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Research paper

Stealth anti-CD4 conjugated immunoliposomes with dual antiretroviral drugs – Modern Trojan horses to combat HIV

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ABSTRACT

Highly active antiretroviral therapy (HAART) is the currently employed therapeutic intervention against AIDS where a drug combination is used to reduce the viral load. The present work envisages the development of a stealth anti-CD4 conjugated immunoliposomes containing two anti-retroviral drugs (nevirapine and saquinavir) that can selectively home into HIV infected cells through the CD4 receptor. The nanocarrier was characterized using transmission electron microscopy, FTIR, differential scanning calorimetry, particle size and zeta potential. The cell uptake was also evaluated qualitatively using confocal microscopy and quantitatively by flow cytometry. The drug to lipid composition was optimized for maximum encapsulation of the two drugs. Both drugs were found to localize in different regions of the liposome. The release of the reverse transcriptase inhibitor was dominant during the early phases of the release while in the later phases, the protease inhibitor is the major constituent released. The drugs delivered via anti-CD4 conjugated immunoliposomes inhibited viral proliferation at a significantly lower concentration as compared to free drugs. *In vitro* studies of nevirapine to saquinavir combination at a ratio of 6.2:5 and a concentration as low as 5 ng/mL efficiently blocked viral proliferation suggesting that co-delivery of anti-retroviral drugs holds a greater promise for efficient management of HIV-1 infection.

1. Introduction

Acquired immune deficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), presents a huge challenge for treatment as it tends to disable the immune system thereby leaving the patient susceptible to many opportunistic infections [1]. According to the 2013 WHO report, about 34 million people have been diagnosed to be HIV positive throughout the world [2]. Highly active retroviral therapy (HAART) is one of the treatment strategies currently employed for treatment of HIV infections where a cocktail of drugs, each active at different stages of the disease, is administered to the patient [3]. However, HAART therapy has not proved very effective and is severely limited due to various factors. Most of the conventional HAART drugs produce various adverse effects such as skin rashes, vomiting, diarrhea and liver disorders [4].

Systemic administration of combinations of various drugs may also result in enhanced elimination of one drug with respect to the other leading to poor bioavailability of drug(s) in the combination. For instance, it has been demonstrated that when the two drugs nevirapine and saquinavir are administered in tandem, selective metabolization of saquinavir is promoted due to the activation of the cytochrome enzyme CYP3A4 [5]. This reduces the bioavailability of saquinavir and necessitates higher dosage of the drug thereby amplifying the possibility of adverse effects [6]. This problem can be circumvented if both drugs are made available to the target cell simultaneously. Administration of higher doses of the drugs in the conventional therapy may evoke the drug resistance mechanism thus lowering the therapeutic efficacy [7]. In addition, most drugs suffer from poor bioavailability due to metabolization or rapid elimination and hence the amount of drug reaching the target CD4 positive cells is inadequate [8]. Currently, frequent doses are administered to counter this effect, but these results in higher levels of adverse effects. Most of the drugs used in HAART are unable to cross the blood–brain barrier and therefore could not annihilate the HIV virions residing in the glial cells of the brain [9]. These disadvantages of the conventional therapy can be overcome by specifically targeting virus-infected cells.

The advent of nanotechnology has opened new vistas in chemotherapeutics by imparting target specificity and enhanced

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therapeutic dosages to the target site. This can result in reduced frequency of administration and side effects while improving bioavailability and therapeutic efficacy. Many attempts have been made to improve the bioavailability of drugs through the use of nanocarriers [10]. A wide range of nanocarriers has been investigated for site-specific delivery of anti-cancer agents and antimicrobial agents [11]. However, not much focus has been directed toward site-specific delivery of anti-retrovirals. Some of the nanocarriers that have been explored for delivery of anti-retroviral drugs are liposomes [12], dendrimers [13], solid lipid nanoparticles [14], chitosan nanoparticles [15] and polymeric nanoparticles [6]. Most of these carriers have been developed for monotherapy i.e., treatment with a single drug. Combinational therapy employing nanocarriers has been reported for PLGA nanoparticles containing ritonavir, efavirenz and lopinavir [16]. A PLGA-based vaginal gel with raltegravir and efavirenz has also been reported [17]. No other reports are available in the context of multiple drug-loaded carriers for treatment of HIV infections.

HAART alone is not solely efficient for the treatment of the HIV because there are other opportunistic infections like histoplasmosis, Kaposi's sarcoma, tuberculosis, and pneumonia that accompany HIV infections due to the decrease in the number of the CD4 positive immune cells. Treatment with liposomal doxorubicin has been employed for reducing the tumor progression in HIV-associated Kaposi's sarcoma, which is currently in the phase II clinical trials [18]. Thus a combined therapy of HAART drugs and other drugs are used for the treatment of HIV infections in conventional therapy. However, effective reduction in the viral load may reduce the risk of development of such opportunistic infections thereby decreasing the need for other types of drugs along with HAART emphasizing the need for development of efficient anti-HIV strategies. Use of targeted nano-carriers containing co-encapsulated anti-retroviral drugs may improve treatment outcomes. However, this facet remains relatively unexplored.

Targeting of the liposomal system to the virus infected cells is a key requisite to ensure undesirable interactions resulting in the loss of efficacy as well as to minimize adverse effects. Many strategies have been attempted to specifically target virus-infected cells. These include conjugation of specific ligands to bind to gp120 in the HIV [19], glycan residues in the viral envelope [20], CXCR4 in the T cells [21], CCR5 in the macrophages, T cells and dendritic cells, HLA receptors in the virus infected cells [22], tuftsin receptors in the macrophages [23], CD4 receptors of both T cells and macrophages [24–27], etc. However, each strategy has met with limited success owing to various factors.

The present study aims to develop a liposomal delivery system encapsulating two drugs. The drugs chosen for the study are a reverse transcriptase inhibitor (nevirapine) that is active in the early life cycle of the HIV life cycle [28] and the protease inhibitor (saquinavir) that is more effective during the late phase of the HIV life cycle [29]. The difference in the hydrophobicity of both drugs is expected to influence their localization within the liposomes and consequently their release profiles. Target-specificity is achieved by introducing an anti-CD4 moiety on the surface of the liposomes to specifically home into cells that are commonly infected with HIV. The antibody employed in the present work is expected to target to both CD4 positive T_H (helper T) cells and also the macrophages, which are the primary hoarding cells for HIV and hence can provide better therapeutic benefits.

2. Materials and methods

2.1. Materials

Sodium dihydrogen phosphate, disodium hydrogen phosphate and HEPES (hydroxyethylpiparazinyl ethane sulfonic acid) were purchased from Merck Chemicals, India. Saquinavir was a kind gift from M/s Hetero drugs, India and nevirapine was a kind gift from M/s Boehringer Ingelheim Ltd., Germany. RPMI 1640 medium was procured from Sigma–Aldrich, USA and CellTiter 96®AQueous one solution was purchased from Promega, USA. Dulbecco's modified Eagle's medium (Sigma–Aldrich, USA), fetal bovine serum (Biological Industries, Israel) were also used in the study. Egg phosphatidyl choline (Egg PC), distearoylphosphatidylethanolamine–poly(ethylene glycol) (DSPE–PEG), NBD-DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1, 3-benzoxadiazol-4-yl) [Triethylamine salt] and maleimide-terminated DSPE–PEG (DSPE–PEG-mal) were purchased from NOF, Japan while cholesterol was procured from Sigma–Aldrich, USA. The anti-CD4 antibody purified from a hybridoma clone producing human monoclonal antibody against CD4 at JNCASR was used for conjugation [30], Traut's reagent (2-iminothiolane) was procured from Sigma–Aldrich, USA, CellTiter 96® AQueous one solution was purchased from Promega, USA.

2.2. Preparation of dual drug loaded anti-CD4 conjugated immunoliposomes

The liposomes were prepared by the thin film hydration method [31,32]. The lipid combination of egg PC:cholesterol:DSPE–PEG of desired composition dissolved in chloroform was purged with nitrogen for removal of solvent and formation of a thin layer of lipid. This was followed by the addition of the hydrophobic drug nevirapine in chloroform and the solvent was removed by purging with nitrogen. Saquinavir dissolved in phosphate buffered saline (PBS) was added to the lipid–nevirapine layer for hydration. The mixture was constantly stirred at 60 °C for half an hour in a water bath followed by extrusion through a polycarbonate membrane with pore size of 0.2 μm to obtain unilamellar liposomes. The liposomes were then centrifuged at 845*g* (3000 rpm) to sediment the unencapsulated nevirapine [31]. The supernatant was then centrifuged at 21,130*g* (15,000 rpm) to sediment the dual drug loaded liposome. The supernatant containing the unencapsulated saquinavir was used to estimate the amount of drug encapsulated in the liposomes [32]. For conjugation of the anti-CD4 antibody to the dual drug loaded liposome was achieved in two steps. Initially, the antibody was thiolated using Traut's reagent with an antibody to reagent ratio of 1:20 (mol/mol) [33]. The reaction was carried out at room temperature for 1 h at pH 8. The unreacted Traut's reagent was removed by dialysis and the thiolated antibody was conjugated with the dual drug loaded liposomes by incubation overnight at 4 °C in HBS (HEPES buffer solution of pH 7.4) [34]. The unconjugated antibody was separated using Centriprep® (Millipore, India) at 9391 g (10,000 rpm). The amount of antibody conjugated to the liposome was estimated using the standard Lowry's method and the colored product obtained was read at 660 nm using multimode reader (Infinite M200, Tecan, Austria). The anti-CD4 conjugated immunoliposomes were freeze-dried (Alpha 2-4 LD *plus*, Christ, Germany) and stored in airtight moisture-free vials at −20 °C until further use.

2.3. Characterization of anti-CD4 conjugated immunoliposomes

Lyophilized unconjugated liposomes or anti-CD4 conjugated immunoliposomes (500 μg) were dispersed in 500 μL of PBS and placed over a copper grid. Then, PBS was removed using filter paper and the sample was allowed to dry followed by imaging using high resolution transmission electron microscopy (JEM 2100F, JEOL, Japan).

The amount of nevirapine and saquinavir encapsulated was determined from the amount of unencapsulated drugs. The unencapsulated nevirapine was pelletized by centrifugation of the extruded liposomes at 3000 rpm (Eppendorf 3340R, Germany).