

1.1 Introduction

Human immunodeficiency virus (HIV) is a lentivirus (a member of the retrovirus family) that causes acquired immunodeficiency syndrome (AIDS), a condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections (1). According to statistics of world health organization in 2013, 35 million people lived with HIV/AIDS worldwide including 3.2 million children (< 15 years old) and 2.4 million people in India. HIV is considered as world's leading infectious killer that has taken lives of approximately 39 million people worldwide since its first discovery in 1981 till 2013 (2). Human retroviruses are of four major types that is human T cell leukemia viruses - HTLV-1 and HTLV-2 and human immunodeficiency viruses - HIV-1 and HIV-2. HTLV-1 and HTLV-2 belong to the subclass of oncovirinae and causes adult T cell leukemia and spastic paraparesis. HIV-1 and HIV-2 from the subclass lentivirinae are responsible for causing acquired immunodeficiency syndrome (AIDS). All of these viruses infect CD4 receptor-bearing T cells. HIV-1 is the most pathogenic virus of these four types, and is mainly responsible for the global Acquired Immuno Deficiency Syndrome (AIDS) pandemic (3). Once HIV enters the body, it attaches itself to CD4 T cells and begins the replication process. Inside the CD4 T cell, the virus translates the RNA instructions into DNA so that the cell can code them using an enzyme known as reverse transcriptase. This enzyme takes the single strand of viral RNA and turns it into a double strand of DNA, which the cell can read. Once the new viral RNA strand moves out of the host DNA, the strand is "cut" into smaller subunits. The protease enzyme makes this process possible. The subunits come together to form new HIV moieties, which move out of the cell to infect other CD4 T cells. This process repeats itself continuously, and after repeated assaults by viral particles, the CD4 host cells die. As the number of CD4 cells decreases, immune system loses its ability to fight life-threatening infections (4).

Drugs used in treatment of HIV need to be taken every day for the rest of a person's life. The aim of antiretroviral treatment is to keep the amount of HIV in the body at a low level. This stops any weakening of the immune system and allows it to recover from any

damage that HIV might have caused already. Following Table 1.1 illustrates the classes of drugs used for AIDS treatment.

Table 1.1 Different classes of drugs used in antiretroviral therapy

Drug class	Mechanism of action	First approved by FDA	Drugs
Nucleoside reverse transcriptase inhibitors (NRTIs)	NRTI interferes with the action of an HIV protein called reverse transcriptase, which the virus needs to make new copies of itself	1987	Zidovudine Abacavir Lamivudine Didanosine Zalcitabine Tenofovir Emtricitabine
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	NNRTIs also stop HIV from replicating within cells by inhibiting the reverse transcriptase protein	1997	Efavirenz Nevirapine
Protease inhibitors (PI)	PIs inhibit protease, which is another protein involved in the HIV replication process	1995	Saquinavir Indinavir Ritonavir Nelfinavir Lopinavir Darunavir
Fusion or Entry inhibitors	Fusion inhibitors prevent HIV from binding to or entering human immune cells	2003	Maraviroc Enfuvirtide
Integrase inhibitors	Integrase inhibitors interfere with the integrase enzyme, which HIV needs to insert its genetic material into human cells	2007	Raltegravir

When several anti-HIV drugs, typically three or four, are taken in combination, the approach is known as Highly Active Antiretroviral Therapy, or HAART. If only one drug is taken, HIV would quickly become resistant to it and the drug would become ineffective. Taking two or more anti-retroviral at the same time vastly reduces the rate at which resistance develops making treatment more effective in the long term. The HAART therapy is prescribed to HIV positive patients even before they develop symptoms of AIDS (5). A combination of three anti-retrovirals consisting of two NRTIs and a third agent is recommended for first-line therapy. The third recommended agent may be selected from NNRTIs or one of several Ritonavir-boosted protease inhibitors. PIs are the drugs that have shown best results and triple combinations of drugs including **a protease inhibitor** are the gold standard of antiretroviral therapy (6). Despite the HAART therapy, the HIV virus has been reported to survive in extremely long-living cells and be reactivated even after years of potent antiretroviral therapy and there exists only very limited options after HAART failure (6). The major limitations behind the current antiretroviral therapy are outlined below:

- Epidemiology reveals that optimal therapeutic results are attained when treatment adherence levels are greater than 95% (no more than two doses missed monthly in a twice-a-day regime); *adherence levels* below 95% could diminish therapeutic effectiveness by 50% (7) (8).
- The frequent administration of several drugs in relatively high doses is a main cause of *patient incomppliance* (9).
- Current therapy is not able to provide a cure mainly because of HIV's ability to persist in latency state in cellular and anatomical reservoir sites (10).
- Problem of current therapy also includes prolonged treatment periods with drugs possessing severe adverse effects, drug resistance, drug-drug interactions, poor drug pharmacokinetics, viral levels rebound after therapy cessation and costs (10).
- *Efflux pumps* are found in the gastrointestinal tract and may account for the remarkable inter-individual variability of several orally-administered ARV drugs among patients (11, 12).

Protease inhibitors (PIs)

Protease inhibitors, being an integral part of the HAART therapy, several drugs having potent antiretroviral efficacy have been discovered in order to tackle with resistance that develops with continuous administration of any one drug. PIs interfere with the HIV replication process. After transcription in the nucleus, viral mRNA enters the cytoplasm and uses the host's cellular machinery to manufacture virus proteins. The viral components then gather at the cell membrane and immature viruses bud off the cell. Core proteins are produced as part of long polypeptides, which must be cut into smaller fragments by the enzyme protease in order to form mature, functional proteins. PIs bind to the site where protein cutting occurs and so prevent the enzyme from releasing the individual core proteins. As a result, the new viral particles are unable to mature or become infectious (13). Table 1.2 lists the FDA approved protease inhibitors for use in HAART therapy:

Table 1.2 Protease inhibitors approved as antiretroviral drugs (14)

Drug	FDA approval	Dosage forms	Adult dose
Saquinavir	1995	Capsule (200 mg) and tablet 500 mg	2000 mg/day (2 doses)
Indinavir	1996	Capsule (100, 200, 300, 400 mg)	2400 mg/day (3 doses)
Ritonavir	1996	Soft capsule (100 mg) and oral solution (600 mg/7.5 ml)	1200 mg/day (2 doses)
Lopinavir	1997	Tablet (400 mg)	800 mg/day with 200 mg Ritonavir (2 doses)
Nelfinavir	1997	Oral powder (50mg/g) and tablets (250 and 625 mg)	2500 mg/day (2 or 3 doses)
Amprenavir	1999	Capsule (50 mg) and oral solution (15mg/ml)	2400 mg/day (2 doses- 24 cap x dose)
Atazanavir	2003	Capsule (100, 150, 200, 300 mg)	400 mg/day (1 dose)
Tipranavir	2005	Capsule (250mg) and oral solution (100 mg/ml)	1000 mg/day (2 doses)

Darunavir	2006	Tablets (75, 300, 400, 600 mg)	1200 mg/day (1 or 2 doses)
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All protease inhibitors share common drawbacks as described below (15):

- The *oral bioavailability* of PIs is generally low, due to metabolism by the cytochrome P450 (CYP) system, particularly CYP-3A4 and to a much lesser extent CYP2D6, CYP2C9 and CYP-2C19.
- PIs are substrates for multidrug resistance influx and efflux transporters expressed in the gastrointestinal tract and liver, which may also reduce bioavailability. (15)
- Molecular weights are fairly high, ranging from 600 daltons for Indinavir to 721 daltons for Ritonavir. Intestinal permeability decreases as molecular weight increases.
- Each has one or more amide groups, as well as other hydrogen bonding groups which directly decrease the intestinal permeability.
- Permeability is also often proportional to lipophilicity, and the HIV protease inhibitors typically have high partition coefficients. However, this results in reduced aqueous solubility at physiologic pH for these high dose compounds.

Darunavir: Darunavir (formerly TMC114) is a potent antiretroviral drug used in first line therapy. It is administered as oral immediate release tablet at adult dose of 600 mg with 100 mg Ritonavir twice a day. This high dose can cause severe life threatening complications like hepatitis and skin rashes (16, 17). Darunavir undergoes hepatic metabolism and is extensively metabolized by CYP enzymes, primarily by CYP3A. It is a substrate of ABC transporters like P-gp and thus have low permeability resulting in increased enzymatic degradation and lower bioavailability (~37 %) (16, 18). To overcome this drawback, it is always administered with Ritonavir which inhibits both, the activity of P-gp as well as the enzymatic degradation (14) (19). But long term use of Ritonavir can cause its resistance and further cross resistance for other protease inhibitors (19). Moreover, it can cause several serious side effects like perioral and peripheral paraesthesiae, liver problems, pancreatitis, heart rhythm problems, severe allergic reactions etc (20). The bioavailability of Darunavir is highly food dependent and is increased in presence of high fat meals (18). Formulations that allow high dose of

Darunavir while being slowly released for extended time periods would require less frequent dosing resulting in decreased dose dependent side effects. Few approaches have been made to increase bioavailability of Darunavir viz. pellet formation of Darunavir using K-pelletisation (21), solid self-microemulsifying drug delivery system of Darunavir (22) and Darunavir tablet prepared by melt extrusion containing poloxamer 188 as solubilizer (23).

Atazanavir: Atazanavir (formerly BMS 232632), unlike other PIs, need to be administered only once a day and shows lesser effects on the lipid profile like lipodystrophy. It is administered as at an adult dose of 300 mg with 100 mg Ritonavir orally once a day. It is rapidly absorbed with a T_{max} of approximately 2.5 hours. Co-administration of food has found to enhance its bioavailability and reduction in pharmacokinetic variability (24). The oral bioavailability is 60-68%. The common adverse effect associated with the use of Atazanavir is the rise in bilirubin levels (25). Atazanavir sulfate loaded Eudragit L 100 nanoparticles have been prepared in literature in order to enhance its oral bioavailability (26).

Bioavailability of a drug molecule can be enhanced by different techniques like particle size reduction, complexation with β -cyclodextrin, prodrug approach, conjugation with vitamin-C, salt formation etc. One approach is nanoparticulate formulation. This system can be prepared using polymers like PLGA, chitosan, Gelatin, polycaprylactone etc or solid-lipids like Compritol, Precirol, Dynasan, glyceryl stearate, hydrogenated castor oil etc. The unique properties of lipids viz., their physiochemical diversity, biocompatibility and proven ability to enhance oral bioavailability of poorly water soluble, lipophilic drugs through selective lymphatic uptake have made them attractive candidates as carriers for oral formulations (27). Lipids are reported to decrease the effect of cytochrome P450 enzymes on the drugs (28) and thus are likely to reduce the metabolism of drugs susceptible to these enzymes and hence this approach can eliminate the need for co-administration of Ritonavir with Darunavir. Lipid carriers are reported to enhance the intestinal permeability by reducing the efflux transport activity at intestinal wall (29). Moreover, lipid-based formulations can increase the solubility and dissolution of lipophilic drugs and facilitate the formation of solubilized species from which

absorption occurs and thus can reduce the food dependent bioavailability (30). The bioavailability of protease inhibitors like Saquinavir (31) and Lopinavir (32) has been increased by incorporating them into solid lipid nanoparticles. **Solid lipid nanoparticles** possess the solid core matrix that can solubilize lipophilic drugs and have diameters in range of 10-1000 nm. Another lipid based carrier called **nanoemulsion** are novel carriers used as drug delivery system. Nanoemulsions with an oil-like lipid matrix are commonly prepared by incorporating drugs into the interior oil phase or into the oil–water interface. Moreover, it can be given to those patients who have difficulty in taking tablets or capsule. Lipid nanoemulsion carrier has been used for oral bioavailability enhancement (33), sustained release (34) and targeting (35). Thus, an approach was done to develop solid lipid nanoparticles (SLNs) and nanoemulsion of Darunavir to increase its oral bioavailability. Figure 1.1 depicts various absorption mechanisms by which lipid nanocarriers improve the oral bioavailability of drug substances.

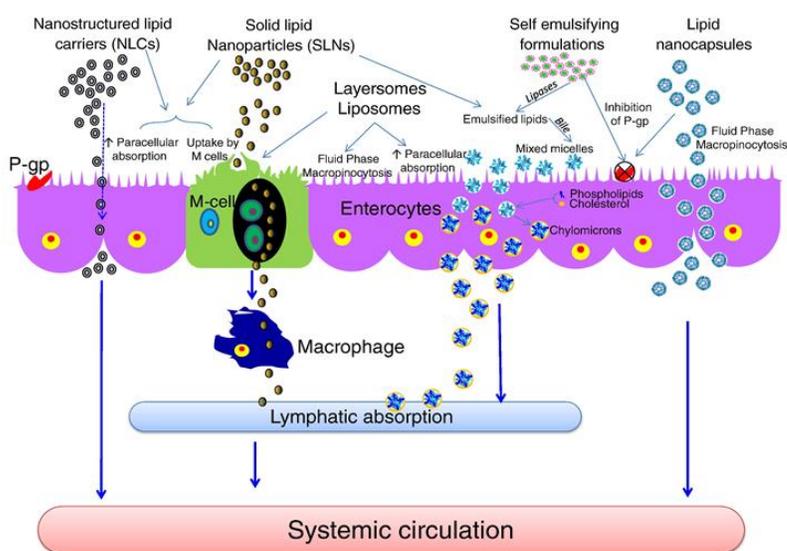


Figure 1.1 Absorption mechanisms implemented by lipidic nanocarriers for improving the oral bioavailability of drug substances (36)

In addition, nanoparticles carry advantage of uptake by the M-cells whereby the first pass metabolism is avoided. The nanoparticles by virtue their size and colloidal properties can be targeted to GALT (Gut associated lymphoid tissue) to deliver high loads of drug to lymphatic tissue and then to systemic circulation. The nanoparticles are reported to be taken up intact by M cells of peyer's patches in the intestine associated

lymphoid tissue (37). M-cells are a part of intestinal epithelium consisting of lymphoid follicles arranged to form distinct structures called as peyer's patches. These cells have sparse microvilli, glycocalyx, absence of mucus and are characterized by their ability of transporting antigens from the intestinal lumen to the cells of the immune system. M-cells can endocytose particles either by fluid phase endocytosis, adsorptive endocytosis or by phagocytosis (Figure 1.2) (38). Lymphocytes and macrophages are present at its basolateral side through which the M-cells can exocytose particles across the basolateral membrane into the lymphoid tissue. Jung et al. (39) suggested that the nanoparticles with a negative charge combine with the hydrophilic surface and promote M-cell uptake.

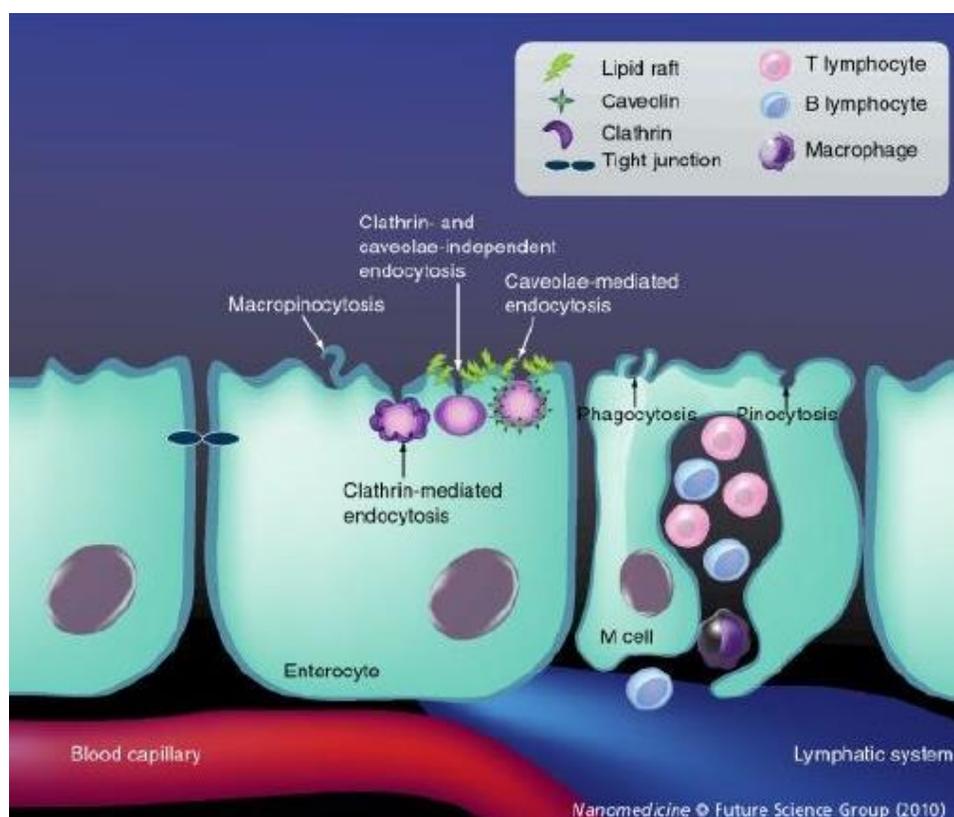


Figure 1.2 Possible transcellular mechanism of nanocarrier uptake by the intestinal barrier (38)

The particle size of SLNs is one of the most important factors in the uptake of nanoparticles by the gastrointestinal region, mainly peyer's patch. Various studies have been done for selection of optimum particle size for enhanced uptake by GITract. Smaller particles of size between 50 nm-500 nm are reported to be taken up readily in comparison to bigger particles (40, 41). Win and Feng established that 100nm polystyrene

nanoparticles experienced a 2.3 fold greater uptake by enterocytes compared with that of 50 nm polystyrene particles (42). In an another study, the uptake of 100 nm carboxylated polystyrene particles by PPs and normal villi was greater in comparison with larger particles (500 nm, 1 μ m and 3 μ m) (43, 44). These studies demonstrate that smaller particle size facilitates uptake by peyer's patch in comparison to larger particles but the difference in the efficacy of uptake of particles in size range 50-500 nm was not clear. Therefore, investigating and determining the optimal size range of particles is necessary for efficient oral delivery. Hence, attempt was made to determine the optimum particle size of solid lipid nanoparticles of Darunavir for enhanced oral bioavailability.

Targeting lymphatic system as HIV reservoirs

Regardless the remarkable progress made in HAART, HIV is able to conserve its replication machinery in anatomical and intracellular sites where the antiretroviral drugs have restricted access. HAART does not eliminate these reservoirs, nor prevent their generation and hence, a rebound in viral plasma levels occurs upon HAART withdrawal (45). CD4⁺ T lymphocytes are the best investigated cellular reservoir. Other cellular reservoirs are the macrophages and follicular dendritic cells (i.e. dendritic-like cells present in the lymph nodes), While the anatomical reservoirs of HIV includes the lymphoid organs [particularly the spleen, lymph nodes, and gut-associated lymphoid tissue (GALT)], the central nervous system, testicles and the female genital tract (46). Nearly 99% of all viral replication occurs in activated and productively infected CD4⁺ T cells of the blood and lymphoid tissues such as the peripheral secondary lymphoid organs, the spleen, lymph nodes, and GALT (47, 48). Lymphoid tissues have a greater extent of infection than the peripheral blood since only 2% of lymphocytes are in the general circulation at any one time and the remainders are distributed among the lymphoid tissues primarily in the Lymph nodes (48). Moreover, virus isolated from lymphoid tissues, blood CD4⁺ T cells and plasma are all equally sensitive to anti-HIV drugs (49), particularly protease inhibitor (50). These data suggest that even at effective plasma drug concentrations, insufficient drug exposure to lymphoid tissue may be one of the key factors in the inability to completely eliminate residual virus. As 98 % of circulating lymphocytes reside in the lymphatic system, the sub-therapeutic drug levels in

the lymphatic system may allow low persistent levels of viral replication and increase the probability of developing and harboring drug resistant virus. Hence, targeting of antiretroviral drug to lymphatics can lead to significant elimination of HIV along with enhanced bioavailability because of avoidance of first pass metabolism.

One of the most exciting areas of nanoparticle engineering or formulation is nanoparticle targeting. The targeting of nanoparticulate formulations focuses on both the development of new diagnostic tools and improving the efficacies of therapeutic agents. Targeting approaches can be broadly classified into two areas; passive and active targeting. The characteristics of nanoparticles like, size and zeta potential determines the passive targeting in the body. Active targeting involves modification of nanoparticles with a targeting moiety. This modification is usually on the corona of the particle, introducing a ligand, which facilitates the homing, binding and internalization of the formulation to the targeted cells. The most successful targeted drug therapies will ultimately prove to be a combination of passive and active targeting. Once retained in a tissue or organ, a nanocarrier displaying a targeting moiety on its surface would have a much higher specificity than the non cell-targeted nanocarrier because it has been enriched twice, once at the organ/ tissue level (i.e., passive targeting) and once at the cellular level (i.e., active targeting) (51). Active targeting of antiviral drug to the HIV-infected Tcells has been done using binding of nanocarrier to ligands like soluble gp 120 antibody fragment (52), CD4 molecule (53), gp120/gp41 complex with a CD4 molecule (54) and synthetic peptides derived from CD4 receptors (55). Thus, solid lipid nanoparticles optimized for enhanced Darunavir bioavailability were utilized for active targeting using synthetic peptide having affinity of binding selectively to CD4 molecules. Similar nanoparticles were developed for Atazanavir sulfate and subsequent attachment to synthetic peptide was done for increased binding affinity to CD4 Tcells.

1.1 Objective of the proposed work

The objective of the proposed investigation was

- To develop and characterize solid lipid nanoparticles and lipid nanoemulsion of Darunavir in order to increase its oral bioavailability.

- To develop and characterize Darunavir loaded peptide attached solid lipid nanoparticles and Atazanavir sulfate loaded peptide attached solid lipid nanoparticles in an attempt to enhance binding of nanoparticles to CD4 Tcells as viral reservoirs.

1.2 Hypothesis

It is hypothesized that the oral bioavailability of Darunavir would increase by incorporating it into lipid carriers- solid lipid nanoparticles and lipid nanoemulsion. The presence of lipid in the system would increase the lymphatic uptake for enhanced bioavailability and avoidance of first pass hepatic metabolism. Additionally, active targeting using peptide would enhance the binding and release of drug (Darunavir and Atazanavir sulfate) to T cells, the HIV reservoirs.

1.3 Work plan

- Literature survey, procurement of API's and excipients
- Procurement of excipients and preformulation studies
- Analytical methods development
- Formulation development of solid lipid nanoparticles of particle sizes -100 nm, 200 nm and 500 nm
- Characterization of Darunavir loaded solid lipid nanoparticles- particle size, zeta potential, TEM, FE-SEM, DSC, FT-IR and *in-vitro* drug release studies.
- *In-vivo* studies- Pharmacokinetic studies- comparison of all three sized particles, marketed formulation and drug suspension. Selection of optimum sized particles (final optimized nanoparticles) giving highest enhancement in bioavailability.
- Cell line studies of optimized Darunavir loaded solid lipid nanoparticles- MTT assay, internalization pathways of SLNs, intestinal permeability in Caco-2 cells
- *In-vivo* studies of optimized Darunavir loaded solid lipid nanoparticles- *In-situ* absorption from stomach and intestine, lymphatic transport studies, biodistribution studies
- Formulation development of lipid nanoemulsion of Darunavir and characterization- globule size, zeta potential, centrifugal stress, short term

stability, TEM. *In-vitro* release studies and *in-vivo* studies (Pharmacokinetics and biodistribution study)

- Formulation development of peptide attached solid lipid nanoparticles of Darunavir and characterization- particle size, zeta potential, qualitative and quantitative estimation of bound peptide. Cell-line study, *in-vivo* studies (Pharmacokinetics and biodistribution study).
- Formulation development of peptide attached solid lipid nanoparticles of Atazanavir sulfate and characterization- particle size, zeta potential, qualitative and quantitative estimation of bound peptide, *in-vivo* studies (Pharmacokinetics and biodistribution study),
- Stability study of formulations as per ICH guidelines.

1.4 References

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