DEVELOPMENT AND CHARACTERIZATION OF LOPINAVIR AND VERAPAMIL HCL-LOADED NANOSTRUCTURED LIPID CARRIERS (NLC)

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DEVELOPMENT AND CHARACTERIZATION OF LOPINAVIR AND VERAPAMIL HCL-LOADED NANOSTRUCTURED LIPID CARRIERS (NLC)

by

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DEDICATION

This thesis is dedicated to my parents, who have given me invaluable educational opportunities, and to my wife, who has been my emotional anchor all the way.

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Arshad Ali Khan

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LIST OF ABBREVIATION & SYMBOLS

ANOVA	Analysis of Variance
AUC	Area Under Curve
C_{max}	Maximum plasma drug concentration
DSC	Differential Scanning Calorimetry
e.g.	*exempli gratia*
GRAS	Generally recognized as safe
HPLC	High Performance Liquid Chromatography
hr	Hour
ICH	International Conference on Harmonization
ID	Internal Diameter
K_e	Elimination rate constant
Kg	Kilogram
LOD	Limit of Detection
LOQ	Limit of Quantification
M	Molar
mg	Milligram
min	Minute
mm	Millimeter
mL	Milliliter
nm	Nanometer
N	Theoretical plates
ng	Nanogram
NLCs	Nanostructured lipid carriers
o/w	Oil in water
PCS	Photon correlation spectroscopy
PdI	Polydispersity index
QC	Quality Control
RE	Relative Error
RH	Relative Humidity
rpm	Rotation per minute
RSD	Relative Standard Deviation
RSM	Response surface methodology

SD	Standard deviation
SEM	Scanning Electron Microscopy
Sec	Second
SLNs	Solid lipid nanoparticles
SMEDDS	Self microemulsifying drug delivery system
SNEDDS	Self nanoemulsifying drug delivery system
TEM	Transmission Electron Microscopy
T_{max}	Time taken to reach maximum plasma concentration
$t_{1/2}$	Half life
USFDA	United States Food and Drug Administration
USP	United States Pharmacopoeia
UV	Ultraviolet
v/v	Volume by Volume
w/v	Weight by Volume
XRD	X-ray powder diffraction
ZP	Zeta potential
µg/mL	Microgram per milliliter
µL	Microliter
˚C	Degree centigrade

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PEMBANGUNAN DAN PENCIRIAN PEMBAWA LIPID NANOSTRUKTUR (NLC) BERMUATAN LOPINAVIR DAN VERAPAMIL HCL

ABSTRAK

Tujuan kajian ini adalah untuk menyediakan pembawa lipid nanostruktur (NLC) lopinavir (tidak larutair) dan verapamil (larut air). NLC disediakan dengan menggunakan lipid pepejal (compritol 888 ATO), lipid cecair (asid oleik), dan surfaktan (poloxamer 188 dan Tween 80). Formulasi NLC disediakan dengan menggunakan teknik penyeragaman ricih tinggi. Reka bentuk faktorial 2^4 penuh digunakan dalam mengoptimumkan formulasi. Kesan empat pembolehubah tidak bersandar, termasuk masa penyeragaman, kepekatan lipid pepejal, cecair lipid dan surfaktan ke atas pembolehubah bersandar iaitu saiz zarah, indeks polisebaran (PDI), potensi zeta (ZP) dan kecekapan % pemerangkapan (EE) telah dikaji. Formulasi NLC bermuatan drug terpilih di beku-kering menggunakan trehalose sebagai agen pelindung. NLC bermuatan lopinavir beku-kering optimum (LOP-3) mempunyai purata saiz zarah, PDI, ZP dan % EE masing-masing 286.8 ± 1.3 nm, 0.413 ± 0.017, -48.6 ± 0.888 mV dan 87.83 ± 2.04%. NLCS bermuatan verapamil beku-kering optimum (VER-9) mempunyai saiz zarah, PDI, ZP dan% EE masing-masing 192.29 ± 2.98 nm, 0.553 ± 0.075, -48.8 ± 0.569 dan 93.26 ± 2.66%. Kajian pelepasan *in vitro* formulasi optimum LOP-3 dan VER-9 dalam bendalir simulasi gastrik (pH 1.2) dan bendalir simulasi usus (pH 6.8) masing-masing menunjukkan pelepasan pantas dan pelepasan bertahan. Kajian diferensial imbasan kalorimetri kedua-dua formulasi (LOP-3 dan VER-9) menunjukkan tiada interaksi kimia antara drug, lipid dan eksipien lain. Kajian sudut lebar X-ray penyerakan mengesahkan keadaan amorfus drug yang dimuatkan dalam kedua-dua formulasi LOP-3 dan VER-9. Imej TEM kedua-dua

formulasi LOP-3 dan VER-9 menunjukkan nanopartikel berbentuk bukan sfera dengan masing-masing saiz <100 dan <200 nm. Imej SEM menunjukkan bahawa trehalose mengurangkan agregasi formulasi LOP-3 dan VER-9. Kajian *in vitro* pengambilan selular formulasi LOP-3, VER-9 dan ampaian drug bebas yang dilakukan menggunakan Caco-2 garis sel, menunjukkan pengambilan drug yang lebih tinggi daripada formulasi NLC berbanding ampaian drug bebas. Profil farmakokinetik formulasi LOP-3 berikutan pemberian intraduodenal kepada tikus jantan Wistar menunjukkan bahawa bioavailabiliti relatif LOP-3 telah meningkat sebanyak 12.25 kali ganda berbanding ampaian lopinavir bebas. Kajian kestabilan jangka pendek formulasi LOP-3 dan VER-9 dilakukan pada empat masa (0, 1, 3 dan 6 bulan) dan pada tiga keadaan penyimpanan yang berbeza (5 ° C ± 3 ° C, 25 ° C ± 2 ° C / 60% RH ± 5% RH dan 40 ° C ± 2 ° C / 75% RH ± 5% RH). Formulasi LOP-3 dan VER-9 stabil pada penyimpanan sejuk (5 ° C ± 3 ° C). Jangka hayat formulasi LOP-3 dan VER-9 masing-masing adalah 18.84 dan 30.55 bulan. Kesimpulan, NLC bermuatan lopinavir dan verapamil HCl telah berjaya dibangunkan untuk penghantaran oral dan boleh sebagai alternatif kepada produk yang ada dipasaran untuk meningkatkan bioavailability oral.

DEVELOPMENT AND CHARACTERIZATION OF LOPINAVIR AND VERAPAMIL HCL-LOADED NANOSTRUCTURED LIPID CARRIERS (NLC)

ABSTRACT

The aim of the present study was to prepare nanostructured lipid carriers (NLCs) of lopinavir (water insoluble) and verapamil (water soluble). NLCs were prepared using solid lipid (e.g. compritol 888 ATO), liquid lipid (e.g. oleic acid), and surfactants (e.g. poloxamer 188 and tween 80). The NLCs formulation was prepared using a high shear homogenization technique. The 2^4 full factorial design was applied in the optimization of formulations. The effect of four independent variables, including homogenization time, solid lipid, liquid lipid and surfactant concentrations on the dependent variables, namely particle size, poly dispersity index (PDI), zeta potential (ZP) and % entrapment efficiency (EE) were studied. The selected drug loaded NLCs formulations were freeze-dried using trehalose as a cryoprotectant. The optimized freeze-dried lopinavir loaded NLCs (LOP-3) had mean particle size, PdI, ZP and % EE of 286.8±1.3 nm, 0.413±0.017, -48.6±0.888 mV and 87.83 ± 2.04 %, respectively. The optimized freeze-dried verapamil loaded NLCs (VER-9) had mean particle size, PdI, ZP and % EE of 192.29 ± 2.98 nm, 0.553 ± 0.075, -48.8±0.569 and 93.26 ± 2.66 % respectively. *In vitro* release studies of optimized LOP-3 and VER-9 formulations in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8) showed a burst and sustained release, respectively. The Differential scanning calorimetry study of both formulations (LOP-3 and VER-9) revealed the absence of chemical interaction between the drug, lipids and other formulation excipients. The wide angle X-ray scattering study revealed the amorphous state of loaded drug in both LOP-3 and VER-

9 formulations. TEM image of both LOP-3 and VER-9 formulations showed non-spherical shape nanoparticles with size of <100 and <200 nm respectively. SEM images showed that trehalose reduced aggregation of LOP-3 and VER-9 formulations. The *In vitro* cellular uptake studies of LOP-3, VER-9 formulations and free drug suspensions performed using Caco-2 cell line showed higher drug uptake from the NLCs formulations than the free drug suspension. The pharmacokinetic profile of LOP-3 formulation following intraduodenal administration of the formulation to male Wistar rats showed that the relative bioavailability of LOP-3 was increased by 12.25-fold compared to the free lopinavir suspension. The short term stability studies were performed for LOP-3 and VER-9 formulations at four time points (0, 1, 3 and 6 months) and three different storage conditions (5°C ± 3°C, 25°C ± 2°C/60% RH ± 5% RH and 40°C ± 2°C/75% RH ± 5% RH). The LOP-3 and VER-9 formulations were found stable in refrigerated storage condition (5°C ± 3°C). The shelf life of LOP-3 and VER-9 formulations were 18.84 and 30.55 months respectively. In conclusion, lopinavir and verapamil HCl loaded NLCs were successfully developed for oral delivery and could be an alternative to the available marketed products to improve the oral bioavailability.

CHAPTER 1

INTRODUCTION AND LITRATURE REVIEW

1.1 Colloidal drug carriers

The new technologies employed in drug discovery lead to find many new powerful substances. The development of new drugs alone is not sufficient to ensure progress in drug therapy. The main and common problems of new drug molecules are poor water solubility and inadequate bioavailability. The size reduction is the method of choice to enhance the solubility and hereafter the bioavailability of poorly water-soluble drugs. However, this method only suitable for class II and IV drugs of biopharmaceutical classification system (BCS). Therefore, to develop a drug carrier system has become an increasing demand to overcome these obstacles.

The ideal properties of the developed carrier system should have higher drug loading capacity, controlled release, possibility of drug targeting and free of acute and chronic toxicity. It should also offer chemical and physical stability for the loaded drug. The feasibility of production scaling up with reasonable overall costs should be available (Barratt, 2000; Mehnert & Mäder, 2001; Mainardes & Silva, 2004; Roger et al., 2010).

The colloidal carriers system, particularly those in the nanosize range, have been increasingly investigated in the last several years because they can fulfill the required properties mentioned above (van de Waterbeemd, 1998; Dressman & Reppas, 2000).

1.1.1 Microemulsions and nanoemulsions

Microemulsions are optically isotropic, transparent or translucent, low-viscous, single-phase liquid solutions. They are thermodynamically stable bicontinuous systems, which are essentially composed of water, oil, surfactant and co-surfactant (Cortesi & Nastruzzi, 1999; Heuschkel et al., 2008; Santos et al., 2008; Flores et al., 2016). Microemulsions exhibit better solubilizing capacities for both hydrophilic and lipophilic drugs than micellar solutions. Due to the high surfactant concentration in microemulsions, they are usually limited to dermal and peroral applications.

In the 1950's, nanoemulsions were introduced for the purposes of parenteral nutrition. They are heterogeneous systems composed of two immiscible liquids in which one one liquid is dispersed as droplets in the other one and the obtained liquid-liquid dispersion shows thermodynamic instability (Yukuyama et al., 2016; Ganta et al., 2014). Nanoemulsions have been used since some decades as drug carriers for lipophilic actives. Several pharmaceutical products based on nanoemulsion system have been introduced to the market e.g. Diazepam Lipuro®, Etomidat Lipuro®, Stesolid®, Valium®, Diazemuls® and NANOSTAT™ (Chime et al., 2014).

The advantages of nanoemulsions over microemulsions in terms of drug delivery are the reduction of the local and systemic side effects, for example lesser pain during injection and hemolytic activity caused by the high emulsifying agent concentration in microemulsions (Yukuyama et al., 2016). However, the lipophilic loaded drug can move from the oil droplets to the aqueous medium due to their partition effects and hence, stability problems arise (Dingler et al., 2008). Furthermore, the possibility of

controlled drug release from nanoemulsions is limited due to the high mobility of the loaded drug which is dissolved in the oily phase. Ammar et al., (2009) highlighted a rapid release of the drugs from nanoemulsions.

1.1.2 Nanocapsules and polymeric nanoparticles

Nanocapsules consist of a barrier made from polymers between the core (usually oil) and the aqueous surrounding environment. Interfacial polymerization and solvent displacement methods are often used for nanocapsules preparation (Letchford & Burt, 2007; Kothamasu et al., 2012). Polymers used in the formulation of nanocapsules include cellulose derivatives, poly methylidene malonate, poly alkylcyanoacrylates, polyanhydrides, polyorthoesters and polyesters for example poly glycolic acid, poly lactid acid and poly ε-caprolactone and their copolymers (Mora-Huertas et al., 2010). Various procedures are applied for polymeric nanoparticles production e.g. solvent evaporation, coacervation technique, interfacial polymerization, degradation by high-shear forces (high pressure homogenization or micro fluidization) and natural proteins or carbohydrates denaturation or desolvation (Mora-Huertas et al., 2010; Kothamasu et al., 2012).

In comparison to liposomes and emulsions, the presence of polymeric barrier in nanocapsules and solid polymeric matrix in polymeric nanoparticles can provide more protection to the loaded drug molecules (Mayer, 2005; Anton et al., 2008; Mora-Huertas et al., 2010). Currently, these carriers have come in the market in the name of some pharmaceutical products e.g. Enantone Depot®, Decapeptyl® and Gonapeptyl Depot®.

1.2 Lipid-based nanoparticles

1.2.1 Liposomes

Liposomes consist of single or multi lipid bilayers of amphiphilic lipids, such as phospholipids, glycolipids and cholesterol (Allen & Cullis, 2013). Liposomes were described in 1965 by Bangham et al. as a cell membrane model (Bangham, 1992). Later on they were used as a carrier system and were introduced to the cosmetic market by Dior in 1986. The first topical liposomal pharmaceutical product in the brand name of Pevaryl®-Lipogel was produced by Cilag. Comprehensive biodisposition studies showed the superiority of the liposomes over the commercial Pevaryl gel, lotion and cream forms. The liposomal products resulted in an increase in drug concentration in the epidermis (7-9 folds), at the site of action (Touitou et al., 1994). The liposomal products displayed less toxicity in contrast to the conventional formulations of the same active pharmaceutical ingredients (Fassas & Anagnostopoulos, 2005; Akbarzadeh et al., 2013). The liposomes size ranges in between few nanometers to some micrometers. There are many methods to prepare the liposomes, e.g. solvent dispersion, detergent dialysis and mechanical dispersion (Elsayed et al., 2007; Samad et al., 2007; Akbarzadeh et al., 2013). The hydrophilic and lipophilic drugs can be encapsulated into the liposomes and due to liposomal composition, this carrier system are also acceptable for intravenous dosage form (Müller et al., 2000; Allen & Cullis, 2013;). During storage, it shows some physical and chemical instability due to unsaturated fatty acid oxidation and phospholipids ester binding hydrolysis. Moreover, due to lack of proper production methods, it cannot produce on large scale easily. All of these points make liposomes not optimal as a pharmaceutical carrier system. To overcome some of the liposomes drawbacks, niosomes were developed. The niosomes

was prepared by saturated hydrocarbon chains with intramolecular ether bindings that increases its chemical stability (Choi & Maibach, 2005; Allen & Cullis, 2013).

1.2.2 Solid lipid nanoparticles (SLNs)

SLNs are prepared by substituting the oil (liquid lipid) of oil in water emulsion by a solid lipid or a blend of solid lipids. This mixture produced solid nanoparticles which stay solid at both body and room temperature (Lucks Stefan, 1993; Parhi & Suresh, 2012). SLNs are comprised of 0.1%w/w to 30%w/w of solid lipid with respect to the amount of whole formulation and usually it stabilized with 0.5%w/w to 5%w/w of surfactant with respect to lipid content. The size of SLNs ranged in submicron region, i.e. extending from around 40 nm to 1 micron (Lucks Stefan, 1993; Parhi & Suresh, 2012).

Moreover, after the preparation at least a part of the solid lipid in SLNs crystallizes in a higher energy modification (α or β`). During storage, these modifications can transform to the low energy, more ordered β modification. Due to this high degree of order, the number of imperfections in the SLNs crystal lattice is small, this leads to drug expulsion (Mehnert & Mäder, 2001; Müller et al., 2002b).

1.2.3 Nanostructured lipid carriers (NLCs)

NLCs have been developed to overcome the drawbacks associated with SLNs. They are considered to be the second generation of lipid nanoparticles. Compared to SLNs, NLCs show a higher loading capacity for active compounds by creating a less ordered solid lipid matrix, i.e. by blending a liquid lipid with the solid lipid, a higher particle

drug loading can be achieved. Therefore, the NLCs have an increased drug loading capacity in comparison to SLNs and the possibility of drug expulsion during storage is less (Müller et al., 2002a; Müller et al., 2002b; Saupe et al., 2005; Naseri et al., 2015; Khan S et al., 2015). NLCs have also a lower water content of the particle suspension and a less tendency of unpredictable gelation (Müller et al., 2002a; Naseri et al., 2015).

1.2.3(a) NLCs composition

NLCs are prepared using combinations of solid and liquid lipids (oils). Herein solid lipids and liquid lipids are mixed together at ratio of 70:30 to 99.9:0.1 to obtain the controlled structured particle matrix. The obtained blends are solid at body and room temperature rather than present as oil (liquid lipids) in the mixture (Müller et al., 2000). The total solid content of NLCs can be enhanced up to 95% (Selvamuthukumar & Velmurugan, 2012)

1.2.3(b) Methods of NLCs preparation

Various methods have been used for the preparation of NLCs. The methods are microemulsion technique (Gupta et al., 2014), emulsification-solvent evaporation (Andalib et al., 2012), multiple emulsion technique (Selvamuthukumar & Velmurugan, 2012), solvent injection (or solvent displacement) (Tiwari & Pathak, 2011), ultrasonication (Uprit et al., 2013), phase inversion (Ming Sun et al., 2014) (Ming Sun et al., 2014), membrane contractor technique (Thatipamula et al., 2011), high-pressure homogenization (Shangguan et al., 2014) and high-shear homogenization (Ustündağ-Okur et al., 2014). High shear-homogenization is very common technique for lipid based nanoformulations. It shows several benefits over

other techniques for example, easy production scale-up, lesser process time and avoidance of organic solvent. For these reasons, it is widely accepted in several productions areas such as pharmaceutical and food industry.

1.2.3(c) Characterization of NLCs

Characterization of NLCs may be a serious challenge because of the small size of the particles resulting in the complexity of the system. Therefore, characterization of the NLCs may be an essential requisite for the management of the quality of the product. Many parameters need to be considered that have direct impact on the stability and release kinetics of NLCs such as particle size, polydispersity index and zeta potential.

1.2.3(d) Stability of NLCs

Among the hurdles in lipid based nanoparticles development, the long-term instability of lipid based nanoparticles dispersions is undoubtedly a major concern. In order to prolong the physical and chemical stability of the NLCs the water removal is a necessary step. The best common method in the pharmaceutical field is freeze drying which allows to change aqueous formulation into dried formulation for sufficient stability during storage (Franks, 1998; Varshosazet et al., 2012). Pharmaceutically, it is also known as lyophilization. It removes water from the frozen sample via sublimation and then desorption of that vapors take place under vacuum.

1.2.3(e) Role of NLCs in different delivery system

NLCs are used as biocompatible carriers for several types of drugs intended for pharmaceutical, cosmetic, and biochemical purposes. During the last one decade

different drugs or active substances have been entrapped into NLCs like lipophilic, hydrophilic molecules, including labile compounds, such as proteins and peptides. Their main feature is that they are prepared with physiologically well-tolerated lipids. The wide range of surfactants and cosurfactants available for the production of such particles make these carriers highly suitable for distinct applications like for topical, oral, parenteral, inhalational and ocular administration.

1.2.3(f) NLCs versus SLNs

As mentioned previously NLCs overcome the disadvantages associated with SLNs, in other words, they provide higher drug loading, faster release rate and storage stability due to a use of blend of solid lipid and liquid lipid in their formulations. Several comparative studies have been documented in literature in which NLCs have served as a better carrier than SLNs. For example, in a study between SLNs and NLCs of simvastatin, it was observed that entrapment efficiency of the drug was improved in NLCs (93.33%) when compared with SLNs (75.81%) due to creation of extra space for loading by liquid lipid (Tiwari & Pathak, 2011). Also, the *in vitro* release patterns in both SLNs and NLCs were similar but NLCs displayed high-percent cumulative drug release in comparison to SLNs in 55 h. A lesser mobility of drug in SLNs (a crystallized system) in comparison to NLCs (disordered arrangement) was responsible for the slower release of drug. Differential scanning calorimetric analysis showed decreased recrystallization index of NLCs in comparison to solid lipids and physical mixture of solid lipid and liquid lipid favoring the formation of disordered arrangement and reduced capacity of solid lipids to recrystallize suggesting their higher long-term stability. The results of *in vivo* studies also suggested NLCs to be superior as they exhibited 2.29-fold increase in oral bioavailability when administered to mice. Similar

results were also noted with lovastatin-loaded NLCs. Study of partitioning behavior of lovastatin in pure solid lipid and mixtures of solid lipid and liquid lipid also depicted higher partitioning of drug in the lipid phase consisting of a mixture of solid lipid (Precirol ATO 5) and liquid lipid (squalene) thus suggesting that higher solubility of drug was favored by the presence of liquid lipid (Chen et al., 2010). Another report on progesterone (Yuan, Wang, et al., 2007) and domperidone-loaded (Thatipamula et al., 2011) SLNs and NLCs also suggested the NLCs as a better vehicle in respect of drug loading and release rate (Poonia, Kharb, Lather, & Pandita, 2016).

1.3 Lipid based nanoparticles as a carrier for oral drug delivery

Lipid based nanoparticles for example SLNs and NLCs can be given by oral route in tablet, capsules, pellets, dispersions and as lyophilized powders ready for reconstitution (Mehnert & Mäder, 2001). The GIT stability of lipid nanoparticles has to be comprehensively studied, the acidic pH and elevated ionic strength in the upper GIT could result in lipid nanoparticles aggregation. So, to verify this, some authors investigated different lipid nanoparticle formulations behavior in simulated gastric fluids. They observed that zeta potential of a minimum 8-9 mV might hinder lipid nanoparticles aggregation (Zimmermann & Müller, 2001). Furthermore, to optimize the release profile of lipid nanoparticles, the knowledge of enzymatic degradation of lipids during *in vivo* drug release studies is essential (Olbrich & Müller, 1999). Since, *in vivo* release media contain lipolytic enzyme (pancreatic lipase) and drug release take place by lipid matrix degradation and solid phase diffusion process. While, *in vitro* drug release take place by solid phase diffusion, due to absence of lipolytic enzyme. Olbrich & Müller (1999), established an enzymatic degradation assay of SLNs using a lipase/colipase complex. In that, it shown that the degradation velocity depends on

the composition of the lipid matrix. In general, degradation velocity increased with decreasing length of the fatty acid chain length when using glycerides as lipid matrix. In addition, the degradation of SLNs based on waxes (e.g. cetylpalmitate) was found to be slower compared to glyceride matrices (Olbrich & Müller, 1999). A prerequisite for the degradation of lipid nanoparticles after oral administration is the anchoring of the lipase/ colipase complex onto the particle surface. Therefore, it was expected that not only the composition of the lipid matrix, but also the nature of the stabilizing surfactant layer would be a determining factor for degradation (Olbrich & Müller, 1999). Compounds such as cholic acid sodium salt are known to promote the anchoring of the lipase colipase complex on surfaces (Borgström, 1975). Sterically stabilizing polymers such as the Poloxamer series are known to prevent or to hinder the absorption of large molecules such as proteins (Blunk et al., 1993).

1.4 The drug absorption of lipid-based nanoparticles via lymphatic circulation

The lymphatic system is part of the circulatory system and is comprised of an intricate network of conduits that carry a clear fluid called lymph. The primary functions of the lymphatic system are to maintain the body's water balance by returning extracellular fluid that has leaked out into the interstitial space back to the systemic circulation and to transport immune cells to the lymph nodes (Iqbal & Hussain, 2009; Miteva et al., 2010). Further, the lymphatic system has specialized roles in specific areas because of its nonuniform structure and function throughout the body. It plays an essential role in absorption of long- chain fatty acids, triglycerides, cholesterol esters, lipid soluble vitamins, and xenobiotics (Iqbal & Hussain, 2009). Drug delivery via the lymphatic system has several major advantages, including circumventing first-pass metabolism in the liver and targeting drugs to diseases that spread through the lymphatic system

(eg, certain types of cancer and human immunodeficiency virus). The lymphatic system also plays an active role in disseminating metastatic cancer cells and infectious agents throughout the body. Cancer cells use the lymph nodes as a reservoir to spread to other areas of the body (Sleeman, 2000, 2006; Pantel & Brakenhoff, 2004; Lee et al., 2005; Iqbal & Hussain, 2009).

There are three ways to deliver drugs through the intestinal lymphatic vessels (Muranishi et al., 1997; Porter & Charman, 1997). First, lymphatic capillaries are comprised of single-layered, nonfenestrated endothelial cells. These cells are arranged in a highly gapped and overlapped manner to form a porous wall in the lymphatic vasculature, which allows for macromolecular targeting to the lymphatic system. Therefore, increased absorption of hydrophilic macromolecules and macroconjugates is possible by opening up the paracellular route with the help of an absorption enhancer (Hiroshi et al., 1981). Secondly, gut-associated lymphoid tissue consists of either isolated or aggregated lymphoid follicles that form Peyer's patches, which provide an entry point for drug to the lymphatics (Figure 1.1 A) (Eldridge et al., 1990; Hawley, Davis, & Illum, 1995; Beier & Gebert, 1998; Wells & Mercenier, 2008). Finally, the primary route for lipid transport is through the intestinal walls via transcellular absorption, paracellular transport, P-glycoprotein, and cytochrome P450 inhibition. Increased production of chylomicrons is associated with delivery of lipophilic compounds into the lymphatic system (Figure 1.1 B) ((Porter & Charman, 1997).

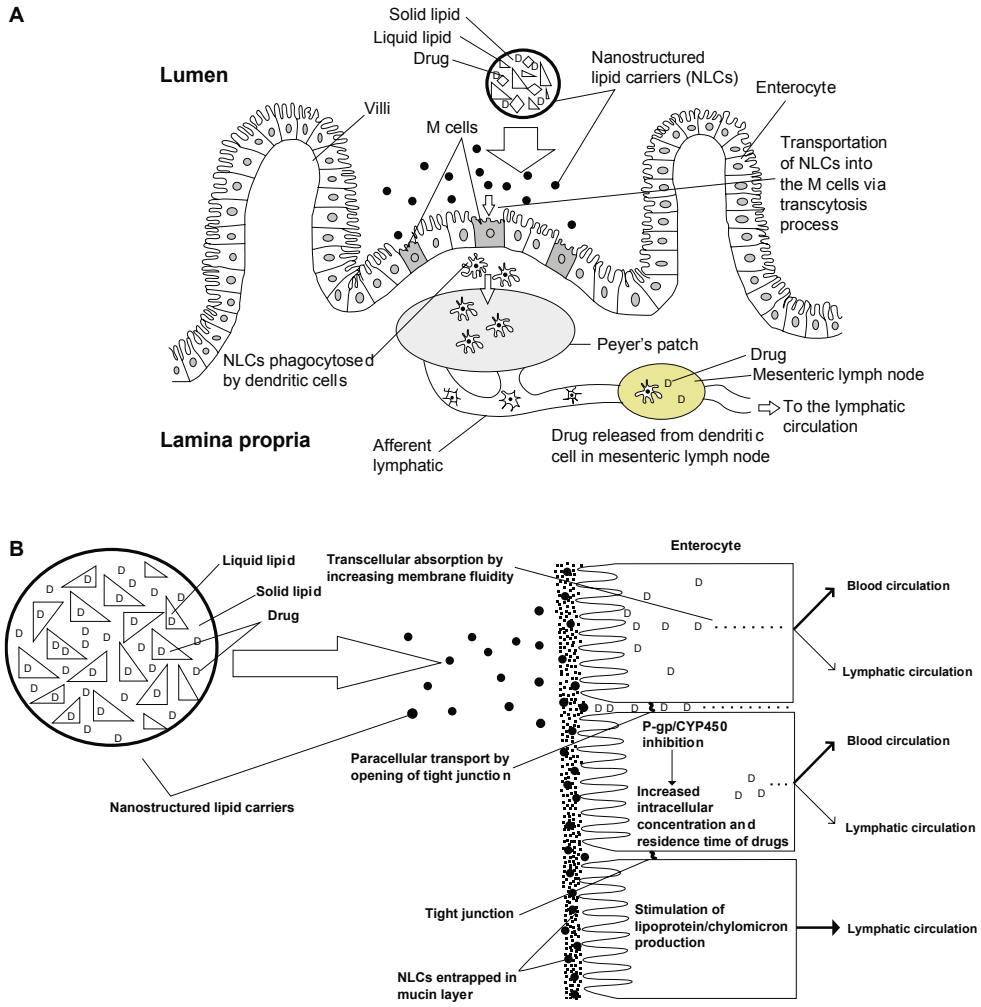

Figure 1.1 A Schematic transverse section of Peyer's patch, illustrating M cell transportation of lipid based formulation (nanostructured lipid carriers) to the lymphatic vessels. B Schematic diagram of the different mechanisms of the intestinal transport of lipid based formulation (nanostructured lipid carriers) through blood and lymphatic circulation. Adopted from Khan et al., (2013).

Abbreviations: P-gp, P-glycoprotein; CYP450, cytochrome P450 enzyme; M cell, membranous cell.

A number of lipid-based formulations, including emulsions, micellar systems, self-emulsifying drug delivery systems, self-microemulsifying drug delivery systems, self-nanoemulsifying drug delivery systems, liposomes, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs) have been investigated as drug carriers for the lymphatic system (table 1.1).

Table 1.1 Formulations that have been used for lymphatic delivery

Formulations	Drugs	References
Emulsion	Penclomedine	(Myers & Stella, 1992)
Emulsion	Ontazolast	(Hauss et al., 1998)
Microemulsion	Puerarin	(Wu et al., 2011)
Microemulsion	Raloxifene	(Thakkar et al., 2011)
Micellar systems	Cyclosporine A	(Takada et al., 1986)
SEEDS	Coenzyme Q 10	(Kommuru et al., 2001)
SMEDDS	Halofantrine	(Holm et al., 2003)
SMEDDS	Nobiletin	(Yao et al., 2008)
SMEDDS	Valsartan	(Dixit et al., 2010)
SMEDDS	Vinpocetine	(Y. Chen et al., 2008)
SMEDDS	Silymarin	(Li et al., 2010)
SMEDDS	Sirolimus	(Minghui Sun et al., 2011)
SNEDDS	Carvedilol	(Singh et al., 2011)
SNEDDS	Valsartan	(Beg et al., 2012)
SNEDDS	Halofantrine	(Holm et al., 2012)
Liposomes	IgG1	(Moghimi & Moghimi, 2008)
Liposomes	Doxorubicin	(Frenkel et al., 2006)
Liposomes	Cefotaxime	(Ling et al., 2006)
Liposomes	9-nitro-camptothecin	(Lawson et al., 2004)
Liposomes	Paclitaxel	(Latimer et al., 2009)
Liposomes	Ovalbumin	(Kojima et al., 2008)
SLNs	Etoposide	(Harivardhan et al., 2005)
SLNs	Methotrexate	(Paliwal et al., 2009)
SLNs	Idarubicin	(Zara et al., 2002a)
SLNs	Tobramycin	(Cavalli et al., 2000; Cavalli et al., 2003)

SLNs	Nimodipine	(Chalikwar et al., 2012)
NLCs	Testosterone	(Muchow et al., 2011)
NLCs	Vinpocetine	(Zhuang et al., 2010)
NLCs	Tripterine	(Zhou et al., 2012)

Abbreviations: SEEDS, Self emulsifying drug delivery system; SMEDDS, Self-microemulsifying drug delivery systems; SNEDDS, Self-nanoemulsifying drug delivery systems; SLNs, Solid lipid nanoparticles; NLCs, Nanostructured lipid carriers.

1.5 Different routes for NLCs lymphatic uptake

Lipid based nanoformulations such as SLNs and NLCs offer a prominent advantage over other nanoparticulate systems because they use physiological lipids and surfactants, which are generally recognized as safe. The commonly used lipids in the lipid based nanoformulations preparation are fatty acids, waxes, monoglycerides, diglycerides, and triglycerides; surfactants such as poloxamer and polysorbate are also widely used. Further, the possibility of avoiding a solvent using high-pressure homogenization can help to prevent the carrier biotoxicity problem in humans (Müller et al., 2000; Wissing et al., 2004). Lipid based nanoformulations involve formation of a relatively rigid core consisting of lipids that are solid at room temperature. Thus, lipid based nanoformulations can help improve stability and provide controlled release and drug targeting (Mehnert & Mäder, 2001). The minute size of these formulations enables efficient uptake of drugs into the intestine, particularly via the lymphatic route, involving particles only 20–500 nm in diameter (Yuan, Wang, et al., 2007). Absorption via the lymphatic route can be used for delivery of cytotoxic agents to overcome the limitations of nonspecificity, drug resistance, and severe toxicity (Wong et al., 2007).

There are mainly three different routes have been explored for lymphatic uptake of NLCs, including the subcutaneous, pulmonary, and duodenal routes.

1.5.1 Subcutaneous route for lymphatic delivery of NLCs

The subcutaneous route is an attractive one for lymphatic delivery of NLCs, with several advantages, including drug accumulation at the site of administration for a longer period of time, low clearance, sustained release, and increased absorption. On subcutaneous administration, NLCs are not directly transported into the bloodstream because capillaries control the permeability of water and small molecules. Instead, the lymphatic capillaries surrounding the subcutaneous injection site absorb the lipid-based nanoparticles. Absorption of NLCs into the lymphatic system depends primarily on the size of the nanoparticles. Larger NLCs accumulate at the injection site, and the drug is slowly released from the NLCs. The free drug can enter the blood circulation via pores on the walls of the capillaries. Smaller NLCs (not more than 100 nm) can easily access the lymphatic capillaries and concentrate in regional lymph nodes (Oussoren, 2001). Thus, based on these advantages, NLCs could be developed as a carrier for lymphatic drug delivery by subcutaneous administration because they have improved physicochemical properties compared with other lipid-based nanocarrier systems.

1.5.2 Pulmonary route for lymphatic delivery of NLCs

Drug administration via the pulmonary route has several advantages compared with the oral and parenteral routes. The pulmonary route avoids first-pass metabolism, reduces systemic toxicity, noninvasive, minimizes the need for continuous dosing,

allows the drugs administered to reach less accessible parts of the lung directly, and enables increased local concentrations of drug (Walker et al., 2009). The pulmonary route shows great potential for the delivery of NLCs into the lymphatic circulation. The particle size of NLCs can be reduced to less than 500 nm, which could increase drug deposition in the lung epithelium because of their diffusional mobility (Jaques & Kim, 2000). NLCs are lipid-based nanoparticles that could be used as a carrier for targeting drugs to small cell lung cancer and human immunodeficiency virus, both of which spread through the lymphatic system and can cross into the systemic circulation (Chambers et al., 2002; Pantel & Brakenhoff, 2004). Thus, NLCs have the potential to provide a drug delivery mechanism via the lymphatic system through the pulmonary route and may have increased effectiveness compared to SLNs.

1.5.3 Intestinal route for lymphatic delivery of NLCs

NLCs have the potential to be an effective method for oral drug delivery, because they can increase solubility and enhance the oral bioavailability of drugs that are either hydrophobic or poorly soluble in water (O'Driscoll & Griffin, 2008). Among the traditional lipid-based formulations, NLCs have become an important alternative to the more traditional colloidal drug carriers (Müller et al., 2002a). Zhuang et al developed drug-loaded NLCs to improve the oral bioavailability of vinpocetine (Zhuang et al., 2010). Both vinpocetine-loaded NLCs and a vinpocetine suspension were orally administered to male Wistar rats. The time taken to reach maximum plasma concentrations (t_{max}) and the peak concentration reached (C_{max}) for the vinpocetine suspension were 30 minutes and 354.29 ± 57.49 ng/mL, respectively, whereas the t_{max} and C_{max} of vinpocetine-loaded NLCs were 1.5 hours and 679.29 ± 135.57 ng/mL, respectively. The Tmax for vinpocetine-loaded NLCs was one hour

longer than the vinpocetine suspension, indicating indirect transport of NLCs into the systemic circulation. The C_{max} for vinpocetine- loaded NLCs was also significantly higher than the vinpocetine suspension. The area under the curve for the vinpocetine-loaded NLCs was 3.2-fold greater than that of the vinpocetine suspension. *In vivo* pharmacokinetic analysis showed a 322% increase in the relative bioavailability of vinpocetine-loaded NLCs compared to the vinpocetine suspension after oral administration. These results suggest that NLCs can improve the oral bioavailability of drug which are poorly soluble in water (Zhuang et al., 2010). One possible reason for the enhanced bioavailability of vinpocetine could be that NLCs are transported in the lymphatic system, so largely avoid first-pass metabolism, which is the main cause for the low bioavailability of vinpocetine (Zhuang et al., 2010).

In another study, Zhou et al., (2012) developed tripterine NLCs and evaluated their potential as an oral drug delivery system. A rat intestinal perfusion model was used to compare the absorption of tripterine-loaded NLCs with that of a tripterine solution. The effective permeability of tripterine NLCs in the duodenum, jejunum, ileum, and colon was 2.1, 2.7, 1.1, and 1.2 times higher, respectively, compared with the tripterine solution. The percentage absorption of tripterine- loaded NLCs in 10 cm of duodenum, jejunum, ileum, and colon was 2.2, 2.3, 1.2, and 1.3 times greater, respectively, than for the tripterine solution (Zhou et al., 2012). These results indicate that NLCs could be used as a carrier to improve the absorption of tripterine in the gastrointestinal tract.

1.6 *In vitro* models for studying lymphatic drug transport

Various *in vitro* models can serve as an alternative to *in vivo* models for studying lymphatic drug transport. In the intestinal permeability model, Caco-2 cells are used to evaluate intracellular lipoprotein-lipid assembly and to examine the effect of lipids and lipidic excipients on incorporation of drug with lipoproteins in lymphatic transport (Seeballuck et al., 2003; Seeballuck et al., 2004; Karpf et al., 2006). In one *in vitro* model, Gershkovich & Hoffman, (2005) described a correlation between the degree of ex-vivo incorporation of a drug into chylomicrons and the extent of intestinal lymphatic drug transport. According to a lipolysis model described by Dahan & Hoffman, (2008) *in vivo* drug absorption could be predicted by evaluating drug release from a lipid-based drug delivery system and estimating precipitation of the drug during lipolysis. Holm & Hoest, (2004) reported an in silico method that established a quantitative relationship between the molecular structure and amount of drug transferred from the intestinal to the lymphatic system.

1.6.1 *In vitro* cellular uptake study via Caco-2 cell monolayer model to evaluate the indirect lymphatic uptake of NLCs

The Caco-2 cell line is a heterogeneous human epithelial colorectal adenocarcinoma cells that derived from human colon carcinoma. These cells have the ability to differentiate and polarized functionally and morphologically as the intestinal enterocytes lining when cultured in specific conditions. These cells resemble as enterocytes in various aspects of its properties such as microvilli, tight junction, some transporters and enzymes. Due to the uniqueness of these cells and lack of proper *in vitro* lymphatic absorption methods these cells have been chosen and used as an

indirect method for the lymphatic uptake study for oral lipid based nanoformulations (Shah et al., 2014).

1.6.2 Characteristics of Caco-2 cells

1.6.2(a) Tight junction

The Caco-2 monolayers form the tight junction when cells become confluent. The integrity of monolayer can be measured by trans-epithelial electrical resistance (TEER) and paracellular markers permeability such as mannitol, inulin and lucifer yellow across the Caco-2 monolayer (Mukherjee et al., 2008). For paracellular transport the pore radius of Caco-2 tight junction is smaller (~4.5 Å) than the human intestinal enterocytes tight junction pore radius (~8 – 13 Å) (Fine et al., 1995; Watson et al., 2001).

1.6.2(b) Drug transporters

Several transporter and enzyme are present in Caco-2 monolayers same as found in intestine. These are H+/di-tripeptide transporter (PEPT1), monocarboxylic acid transporter 1 (MCT1), organic anion-transporting polypeptide 2B1 (OATP-B), organic cation/carnitine transporter (OCTN2) and apical Na+-dependent bile acid transporter (ASBT), all are expressed on apical membrane. Also, the efflux transporter P-glycoprotein (MDR1) and multidrug resistance-associated protein 2 (MRP2) are present on apical membrane (Figure 1.2). These efflux transporter promotes the elimination of solute from intracellular site to the basolateral area, as a result to enhance the drug absorption (Sun et al., 2008).

1.6.2(c) Metabolic enzymes

The Caco-2 monolayers express several metabolic enzymes, for example cytochrome P450 1A (CYP1A), UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs) and sulfotransferases (SULTs) (figure 1.2). However, the Cytochrome P450 3A4 (CYP3A4) usually absent or weakly present in Caco-2 monolayers (Sun et al., 2008).

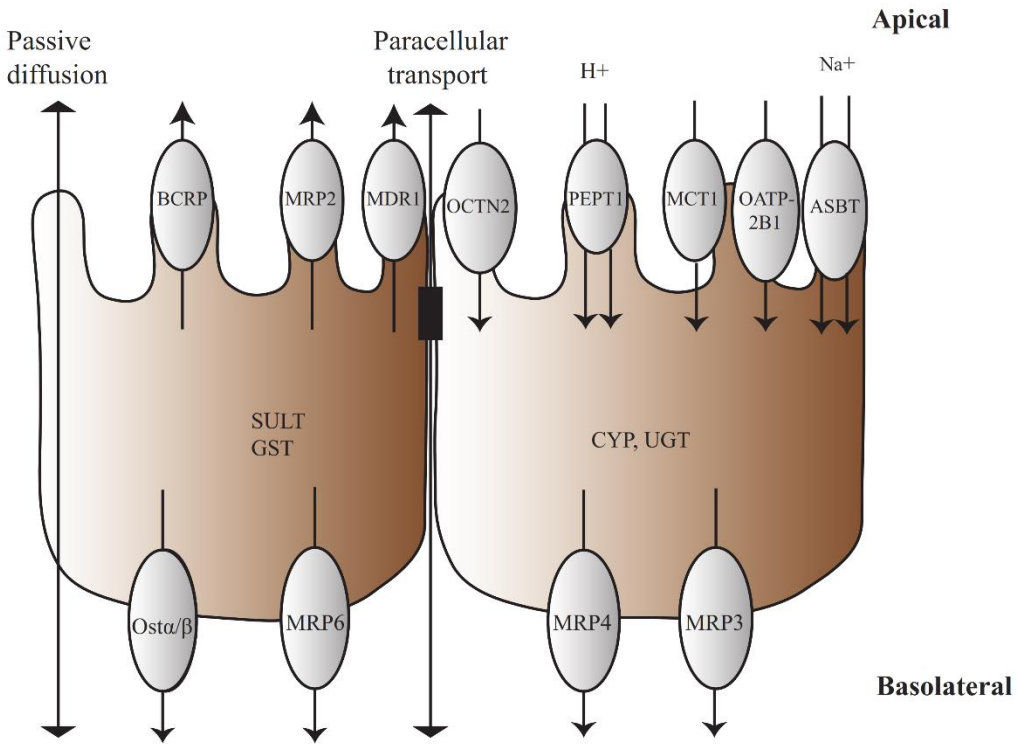

Figure 1.2 Drug transporters and metabolic enzymes present in Caco-2 cell monolayer

Abbreviations: PEPT1, H+/di-tripeptide transporter; OATP-B, organic anion-transporting polypeptide 2B1; MCT1, monocarboxylic acid transporter 1; ASBT, Na+-dependent bile acid transporter; OCTN2, organic cation/carnitine transporter; MDR1, or P-glycoprotein, P-gp, multidrug resistance protein 1; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; MRP4, multidrug resistance-associated protein 4; MRP6, multidrug resistance-associated protein 5; BCRP, breast cancer resistance protein; OSTα/β, organic solute transporters; CYP, cytochrome P450; SULTs, sulfotransferases; UGTs, UDP-glucuronosyltransferases; GSTs, glutathione S-transferases.