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Development and in vivo evaluation of functionalized ritonavir proliposomes for lymphatic targeting

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ABSTRACT

Aims: The aim of the present work was to prepare, characterize, and evaluate proliposomes containing lipophilic prodrug ritonavir for targeting towards CD4+ T cells in the lymphatic system.
Materials and methods: The liposomes were prepared by lipid thin film hydration method and lyophilized in the presence of cryoprotectant mannitol to obtain proliposomes. The optimized proliposomes by Central Composite Design, were surface modified with biotin. The proliposomes were evaluated for particle size, zeta potential, polydispersity index (PDI), entrapment efficiency, *in vitro* drug release, *in vivo* pharmacokinetics and biodistribution studies.
Key findings: The mean particle size was found to be in the range of 126.6 to 306.2 nm with PDI of 0.340–1.00. The entrapment efficiency was found to be in the range of 18.9 to 86.2%. The formulations showed a zeta potential in the range of −18.1 to −20.2 mv. Biotinylated proliposomes (LIP-5B) were in the size of 149.8 ± 6.8 nm with entrapment efficiency 61.6%. The % CDR of pure drug, conventional, biotinylated proliposome in 3 h was found to be 58.3, 82.04, and 95.9% respectively. *In vitro* drug release and *in vivo* pharmacokinetics of the pure drug, optimized conventional proliposomes (LIP-5) and biotin proliposomes (LIP-5B) were executed.
Significance: The AUC for the liposomes were found to be much higher in the spleen and thymus compared to that in the plasma which indicated that the developed formulations enhance the bioavailability and target specificity compared to that of the pure drug thereby enhancing bioavailability at target site.

1. Introduction

Acquired immune deficiency syndrome (AIDS) is a syndrome caused by HIV (Human immunodeficiency virus) which alters the immune system making people more vulnerable to disease and infections. Currently, no treatments are available to cure AIDS/HIV completely and still it is an ongoing research field. HIV mainly attacks $CD4^+$ T cells in the lymphatic system, and the viral load will remain very high in the lymphatic system. Macrophages are the major reservoirs of the HIV, and the virus persists in these cells to give rise to the new virus. Highly active antiretroviral therapy (HAART) which has greatly reduced the mortality among AIDS patients, but the mortality rate has remained approximately five times higher in patients with AIDS than in HIV-infected patients without AIDS [1,2]. The antiviral drug therapy mainly aims at reducing the viral load and restoration of immunity. The lymphatic system is the site or target for many diseases such as metastitial tuberculosis, acquired immune deficiency syndrome, cancer, and filariasis. Most studies of HIV-1 infection are focused on blood because of ease of acquisition. But since lymphatic system is the main reservoir site for HIV-1 infection, lymph nodes play a major role in the propagation of the virus. Levels of HIV in lymph nodes and lymphatic system are 100–10,000 times greater than that of blood. The lymphatic virus remains unaffected by HAART, interruption in the therapy leads to replenishing the systemic infection [3]. Hence drugs which effectively reduce the viral load in the lymphatic system will be advantageous in reducing the mortality [2,4].

The eradication of virus at lymph nodes can be achieved by using nanotechnology based drug delivery systems mainly by various lipid drug delivery systems such as liposomes, niosome, and solid lipid nanoparticles, etc. The main advantage of using nanocarriers in anti-HIV therapy is that it can avoid presystemic metabolism and also enhance the bioavailability of lipophilic drugs. The particle size, shape, and the surface charge modulate the bioavailability of the drugs through altered internalization of the drug into cells. Nanoparticles can circumvent the P-glycoprotein (P-gp) efflux transporter that is present on the HIV target cells and thus escape this bioelimination process. Nanocarriers will

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stimulate the immune systems and helps in targeting the drug in disease associated with biosystems such as lymphatic tissues [5]. In the similar line, solid lipid nanoparticles of lopinavir, efavirenz and stavudine and lopinavir loaded PLGA nanoparticles are studied for lymphatic targeting [1,6–8].

In the present study proliposomes of protease inhibitor ritonavir were prepared with the aim of delivering the drug to the intestinal lymphatic system. Ritonavir has got a variable bioavailability of 40–50% due to its poor aqueous solubility [9]. Protease inhibitors extensively bind to plasma proteins and are the main substrate of P-gp efflux transporter which limits the absorption of these drugs into the target sites [10]. Ritonavir is marketed in the form of soft gelatin capsules, tablet and oral solutions with the brand name Norvir. Ritonavir is widely used in combination with other protease inhibitors such as lopinavir. It was observed that the blood levels of lopinavir and darunavir were increased in the presence of low dose of ritonavir. Hence both are used in combination with ritonavir. Ritonavir is considered a booster for lopinavir [11]. Many researchers are working on protease inhibitors to increase their efficacy. Sudhakar et al. formulated injectable stealth liposomes of ritonavir to improve its bioavailability [9]. Solid dispersions of ritonavir using different career systems were also attempted to increase its oral bioavailability [12–15]. Formulation of ritonavir into proliposomes is considered to be beneficial in improving the bioavailability and targeting the drug to lymphatic tissues, more importantly, the drug can be delivered orally. Lipid-based nanoparticles are extensively used for passive targeting to lymphatic systems such as lymph node, lymph vessels, tonsils, spleen, bone marrow and thymus gland [4]. The mechanism of drug transport into the intestinal lymphatics includes paracellular transport, through M-cells of Payer patches and transcellular route [1].

Proliposomes are the lyophilized liposome vesicles of nanometer scale in which aqueous core is entirely enclosed with a concentric bilayer of lipids. Liposomes are biocompatible, biodegradable and non-toxic, widely used for drug delivery applications [16–18]. Proliposomes are attractive substitutions to conventional liposomes for oral delivery due to various advantages, such as protection of drug molecules from GI degradation, improved drug solubilization, permeation across the GI barrier and enhanced bioavailability [19]. These also possess good biocompatibility and are suitable for the delivery of both hydrophilic and hydrophobic drugs. Proliposomes are highly efficient in transporting the lipophilic drugs like protease inhibitors through the lymphatic region due to its high resistance in gastric environment and fine particle size. There are few literatures available on delivery of drugs such as lercanidipine, paclitaxel, quercetin, and lopinavir using proliposomes approach [19–22]. By considering all these aspects, proliposomes of ritonavir were prepared. The developed optimized proliposomes of ritonavir were coated with biotin to enhance the therapeutic efficacy. There are few literatures available on subcutaneous (s.c.) administration of biotinylated liposomes followed by adjacent s.c. injection of avidin for lymphatic delivery of drugs [23,24]. Biotinylated liposomes of insulin were observed to permeate through GI tract by facilitated absorption mechanism upon oral administration [25]. Based on this available literature an attempt was made to coat the liposomes with biotin to convert into biotinylated proliposomes, to enhance the uptake into the intestinal lymphatic tissues. The formulations were characterized by various physicochemical parameters, and lymphatic tissue distribution studies were performed.

2. Materials and methods

2.1. Materials

Ritonavir was obtained from Hetero Drugs Limited, Hyderabad, India. Soyaphosphatidyl choline, cholesterol and Triton-X 100 were purchased from Sigma, MO, USA. Biotin was procured from Hindustan Chemicals and Pharmaceuticals, Bhandup West, Mumbai, India. All other chemicals used were of analytical or HPLC grade.

2.2. Analysis of ritonavir

The ritonavir present in the formulations, plasma, spleen, and thymus was analyzed using HPLC method. HPLC analysis was carried out using Shimadzu LC-2010HT assembly equipped with a UV detector. The samples (20 μl and 100 μl each for analytical and bioanalytical sample analysis respectively) were injected into Grace smart C_{18} (250 mm × 4.6 mm, 5 μ) column maintained at 40 °C. Acetonitrile: 10 mM phosphate buffer pH 4.8 (70:30% v/v) at a flow rate of 1.0 ml/min was used as the mobile phase for eluting ritonavir which was detected at a wavelength of 240 nm. Before analysis of the drug in plasma and tissue (spleen and thymus) homogenate, the drug was extracted from the same by protein precipitation method. 100 μl of rat plasma/tissue homogenate was mixed with 10 μl of IS (efavirenz, 2.5 μg/ml). 400 μl of chilled acetonitrile was added and mixed for a minute. The resulting solution was vortexed for 5 min and centrifuged at 10000 rpm, 4 °C for 5 min. The supernatant was separated, and 50 μl was injected to HPLC. The retention time for ritonavir was found to be 6.9 min, and that for efavirenz (Internal Standard) was 9.1 min. HPLC method was validated as per USFDA guidelines.

2.3. Design of Experiments (DoE) in formulation optimization

DoE was used in the formulation development of liposomes to control the process variables. Target product profile (TPP) was set to enumerate product attributes, those linked to the quality of the product. Table 1 enumerates TPP for the development of ritonavir liposomes. Some of the critical material attributes and critical process parameters affecting the product performance were identified as lipid concentration, amount of drug, rotation of rotavapor, temperature, rehydration medium, sonication time and sonication amplitude. As per the preliminary study and literature data, the sonication time and sonication amplitude were optimized. Further based on prior knowledge, the most popular face centered response surface design, Central Composite Design (CCD) was used with a group of star points that allows the estimation of curvature [26]. In this study, CCD was used with two input variables (X_1, X_2) relating to the criticality of the material attributes at three levels (−1, 0, +1). The variables and levels included X_1: Lipid (SPC:cholesterol) ratio at 2:1, 5:1, 8:1 levels and X_2:Lipid drug ratio at 5:1, 7.5:1, 10:1 levels. The X_1: Lipid (SPC:cholesterol) ratio 5:1 and X_2:Lipid drug ratio 7.5:1 were the center points in the study. The output response variables were Vesicle size (Y_1), Poly dispersity index (PDI, Y_2), Zeta potential (Y_3) and Entrapment efficiency (Y_4). The

Table 1
Target Product Profile (TPP) for proliposome formulation design.

TPP elements	Target	Product attributes (CQAs)
Dosage form	Proliposomes filled in the capsule	Identity, assay, uniformity of content
Microbial quality	No contamination	Test for microbial growth
Product performance	Robust manufacturing	Entrapment efficiency, vesicle size, PDI, zeta potential, morphology, solid state characteristics, in vitro drug release
PK target-efficacy and safety	Bio-performance: better pharmacokinetic profile of lymphatic tissues compared to plasma	Pharmacokinetic parameters (C_{max}, T_{max}, AUC, MRT)
Stability	Stable for at least 3 months at accelerated temperature as per ICH guidelines	Assay/drug content, redispersibility (measured by vesicle size, PDI)