1 mg) was mixed with potassium bromide at 1: 100 ratio and a pellet was prepared using high pressure hydraulic press (Riken Seiki, Japan). Spectra were recorded within the range of 4000–400 cm^{-1} at a spectral resolution of 2 cm^{-1}.

Differential scanning calorimeter (DSC-60, Shimadzu, Japan) was used to study the thermal transitions of free IM, blank LbL-AuNP and IM loaded LbL-AuNP. Sample (3 mg) was placed in an aluminum pan and sealed with a lid. Thermograms were recorded at a heating rate of 10 °C per min from ambient temperature up to 450 °C.

2.4. In vitro release of IM from LbL-AuNP

In vitro release of IM from AuNP-CS/SA/CS was studied using a dialysis membrane (molecular weight cut-off of 12–14 kDa, Spectrum Labs, USA) placed in Franz diffusion cell apparatus. Phosphate buffered saline (PBS, pH 7.4) was used as receptor medium. Samples (0.3 ml) were withdrawn from receptor compartment at predetermined time intervals and replaced with fresh PBS maintained at 37 °C. *In vitro* diffusion of equivalent concentrations of free IM across dialysis membrane was also performed. IM release was analyzed using RP-HPLC method (Labala et al., 2015).

2.5. Cell viability studies

B16F10 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. Cells were incubated at 37 °C and 5% CO_2. For cell viability study, cells (1×10^4) were seeded in a 96-well plate 24 h before incubation