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Adenosine conjugated lipidic nanoparticles for enhanced tumor targeting

Rajan Swami [a], Indu Singh [a], Manish Kumar Jeengar [b], V.G.M. Naidu [b], Wahid Khan [a,*], Ramakrishna Sistla [c,*]

[a] Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad 500037, India
[b] Department of Pharmacology, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad 500037, India
[c] Medicinal Chemistry and Pharmacology Division, Indian Institute of Chemical Technology (IICT), Hyderabad 500007, India

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ABSTRACT

Delivering chemotherapeutics by nanoparticles into tumor is impeded majorly by two factors: nonspecific targeting and inefficient penetration. Targeted delivery of anti-cancer agents solely to tumor cells introduces a smart strategy because it enhances the therapeutic index compared with untargeted drugs. The present study was performed to investigate the efficiency of adenosine (ADN) to target solid lipid nanoparticles (SLN) to over expressing adenosine receptor cell lines such as human breast cancer and prostate cancer (MCF-7 and DU-145 cells), respectively. SLN were prepared by emulsification and solvent evaporation process using docetaxel (DTX) as drug and were characterized by various techniques like dynamic light scattering, differential scanning calorimeter and transmission electron microscopy. DTX loaded SLNs were surface modified with ADN, an adenosine receptors ligand using carbodiimide coupling. Conjugation was confirmed using infrared spectroscopy and quantified using phenol–sulfuric acid method. Conjugated SLN were shown to have sustained drug release as compared to unconjugated nanoparticles and drug suspension. Compared with free DTX and unconjugated SLN, ADN conjugated SLN showed significantly higher cytotoxicity of loaded DTX, as evidenced by *in vitro* cell experiments. The IC50 was 0.41 μg/ml for native DTX, 0.30 μg/ml for unconjugated SLN formulation, and 0.09 μg/ml for ADN conjugated SLN formulation in MCF-7 cell lines. Whereas, in DU-145, there was 2 fold change in IC50 of ADN–SLN as compared to DTX. IC50 was found to be 0.44 μg/ml for free DTX, 0.39 μg/ml for unconjugated SLN and 0.22 μg/ml for ADN–SLN. Annexin assay and cell cycle analysis assay further substantiated the cell cytotoxicity. Fluorescent cell uptake and competitive ligand-receptor binding assay corroborated the receptor mediated endocytosis pathway indicated role of adenosine receptors in internalization of conjugated particles. Pharmacokinetic studies of lipidic formulations depicted significant improvement in pharmacokinetic parameters than marketed formulation. ADN conjugated SLN proved to be an efficient drug delivery vehicle. Hence, ADN can be used as a potential ligand to target breast and prostate cancer.

1. Introduction

Identifying cancer hallmarks helps in targeting cancer cells in better ways. Over expression of certain receptors on tumor surface enables them to become hyper responsive to the ambient levels of growth factor that normally would not trigger proliferation (Hanahan and Weinberg, 2011; Wang, 2014). With this advancement of understanding of receptor's role in cancer biology it becomes mandatory to use such information to target and eradicate cancer. There are many receptors such as transferrin receptors, folic acid receptors, estrogen receptors and adenosine receptors *etc.*, which are over expressed on tumor surface. Their respective ligands can be used to actively target drug encapsulated nanoparticles to cancer cells which further increases the efficacy of these nanoparticles (Sagnella et al., 2014).

Adenosine receptors are over expressed on many tumor cell surfaces like prostate, breast melanoma and brain tumors *etc.* (Fishman et al., 2002; Jajoo et al., 2009; Merighi et al., 2003; Wei et al., 2013). Adenosine (ADN) is a purine nucleoside composed of a

* Corresponding authors. Tel.: +91 8790037913 (W. Khan)/+91 4027193753 (R. Sistla).
E-mail addresses: wahid@niperhyd.ac.in (W. Khan), sistla@iict.res.in, rksistla@yahoo.com (R. Sistla).

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molecule of adenine attached to a ribose sugar molecule (ribofuranose) moiety *via* a β-N9-glycosidic bond, has been reported to stimulate or inhibit the release of angiogenic factors depending on the cell type examined. It was documented that ADN and other agonists of adenosine receptors may be useful for eradicating or suppressing the growth of tumor malignancies (Fishman et al., 2002; Merimsky et al., 2003).

In different approaches, many polymer chains were synthesized, where ADN is part of the basic backbone of polymer to play a major role in internalization of DNA cargo in tumor cell *via* adenosine receptors mediated endocytosis (Chung et al., 2011) and decreases in endocytosis was evident when cells were pre-treated with ADN. Decrease was attributed to blockage of adenosine receptors. Hence, considering these reports as basis, it can be assumed that ADN can be used as ligand to target over expressed adenosine receptors on cancer cells and this present study pioneers to investigate the targeting potential of ADN for breast and prostate tumors.

Solid lipid nanoparticles (SLN) are efficient way to deliver such drugs because SLN offers advantages of polymeric nanoparticles, fat emulsion or liposomes with simultaneously avoiding their disadvantages (Akbarzadeh et al., 2013; Mehnert and Mader, 2001; Mueller et al., 2000). SLN found renowned attention in tumor targeting because of their lymph targeting potential which further helps in increasing bioavailability and targeting to breast cancers *etc*. (Singh et al., 2014). Modification of large surface area of SLN with charge modifying lipids such as stearic acid (SA), stearylamine *etc*. can aid in conjugation of ligand on surface (Sood et al., 2013). Consequently, contributes another advantage to SLN for site specific drug delivery.

Docetaxel (DTX) is a semi-synthetic, taxane derived, highly potent anticancer drug. It has shown broad spectrum antitumor activity against prostate, breast, pancreatic, lung, gastric and hepatic carcinomas (Xu et al., 2009; Zhao and Astruc, 2012). DTX binds irreversibly with actin and stabilizes the microtubule assembly which is responsible for inhibition of cell division and finally cell death (Musumeci et al., 2006). Taxotere® marketed formulation for DTX considered to be first line drug for prostate and breast cancer, however their side effects; that may preclude or at least limit their potential clinical application (Schrijvers et al., 1993).

The present study was conducted to establish the efficiency of adenosine receptor ligand namely ADN to target adenosine receptors over expressed cancer cell lines. Prepared ADN nanoconjugates were physicochemically characterized studied for *in vitro* cytotoxicity and their pharmacokinetic parameters.

2. Materials and methods

2.1. Materials

Docetaxel (DTX) was a kindly provided by Therdose Pharma, Hyderabad, India as a gift sample. Adenosine (ADN) and glyceryl monostearate (GMS) were procured from Alfa Aesar, USA and soya lecithin (SL), stearic acid (SA), Tween 80, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC), octadecylamine (ODA) and cellulose dialysis tubing (molecular weight cut off 14,000 Da) were procured from Sigma–Aldrich (Germany). Chloroform, methanol, acetonitrile were of HPLC grade (Merck, India). MCF-7, DU-145 cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. MEM, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], trypsin, EDTA were purchased from Sigma Chemicals Co. (St. Louis, MO), Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum was purchased from Gibco, USA, 96 well flat bottom tissue culture plates were purchased from Tarsons Products Pvt., Ltd., Mumbai, India.

2.2. Preparation of SLN

DTX loaded unconjugated SLN (DSLN) were prepared using emulsification and solvent evaporation method (Trotta et al., 2003). Briefly, DTX and lipid phase containing SA, SL and GMS (1:2:6, w/w ratio) were dissolved in chloroform to get oil phase in organic solvent. Aqueous phase was prepared by dissolving 1.5% w/w Tween 80. Lipid phase containing organic solvent was added to aqueous phase and homogenized (Ultra Turrax T25, Germany) for 5 min at 11,000 rpm. Emulsion, thus obtained, was sonicated (Vibra cell, Sonics, USA) for 20 min. The nanoemulsion was kept under stirring for 3 h followed by characterization for size, zeta potential. These nanoparticles were further used for conjugating ADN on surface.

2.3. Surface conjugation

ADN was conjugated on the surface of preformed DSLN using carbodiimide chemistry (Kulhari et al., 2014). 20 mg DSLN was dispersed in phosphate buffer (pH 7.4) and were incubated with NHS and EDC (1:5 w/w ratios). The dispersion was kept under gentle stirring for 2 h at room temperature. To this, 600 μg ADN was added, mixed well and kept for further stirring for 4 h. ADN conjugated DTX loaded SLN (ADN–SLN) were collected after centrifugation (Sigma Laborentrifugen GMBH, Germany) at 12,000 rpm for 30 min and washed thrice with distilled water to remove unconjugated ADN in supernatant. Prepared ADN–SLN pellets were recollected and freeze dried (Skadi, Europe). Freeze dried nanoparticles were characterized for qualitative estimation of conjugation using Fourier transform infrared spectroscopy (FTIR).

Conjugation efficiency was deduced from estimating the free or unconjugated ADN in supernatant (indirect method). Saturated phenol sulfuric acid method (Dubois et al., 1956) was employed to calculate amount of adenosine attached to DSLN. Briefly, 2 ml aliquot of carbohydrate solution (supernatant ADN–SLN conjugation process) was mixed with 1 ml of 5% aqueous solution of phenol in a test tube. Subsequently, 5 ml of concentrated sulfuric acid was added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they were vortexed for 30 s and placed for 20 min in a water bath at 37 °C for color development. Reference solutions were prepared in identical manner as above, except 2 ml aliquot of carbohydrate solution, which was replaced with water. Absorbance of samples was taken at 480 nm using UV spectrophotometer (Jasko, Japan). Conjugation efficiency was expressed as percentage of ADN bound to DSLN.

2.4. In vitro characterization of nanoparticles

2.4.1. Particle size and zeta potential

Particle size and zeta potential of blank SLN, DSLN and ADN–SLN were measured by photon correlation spectroscopy using Zetasizer, Nano ZS (Malvern Instruments, UK). Furthermore, transmission electron microscope (TEM, JEOL, Japan) was used to evaluate the surface morphology of ADN–SLN.

2.4.2. Entrapment efficiency

Entrapment efficiency was determined by measuring the concentration of unloaded drug in supernatant collected after centrifugation of nanoparticle dispersion and analysis was done using high performance liquid chromatography (HPLC) with photodiode array detector (Waters, USA). An octadecylsilane column (Inertsil, 250 mm × 4.6 mm, 5 μm) was used for analysis and column temperature was maintained at 25 ± 5 °C. The mobile phase, acetonitrile (60%) and water (40%), was pumped at a flow