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Synthesis, characterization and targeting potential of zidovudine loaded sialic acid conjugated-mannosylated poly(propyleneimine) dendrimers

Virendra Gajbhiye [a,*], Narayanan Ganesh [b], Jaya Barve [b], Narendra K. Jain [a,*]

[a] Pharmaceutics Research Laboratory, Dept. of Pharmaceutical Sciences, Dr. H. S. Gour University, Sagar (MP) 470 003, India
[b] Department of Research, JawaharLal Nehru Cancer Hospital and Research Centre, Idgah Hills, Bhopal (MP), India

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ABSTRACT

The present investigation was aimed at exploring dual targeting of anti-HIV drug, zidovudine (ZDV) via sialic acid conjugated-mannosylated poly(propyleneimine) (PPI) dendritic nano-constructs. Fourth generation PPI dendrimers, sialic acid conjugated PPI dendrimers (SPPI), mannose conjugated PPI dendrimers (MPPI) and dual ligand system i.e. sialic acid conjugated-mannosylated PPI dendrimers (SMPPI) were synthesized and characterized by FT-IR and ^{1}H NMR spectroscopies and were further confirmed by size exclusion chromatography and differential scanning calorimetry. Various parameters like drug loading, pH dependent *in vitro* release, hemolytic toxicity, macrophage uptake and cytotoxicity concerning PPI, SPPI, MPPI and SMPPI dendrimers were evaluated. ZDV loaded SMPPI, SPPI and MPPI have shown reduced hemolytic toxicity, cytotoxicity and *in vitro* drug release at pH 7.4. Extremely significant ($P<0.001$) increase in cellular uptake of ZDV by macrophage cells was observed in case of SMPPI as compared to PPI and free drug. The *in vivo* blood level and tissue distribution studies in albino rats also demonstrated potential of dual targeted system towards sialoadhesin and carbohydrate receptors. The drug concentration in lymph nodes was increased to about 28 times in case of SMPPI (1335 ± 17.6 ng/g) as compared to free drug (48 ± 5.8 ng/g) at 6th hr. The results suggested that such dual ligand dendritic system (SMPPI) hold potential to enhance biocompatibility and site specific delivery of antiretroviral drug, ZDV.

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1. Introduction

According to the Joint United Nations Program on HIV/AIDS (UNAIDS), till date an estimated more than 33 million people are living with AIDS worldwide (UNAIDS, 2009). It is now a leading cause of death worldwide and thousands of new HIV infections arise daily around the world and more than 90% of these are in developing countries. Most markedly, crucial immune cells called CD4+ T lymphocyte cells are disabled and killed by HIV during the typical course of infection. Apart from these major targets, the cells of the mononuclear phagocyte system (macrophages, monocytes) also play a significant role as a reservoir for HIV. In organs like lung and the brain, HIV is located primarily in macrophage (Mac) cells (i.e. alveolar Mac and microglia, respectively) (Levy, 1993; Weiss, 1993; Dutta et al., 2007). HIV replication is proliferation dependent in the case of CD4+ T cells, which leads to cell death. On the contrary both HIV I and II replication can take place in a mature non-proliferating Mac while in immunological active state (Gartner et al., 1986; Nicholson et. al., 1986, Von-Briesen et. al., 1990). In addition, researchers have also postulated that despite of having a function in the pathogenesis of the disease, cells of the Mac family also act as vectors for the transmission of HIV. Moreover, placental macrophages are the candidates to act as a vector of vertical transmission HIV-1 (Kesson et. al., 1994). Milman and Sharma (1994) supplemented important role of Mac that the transmitted HIV variant infect Mac for mucosal transmission. Owing to the important role of cells of the monocyte/macrophage (Mo/Mac) family in the pathogenesis of HIV as depicted above, the effective anti-HIV therapy must reach Mo/ Mac in addition to other target cells (Bender et. al., 1996).

Till date there is no specific and curable therapy against AIDS. The rate of spread of the infection is alarming and thus invokes a need for global mobilization against AIDS and also justifies the need for thinking about the present status of AIDS therapy. Antiretroviral drugs have been developed that can suppress AIDS Virus. Recently, scientists have combined the drugs into regimens that successfully attack the virus at multiple places in its life cycle and people with HIV could expect to live long and well. The new chemotherapy which uses drugs against HIV in carefully-planned combinations is known as Highly Active Anti-Retroviral Therapy (HAART). The main classes of HAART are nucleoside analogue reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PIs) and entry inhibitors. The drugs most widely used are also favorable for resistance profile. Several studies in HIV infected patients have shown treatment failure and adverse effects associated with fluctuating

* Corresponding author.
E-mail addresses: cme_virendra@yahoo.co.in (V. Gajbhiye), jnarendr@yahoo.co.in (N.K. Jain).

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plasma concentration of anti-HIV drugs. Drug distribution and interaction with non-target site results in treatment failure and adverse effects, respectively. One of the most widely used NRTIs is zidovudine (ZDV). ZDV is also associated with adverse effects like hematologic toxicity including neutropenia and severe anemia, particularly in patients with advanced HIV-1 disease, symptomatic myopathy, lactic acidosis and severe hepatomegaly with steatosis, including fatal cases. Hence, administration of ZDV directly to the HIV infected cells is highly desirable. Decreasing the required drug doses and preventing the interaction of drug with non-target site or non-infected cells could increase the therapeutic potential and may also reduce the adverse effects.

Dendrimers have fascinated escalating attention for their applications in targeted and controlled drug delivery due to properties like three-dimensional macromolecules with multivalency, monodisperse, host-guest entrapment properties, defined molecular weight, large number of peripheral end groups and interior cavities (Gajbhiye et. al., 2007; Gajbhiye et. al., 2009a,b; Gajbhiye and Jain, 2011). Sialic acid binding receptors [Sialoadhesin (Sn), prototypic member of the sialic acid binding lectins family called Siglecs] are present on the macrophages and over expressed in HIV infection (Varki and Angata, 2006). Apart from sialic acid, conjugation of mannose sugar to dendrimers could also target lectin receptors present on the surface of Mo/Mac. Drug loaded PPI dendrimers conjugated with both sialic acid and mannose would efficiently target these dendritic carriers to the sialoadhesin and carbohydrate receptors (dual targeting) on macrophages and are expected to improve the safety and efficacy of the drug. The present investigation was aimed at developing and exploring the use of sialic acid conjugated-mannosylated PPI (SMPPI) dendritic architecture for dual targeted delivery of anti-HIV drug, ZDV.

2. Materials and methods

Sialic acid was a benevolent gift from New Zealand Pharmaceuticals Ltd, (New Zealand). ZDV was a benevolent gift from Matrix Laboratories Ltd. (Hyderabad), India. Ethylene diamine, Acrylonitrile and N-hydroxysuccinimide (NHS) were purchased from CDH (Mumbai), India. N'-Dicyclohexyl carbodiimide (DCC) was purchased from Himedia, (Mumbai), India. Raney Nickel was purchased from Merck (Mumbai), India. D-mannose was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were reagent grade and purchased from CDH, (Mumbai), India.

2.1. Synthesis of 4.0G PPI dendrimers

PPI dendrimers (4.0G) was synthesized by divergent method as reported by us earlier (Gajbhiye et. al., 2008, 2009a,b). Briefly, double Michael addition reaction was used to produce half generation (–CN terminated) by adding acrylonitrile to the initiator core, ethylenediamine (EDA). It was followed by heterogeneous hydrogenation by means of Raney Nickel catalyst to synthesize full 1.0G generation ($-NH_2$) dendrimers. The reaction sequence was repeated iteratively to fabricate PPI dendrimers up to fourth generation (4.0G PPI). The 4.0G PPI was purified by dialysis against double distilled water in a dialysis tube (MWCO 5 KDa, Sigma, USA) to remove lower generation dendrimers and un-reacted chemicals. FT-IR spectroscopy was carried out using Perkin–Elmer FT-IR spectroscope (USA). ^{1}H NMR spectroscopy of the dendrimer sample was carried out at 300MHz in $CDCl_3$ (Bruker DRX, USA).

2.2. Synthesis of mannosylated 4.0G PPI dendrimers

Mannose conjugation to 4.0G PPI dendrimers was carried out by the method reported by our group earlier (Kumar et. al., 2006). Briefly, D-mannose (8 mM) was dissolved in 0.1M sodium acetate buffer (pH 4.0) to isomerize the ring form of mannose into open chain form and then it was added to lyophilized 4.0G PPI dendrimers (0.1 mM). The mixture was agitated at 60°C temperature for 48 hr to ensure the completion of reaction. Resulting solution was concentrated under vacuum. Mannosylated dendrimers (MPPI) were purified by dialysis against double distilled water in a dialysis tube (MWCO 12 KDa, Sigma, USA) to remove un-reacted mannose, salts and partially mannosylated dendrimers followed by lyophilization (Heto Drywinner, Germany). FT-IR and ^{1}H NMR spectroscopies of mannosylated dendrimers were carried out as done for 4.0G PPI dendrimers.

2.3. Synthesis of sialic acid conjugated 4.0G PPI dendrimers

The COOH group of sialic acid was activated by EDC [1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide]. The activated form of sialic acid (12 mM) was added to 4.0G PPI dendrimers (0.1 mM). Both the above steps were carried using dichloromethane as solvent. The mixture was agitated at ambient temperature for 48 hr to ensure completion of reaction. Resulting solution was concentrated under vacuum. Sialyated (sialic acid anchored) dendrimers were purified by dialysis against double distilled water in a dialysis tube (MWCO 12 KDa, Sigma, USA) to remove unreacted sialic acid, biproduct and partially sialyated dendrimers followed by lyophilization (Heto Drywinner, Germany). FT-IR and ^{1}H NMR spectroscopies of sialyated dendrimer were carried out as done for 4.0G PPI dendrimers.

2.4. Synthesis of mannosylated-sialic acid conjugated 4.0G PPI dendrimers

The 4.0G PPI dendrimer contains 64 terminal NH_2 groups. Firstly, mannose conjugation was carried out for 32 terminal NH_2 groups. A calculated amount of mannose for 32 NH_2 terminal groups of 4.0G PPI dendrimers was added and the reaction was carried out as described above under synthesis of mannosylated dendrimers. The dialysis tube used was of MWCO 10 KDa (Sigma, USA). The extent of mannosylation was characterized by Size exclusion chromatography (SEC). The partially mannosylated dendrimers was subjected to sialic acid conjugation as described above under synthesis of sialic acid conjugated dendrimers. The mannosylated-sialic acid conjugated 4.0G PPI dendrimers was also characterized for extent of sialic acid conjugation by SEC. The SEC was used to determine exact molecular weight of the partially mannosylated PPI dendrimers as well as mannosylated-sialic acid conjugated PPI dendrimers. This experiment was performed using SEC system consisting refractive index detector. The analysis was done at room temperature using two serially align TSK-GEL columns G3000PW and G4000PW. The isocratic mobile phase was PBS (pH 7.4) at a flow rate of 1mL/min. Sample concentration was kept 1 mg/mL in PBS, and 100 μL was injected. Molecular weights of partially mannosylated PPI dendrimers and mannosylated-sialic acid conjugated PPI dendrimers were determined using Astra V software (Wyatt Technology Corporation).

2.5. Drug loading in formulations

The known molar concentrations of 4.0G PPI dendrimer, MPPI, SPPI and SMPPI were dissolved separately in methanol and mixed with methanolic solution of ZDV (100 mol). The mixed solutions were incubated with slow magnetic stirring (50 rpm) using teflon bars for 24 hr. The methanol was evaporated and dialyzed twice in cellulose dialysis bag (MWCO 1000 Da Sigma, USA) against PBS (pH 7.4) under sink conditions for 1 hr to remove free drug from the formulations. The free drug was then estimated

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