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Research paper

Uptake of phenothiazines by the harvested chylomicrons *ex vivo* model: Influence of self-nanoemulsifying formulation design

Gul Shahnaz [a], Markus Hartl [b], Jan Barthelmes [a], Katharina Leithner [a], Federica Sarti [a], Fabian Hintzen [a], Deni Rahmat [a], Willi Salvenmoser [c], Andreas Bernkop-Schnürch [a,*]

[a] Department of Pharmaceutical Technology, University of Innsbruck, Innsbruck, Austria
[b] Institute of Biochemistry, University of Innsbruck, Innsbruck, Austria
[c] Institute of Zoology, Leopold-Franzens University, Innsbruck, Austria

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ABSTRACT

The aim of this study was to examine the potential of self-nanoemulsifying drug delivery systems (SNEDDS) on the uptake of the lipophilic and poorly water soluble phenothiazines thioridazine and chlorpromazine with the isolated plasma derived chylomicron (CM) *ex vivo* model. The multi-component delivery systems were optimized by evaluating their ability to self-emulsify when introduced to an aqueous medium under gentle agitation. The uptake of phenothiazines by isolated plasma derived chylomicrons was investigated with short chain triglyceride (SCT) SNEDDS, medium chain triglyceride (MCT) SNEDDS, and long chain triglyceride (LCT) SNEDDS. SNEDDS were also evaluated for their stabilities, dispersibilities, percentage transmittances and by particle size analyses. For thioridazine a 5.6-fold and for chlorpromazine a 3.7-fold higher CM uptake could be observed using a LCT-SNEDDS formulation compared to the drugs without formulation. In contrast, *ex vivo* uptake by isolated CM was not significantly increased by SNEDDS formulations based on MCT and SCT. Compared with isolated CM, the CM sizes were increased 2.5-fold in LCT-SNEDDS, whereas in MCT-SNEDDS or SCT-SNEDDS only a small, non-significant ($P < 0.05$) increase in CM size was observed. These results show that distinct SNEDDS formulations containing phenothiazines are efficiently uptaken by plasma derived chylomicrons *ex vivo*.

1. Introduction

Following oral administration, highly lipophilic drugs (typically log $P > 5$, lipid solubility > 50 mg/g) are taken up by the systemic circulation via the intestinal lymphatic system. The key mechanism of the intestinal lymphatic transfer is the association of lipophilic drugs with chylomicrons (CM), likely taking place in the intercellular space and in enterocytes with subsequent uptake by the intestinal lymphatic system [1–4]. The foremost reason for the broad interest in lymphatic transport is the potential to utilize this pathway in order to increase the oral bioavailability of highly lipophilic drugs. Lymphatic transport may contribute to the increase in the oral bioavailability of lipophilic compounds by a number of potential mechanisms: additional pathway of transfer of lipophilic compounds to the blood, bypass of the hepatic first-pass metabolism, and reduction in the intestinal first-pass metabolism [3]. The physicochemical properties of molecules required for lymphatic transport of drugs are thought to be high lipophilicity (log $P > 5$) and reasonable solubility in long chain triglycerides (LCT) [5].

The aim of the present study was to examine the impact of different self-nanoemulsifying drug delivery system (SNEDDS) on the uptake of lipophilic, poorly water soluble drugs utilizing the isolated CM *ex vivo* model [5]. SNEDDS are isotropic mixtures of oil, surfactant and co-surfactant which form fine oil-in-water (o/w) nanoemulsions upon introduction in the aqueous phase under gentle agitation. Furthermore, the influence of the chain length of the lipid-based SNEDDS formulations on the size of chylomicrons and on the uptake extent of the drugs with the harvested CM *ex vivo* model had to be evaluated. For this investigation we selected the two structurally related phenothiazines chlorpromazine and thioridazine. Phenothiazines have been used since the 1950s as tranquilizers and antipsychotic drugs. They have also been reported to have anti-infective [6] and anti-cancer activities [7]. Chlorpromazine (log $P = 5.6$) and thioridazine (log $P = 5.9$) are highly lipid-soluble compounds. When administered orally, they are incompletely absorbed with a bioavailability of approximately 30–50%. Here, we show that enhanced drug *ex vivo* uptake using SNEDDS is determined by the physicochemical properties of the drug and its affinity to the lipidic constituents that compose the chylomicron core. Because this process may be similar to SNEDDS uptake *in vivo*,

* Corresponding author. Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Josef-Möller-Haus, Innrain 52c, A-6020 Innsbruck, Austria. Tel.: +43 512 507 5383; fax: +43 512 507 2933.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

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the *ex vivo* approach might represent a suitable model system for lipophilic drug delivery investigation.

2. Materials and methods

2.1. Materials

Captex 355 (Abitec Corporation, Janesville, WI, USA) and ethanol (CRS, Australia) were used as purchased. Triacetin, Tween 85, linseed oil, olive oil, glycerol, ammonium acetate, acetonitrile, NaCl, KBr, free bases of chlorpromazine (CPZ) and thioridazine (THD) were purchased from Sigma–Aldrich, Austria. All chemicals were of the highest purity available and used as received. All solvents were high performance liquid chromatography (HPLC) grade.

2.2. Methods

2.2.1. Drug solubility tests in nanoemulsion components

The solubility of phenothiazines in various oils (triacetin, linseed oil, olive oil, Captex 355), surfactants (Tween 85), and co-surfactants (ethanol) was determined by mixing excess amount of phenothiazines with each of the individual components. The drug was added to a 5-ml capacity stoppered glass vial and mixed for 10 min with each component using a vortex mixer. The mixture vials were then kept at 37 ± 1.0 °C in an isothermal shaker (Memmert, Germany) for 72 h until homogeneity. The homogenate samples were then centrifuged at 18,000 rpm for 30 min at 4 °C. The supernatant was removed by pipetting and the drug concentration was determined by HPLC at 254 nm as described later.

2.2.2. Preparation of SNEDDS formulations

Approximately 3 g of each formulation was prepared by firstly weighing the drugs into 12-ml Teflon-lined screw-capped glass conical tubes, followed by addition of various proportions of glycerides and surfactants. The components were mixed by gentle stirring and heated in a 50 °C water bath until drug dissolvement. The mixture was cooled to ambient temperature, ethanol was added, and the mixture was stirred to ensure uniformity. The formulation was equilibrated at ambient temperature for at least 48 h and examined for signs of turbidity or phase separation prior to self-emulsification and particle size studies.

2.2.3. Thermodynamic stability tests

Thermodynamic stability tests were performed with selected nanoemulsion formulations shown in Table 1. Nanoemulsions were centrifuged at 18,000 rpm for 30 min at 4 °C. Formulations that did not show any phase separation were subjected to six heating and cooling cycles in which the samples were incubated for each 48 h at 4 °C and 45 °C. The formulations, which were stable at these temperatures, were then subjected to three freeze–thaw cycles between -21 °C and + 25 °C. The physical stabilities of SNEDDS formulations were evaluated by monitoring time-dependent changes in the physical characteristics e.g. drug precipitation. The chemical stabilities of the phenothiazines in the SNEDDS formulations were analyzed by HPLC. The formulations which survived physical, chemical and thermodynamic stability tests monitored by visual observations were selected for further characterization.

2.2.4. Dispersibility tests

The efficiency of self-emulsification was assessed using the standard European Pharmacopoeia (Ph. Eur., 2008) dissolution apparatus 2 (Erweka, Germany). One milliliter of each formulation was added drop wise to 200 ml of simulated intestinal fluid (SIF) pH 6.8 without enzymes at 37 °C as described (Ph. Eur., 2008). Gentle agitation was provided by a standard stainless steel dissolution paddle rotating at 60 rpm. The lipid-based formulations were assessed visually according to the rate of emulsification, apparent stability of the resultant emulsion, final appearance of the emulsion, and the particle size of the emulsion. Precipitation was evaluated by visual inspection of the resultant emulsion after 24 h. The formulations were then categorized according to their appearance as whitish, dull white, and milky, and stable (no precipitation after 24 h) or unstable (showing precipitation within 24 h). Stable formulations with small particle size that had passed the dispersibility tests were selected for further studies.

Table 1
Formulation of optimized lipid-based self-nanoemulsifying drug delivery systems (SNEDDS) for the lipophilic drug thioridazine used for association with plasma derived harvested chylomicrons in the *ex vivo* model.

Formulation composition	% Ingredients (w/w)	Drug contents (m/m)	% Transmittance (T)	Zeta potential (mV)
SCT_{-O}				
Drug	1	92.3%	1.5	−17.1
Tributyrin	40			
Tween 85	48			
Glycerol	6			
Ethanol	5			
MCT_{-F}				
Drug	2	82.7%	0.1	−14.2
Captex 355	35			
Tween 85	50			
Glycerol	8			
Ethanol	5			
LCT_{-N}				
Drug	2	85.5%	0.0	−22.6
Linseed oil	37.5			
Olive oil	17.5			
Tween 85	40			
Ethanol	3			

2.2.5. Characterization of SNEDDS formulations

2.2.5.1. Determination of drug contents. For determination of drug entrapment efficiencies, the phenothiazines were extracted from LCT-SNEDDS (LCT_{-N}), MCT-SNEDDS (MCT_{-F}) and SCT-SNEDDS (SCT_{-O}) formulations using one part of each formulation and nine parts of 100% methanol (v/v) followed by centrifugation at 18,000 rpm for 30 min. The supernatant was diluted 2.5-fold with methanol before being injected in the HPLC-UV chromatographic system as described later.

2.2.5.2. Percentage transmittance. A total of 1 ml of the SNEDDS formulation was diluted 10-fold with distilled water. Percentage transmittance was measured spectrophotometrically (UV-1202, Shimadzu Co., Kyoto, Japan) at 560 nm using water as a blank.

2.2.5.3. Emulsion droplet size and zeta potential analysis. The droplet sizes of the phenothiazine emulsions were determined by photon correlation spectroscopy that analyses the fluctuations in light scattering due to the Brownian motion of the particles using a PSS Nicomp™ 380 ZLS particle sizing system (Nicomp, Santa Barbara, CA, USA). The employed laser was a HeNe laser with a wavelength of 635 nm. Scattered light was detected at a 90° angle. The Zetasizer PSS ZPW388 v1.65 software was used for data acquisition. The zeta potential of the SNEDDS formulation was investigated by measuring the electrophoretic mobility using a PSS Nicomp™ 380 DLS/ZLS device. The size was calculated assuming solid particles, and the number of weighting distribution was fitted into a curve.

2.2.6. Screening of isolated plasma derived CM emulsion

Human blood plasma was kindly provided by the Blood bank of the University Clinic Innsbruck. Chylomicrons (CM) were separated

from 4-ml aliquots of human plasma by density gradient ultracentrifugation [8]. Standard solutions with densities of 1.006, 1.019, and 1.063 g/ml were prepared using appropriate amounts of NaCl and KBr as described [9]. All standard solutions contained 0.01% (w/v) EDTA and were kept at 4 °C up to four weeks. Four milliliters of plasma were adjusted to a density of 1.1 g/ml by adding the appropriate amount of KBr (0.57 g), and then a density gradient was built in 12-ml polyallomer tubes by placing 4 ml of plasma (1.1 g/ml) at the bottom, followed by addition of 3 ml of a 1.063 g/ml NaCl solution, 2.5 ml of a 1.019 g/ml NaCl solution, and 2 ml of a 1.006 g/ml NaCl solution which were sequentially layered on top of the plasma. Samples were centrifuged at 40,000 rpm for 33 min at 15 °C using a SW41 rotor (Beckman L-70 ultracentrifuge). After the run, the top 1-ml fraction with a density of 1.006 g/ml representing the CM fraction with a particle flotation rate (*Sf*) > 400 at a solvent density of 1.063 g/ml [5] was collected using a Pasteur pipette. The final standard CM emulsion was kept at 4 °C for maximal 24 h depending on the drug incubation experiment.

2.2.7. Characterization of harvested chylomicrons

2.2.7.1. Refractive index and density measurements. The refractive indices of control NaCl and KBr gradient solutions before and after ultracentrifugation were determined to evaluate the density in each region of the preparative gradient fractions tube, including the 1-ml top-gradient fraction representing the harvested chylomicrons. Initially, the top and the bottom contents of the gradient tube had densities of 1.006 g/ml and 1.063 g/ml, respectively. The refractive index (n) of gradient fractions and harvested chylomicrons was measured using an Abbe 5 refractometer (Bellingham and Stanley) and the density (ρ) calculated according to the equation $\rho = (10{,}8601 \times n) - 13{,}4974$.

2.2.7.2. Transmission electron microscopy. Air-dried dual stained (negative and positive staining) harvested chylomicrons were analyzed by energy filter transmission electron microscopy (EFTEM). Chylomicrons were mixed 1:l (v/v) with 1% osmium tetroxide and fixed for 2–5 min. The fixed sample was then applied to a carbon film on mica, and the film with the adherent sample was picked up with a Formvar-treated grid. Excess sample was removed by the filter paper, but the grid was not allowed to dry before being stained with 1% phosphotungstic acid/0.1% sucrose, or 0.5% phosphotungstic acid/0.05% sucrose. The grid was air-dried prior to loading into the electron microscope (Zeiss Libra 120 EF-TEM) at 80 kV. Image analysis was achieved using an Olympus SiS iTEM 5.0 and TRS 2048 high speed camera.

2.2.7.3. Dynamic light scattering. The hydrodynamic diameter of harvested chylomicron vesicles was determined by dynamic light scattering (DLS) using a PSS Nicomp™ 380 DLS/ZLS using a HeNe laser source. Scattered light was detected at a 90° angle. The refractive index (1.33) and the viscosity (0.93 Pa s) of distilled water were used at 23 °C for measurements. Zetasizer PSS ZPW388 v1.65 software was used for data acquisition. The size was calculated assuming solid particles and the number of weighting distribution was fitted into a curve.

2.2.7.4. Measurement of the zeta potential. The zeta potential of the harvested chylomicrons vesicle size was investigated by measuring the electrophoretic mobility using a PSS Nicomp™ 380 DLS/ZLS device. All measurements were carried out at room temperature. One milliliter of the sample solution was loaded into the electrode cell and measured. The zeta potential used for data analysis was the average of five measurements.

2.2.8. Uptake of phenothiazines by harvested chylomicrons

The studies of phenothiazine uptake by harvested chylomicrons were performed as described previously [5] except that all incubation experiments were carried out at physiological pH using simulated intestinal fluid (pH 6.8) as experimental medium for the chylomicron emulsion. Briefly, the pure drug stock solutions of tested phenothiazines (0.1 mg/ml) were prepared in 20% (v/v) ethanol. An appropriate volume of drug stock solution was added to 2 ml volumes of chylomicron emulsion in simulated intestinal fluid (pH 6.8) to reach a final concentration of 15 μM. Similarly, appropriate volumes of LCT-SNEDDS (LCT_{-N}), MCT-SNEDDS (MCT_{-F}) and SCT-SNEDDS (SCT_{-O}) formulations were added to 2 ml volumes of chylomicron emulsion in simulated intestinal fluid (pH 6.8) to reach a final concentration of 15 μM. The chylomicron emulsion was incubated with SNEDDS formulation or with drug stock solution at 37 °C for 6 h under constant mixing using a magnetic stirrer. After the incubation, the chylomicron emulsion was adjusted to a density of 1.1 g/ml by adding an appropriate amount of KBr solution to a final volume of 4 ml. Subsequently, a density gradient was built in a 12-ml polyallomer tube and chylomicrons with incorporated phenothiazines were separated by density gradient ultracentrifugation (as described in Section 2.2.6). The buoyant densities (ρ) of plasma lipoproteins separated by ultracentrifugation in KBr gradients are conventionally reported as <0.95 g/ml, 0.95–1.006 g/ml, 1.006–1.063 g/ml, and 1.063–1.21 g/ml for chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins, (LDL), and high density lipoproteins (HDL), respectively. Chylomicrons with a buoyant density lower than that of incubated SNEDDS formulations floated as milky cream layer on the top of the gradient centrifuge tube (data not shown). The 1-ml top-gradient fraction representing chylomicrons loaded with phenothiazines was collected using a Pasteur pipette and kept at −80 °C until analysis.

To monitor possible diffusion of free phenothiazines through the density gradient, control experiments were performed testing SNEDDS formulations and pure drug solutions in simulated intestinal fluid without chylomicron emulsion. Chylomicrons loaded with phenothiazines were characterized by density measurements, transmission electron microscopy, dynamic light scattering, and zeta potential measurement.

2.2.9. HPLC–UV

The amount of chylomicron phenothiazine uptake was analyzed by high performance liquid chromatography (HPLC) using a Merck HITACHI device equipped with an L-2200 autosampler as described previously [10] with following modifications. Separation of drugs was carried out by using the columns Econosphere CN 5u (C18, 5 mm, 4.6 mm i.d. × 250 mm) at 40 °C. Eluents were (A) 30 mM ammonium acetate in water including 0.05% triethylamine pH 5.86 (adjusted with acetic acid) and (B) methanol. Isocratic elution using 10% of eluent A and 90% of eluent B was performed with a flow rate of 1.8 ml/min and running time of 20 min. Products were detected by absorbance measurements at 220–300 nm with a diode array absorbance detector (Perkin Elmer 235 C). The injection volume was 10 μl. The absorption maximum was found to be at 254 nm.

2.2.10. Statistical data analysis

For statistical analysis, analysis of variance and Student's *t*-test were used. A probability (P) of less than 0.05 ($P < 0.05$) was considered to be statistically significant. All results are presented as mean value ± standard deviation (SD).

3. Results and discussion

3.1. Drug solubility tests in nanoemulsion components

The self-nanoemulsifying formulations consisting of oil, surfactants, co-surfactants, and the drug should form a clear and

monophasic liquid at ambient temperature when introduced into an aqueous phase, and should have good solvent properties to allow presentation of the drug in solution. To test drug solubility in single nanoemulsion components, chlorpromazine and thioridazine were tested in various vehicles (Fig. 1). In general, linseed oil, Tween 85, glycerol, ethanol, and olive oil provided higher solubility compared to the other vehicles.

3.2. Characterization and evaluation of SNEDDS formulations

Excipients with a definite regulatory status were chosen to formulate the lipid-based formulations. The triglyceride component of the formulations was either a short chain glyceride (SCT), a medium chain glyceride (MCT), or a long chain glyceride (LCT) lipid. The short chain glyceride examined in this study was triacetin which is a triester of glycerol and acetic acid. Medium chain glycerides (mono-, di-, and triglycerides) are commonly used in lipid-based formulations [11]. The glyceride employed in this study was Captex 355, a C8:C10 triglyceride. The long chain glycerides examined in this study were the C18 triglycerides from linseed oil and olive oil. The formulation components of LCT-SNEDDS (LCT_{-N}), MCT-SNEDDS (MCT_{-F}) and SCT-SNEDDS (SCT_{-O}) encapsulating thioridazine (THD) are listed in Table 1 including drug content and percentage transmittance (λ max 560 nm) after 10-fold dilution.

3.2.1. Stability tests

Nanoemulsions are thermodynamically stable systems that are formed at a particular concentration of oil, surfactant and water. It is the thermostability that distinguishes nano- or microemulsions from emulsions that have kinetic stability and will eventually phase separate [12]. Therefore, SNEDDS formulations were tested for their thermodynamic stabilities i.e. by testing their permanent phase separation, creaming, cracking, and coalescence using centrifugation, heating–cooling cycles, and freeze–thaw cycles (data not shown). Only those formulations that showed thermodynamic, physical, and chemical stability were selected for further investigations.

3.2.2. Dispersibility tests

Self-nanoemulsifying systems form fine oil–water emulsions with only gentle agitations upon their introduction into aqueous media. Free energy of nanoemulsion formation depends on the extent to which the surfactant lowers the surface tension of the oil–water interface and the change in dispersion entropy [13]. Considering the stability, safety, and biocompatibility of the excipients, a SNEDDS consisting of a nonionic surfactant (Tween 85) and a co-surfactant (glycerol) was selected for the development of short chain triglyceride (SCT) SNEDDS encapsulating the phenothiazines THD and CPZ. The results of self-emulsification, precipitation, and particle size studies for SCT-SNEDDS encapsulating THD in simulated intestinal fluid (SIF) are shown in Table 2. Ethanol can be assumed to act as a cosolvent for phenothiazines (cf. Table 1) increasing the solubilization capacity of the vehicle Tween 85. An increase in the amount of the triacetin oil phase resulted in a proportional particle size increase because of the simultaneous decrease in the surfactant/co-surfactant (S/CoS) ratio. Increase in the S/CoS ratio led to a decrease in mean droplet size. Formulation code SCT_{-O} as shown in Table 2, with the highest proportion of surfactant (48% w/w) showed the lowest mean particle size. This could be due to an increased surfactant proportion relative to co-surfactant. It is well known that the addition of surfactants to nanoemulsion systems causes the interfacial film to stabilize and to condense, whereas the addition of co-surfactant causes the film to expand. Therefore, the relative proportion of surfactant to co-surfactant has different effects on the particle size.

Comparing the individual SCT-SNEDDS formulations, the medium chain triglyceride (MCT) SNEDDS based on Captex 355 (oily phase) required at least 50% w/w Tween 85 to provide efficient emulsification in the simulated intestinal fluid (SIF) as shown in Table 3. As observed with the SCT-SNEDDS formulations (cf. Table 2), the usage of the highest surfactant proportion relative to co-surfactant resulted in the formation of the lowest mean MCT-SNEDDS particle size (186 nm) encapsulating the phenothiazine. The formulation code MCT_{-F} demonstrates a crucial role of the S/CoS ratio (Table 3).

For the long chain triglyceride (LCT), SNEDDS formulations comprised of linseed oil/olive oil combinations, the solubility of phenothiazines could be enhanced by increasing the proportion of linseed oil because the solubility of phenothiazines was twofold higher in linseed oil compared to olive oil (cf. Table 1). However, this strategy led to a less efficient emulsification due to an overall olive oil content reduction in the formulation. But a 2:1 (w/w) ratio of linseed oil to olive oil afforded a good balance between

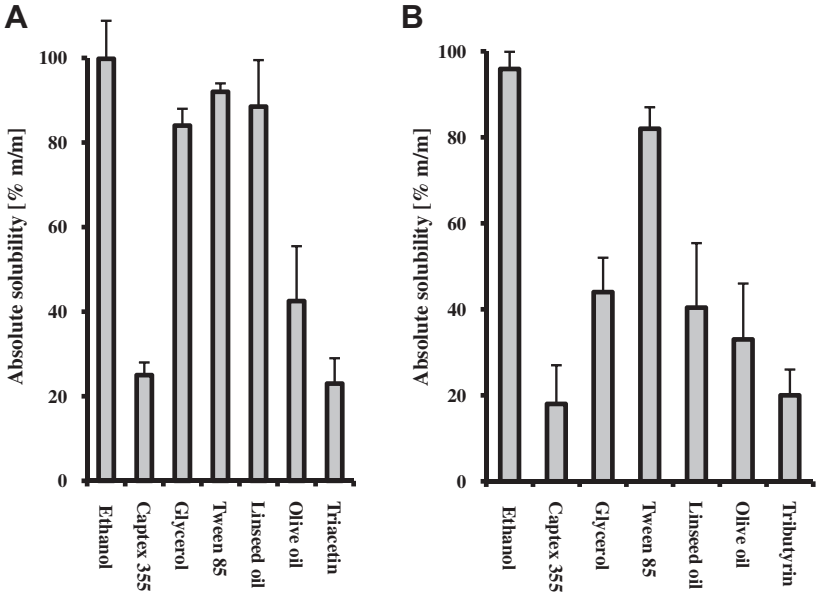

Fig. 1. Solubility of (A) thioridazine and (B) chlorpromazine in various nanoemulsion components. Indicated values are means ± SD of at least three experiments.

Table 2
Effect of formulation composition on the dispersibility and particle size (mean ± SD, $n = 3$) of short chain glyceride formulations (SNEDDS) for the lipophilic drug thioridazine when added to the dispersion medium SIF (simulated intestinal fluid) pH 6.8.

Code	Composition (% w/w)					Dispersion time (sec)	Appearance	Precipitation	Particle size (nm)
	Triacetin	Tween 85	Ethanol	Drug	Glycerol				
SCT_{-A}	65	20	5	1	9	126 ± 31	Dull white	Unstable	873 ± 14
SCT_{-B}	60	25	5	1	9	120 ± 23	Dull white	Unstable	792 ± 21
SCT_{-C}	55	30	5	1	9	118 ± 9	Dull white	Unstable	439 ± 17
SCT_{-D}	50	35	5	1	9	99 ± 4	Dull white	Unstable	335 ± 18
SCT_{-E}	45	40	5	1	9	59 ± 2	Dull white	Stable	278 ± 32
SCT_{-F}	40	45	5	1	9	43 ± 5	Dull white	Stable	204 ± 23
SCT_{-G}	60	20	5	1	14	98 ± 13	Dull white	Unstable	703 ± 37
SCT_{-H}	55	25	5	1	14	75 ± 11	Dull white	Unstable	573 ± 43
SCT_{-I}	50	30	5	1	14	73 ± 8	Dull white	Unstable	328 ± 27
SCT_{-J}	45	35	5	1	14	60 ± 4	Dull white	Unstable	317 ± 30
SCT_{-K}	40	40	5	1	14	55 ± 7	Dull white	Stable	285 ± 28
SCT_{-L}	55	33	5	1	6	57 ± 6	Dull white	Unstable	292 ± 41
SCT_{-M}	50	38	5	1	6	53 ± 5	Dull white	Stable	264 ± 37
SCT_{-N}	45	43	5	1	6	49 ± 7	Dull white	Stable	193 ± 19
SCT_{-O}	40	48	5	1	6	30 ± 4	Dull white	Stable	159 ± 15

Table 3
Effect of formulation composition on the dispersibility and particle size (mean ± SD, $n = 3$) of medium chain glyceride formulations (SNEDDS) for the lipophilic drug thioridazine when added to the dispersion medium SIF (simulated intestinal fluid) pH 6.8.

Code	Composition (% w/w)					Dispersion time (s)	Appearance	Precipitation	Particle size (nm)
	Captex 355	Tween 85	Ethanol	Drug	Glycerol				
MCT_{-A}	60	25	5	2	8	143 ± 21	Whitish	Unstable	958 ± 59
MCT_{-B}	55	30	5	2	8	112 ± 15	Whitish	Unstable	932 ± 21
MCT_{-C}	50	35	5	2	8	98 ± 26	Whitish	Unstable	542 ± 36
MCT_{-D}	45	40	5	2	8	74 ± 27	Whitish	Unstable	483 ± 17
MCT_{-E}	40	45	5	2	8	67 ± 19	Whitish	Stable	231 ± 24
MCT_{-F}	35	50	5	2	8	17 ± 4	Whitish	Stable	186 ± 20
MCT_{-G}	55	25	5	2	13	109 ± 31	Whitish	Unstable	987 ± 23
MCT_{-H}	50	30	5	2	13	83 ± 18	Whitish	Unstable	801 ± 29
MCT_{-I}	45	35	5	2	13	76 ± 25	Whitish	Unstable	595 ± 33
MCT_{-J}	40	40	5	2	13	63 ± 17	Whitish	Unstable	411 ± 28
MCT_{-K}	35	45	5	2	13	41 ± 12	Whitish	Stable	301 ± 25
MCT_{-L}	50	33	5	2	10	77 ± 10	Whitish	Unstable	915 ± 18
MCT_{-M}	45	38	5	2	10	66 ± 11	Whitish	Stable	431 ± 30
MCT_{-N}	40	43	5	2	10	43 ± 16	Whitish	Stable	302 ± 23
MCT_{-O}	35	48	5	2	10	37 ± 14	Whitish	Stable	294 ± 39

phenothiazine loading and efficient emulsification as shown in Table 4. The components oily phase and surfactant used for developing LCT-SNEDDS formulations revealed a high solubilization capacity for phenothiazines as shown by solubility studies (cf. Table 1). For LCT-SNEDDS formulations, nanoemulsion resulted when Tween 85 concentrations were above 40% (w/w) without co-surfactant as shown in Table 4 (formulation code LCT_{-N}). However, further increase in the Tween 85 concentration typically

Table 4
Effect of formulation composition on the dispersibility and particle size (mean ± SD, $n = 3$) of long chain glyceride formulations (SNEDDS) for the lipophilic drug thioridazine when added to the dispersion medium SIF (simulated intestinal fluid) pH 6.8.

Code	Composition (% w/w)				Dispersion time (s)	Appearance	Precipitation	Particle size (nm)
	Olive oil: linseed oil (1:2 w/w)	Tween 85	Ethanol	Drug				
LCT_{-A}	70	20	8	2	153 ± 36	Milky	Unstable	925 ± 82
LCT_{-B}	65	25	8	2	139 ± 25	Milky	Unstable	891 ± 105
LCT_{-C}	60	30	8	2	97 ± 18	Milky	Unstable	782 ± 69
LCT_{-D}	55	35	8	2	58 ± 13	Milky	Stable	298 ± 37
LCT_{-E}	50	40	8	2	36 ± 20	Milky	Stable	203 ± 96
LCT_{-F}	45	45	8	2	98 ± 36	Milky	Unstable	587 ± 161
LCT_{-G}	65	23	5	2	137 ± 23	Milky	Unstable	743 ± 83
LCT_{-H}	60	28	5	2	72 ± 14	Milky	Unstable	439 ± 107
LCT_{-I}	55	33	5	2	93 ± 10	Milky	Unstable	365 ± 95
LCT_{-J}	50	38	5	2	84 ± 21	Milky	Stable	281 ± 34
LCT_{-K}	45	43	5	2	57 ± 17	Milky	Stable	529 ± 42
LCT_{-L}	65	30	3	2	63 ± 24	Milky	Unstable	253 ± 60
LCT_{-M}	60	35	3	2	58 ± 19	Milky	Stable	230 ± 28
LCT_{-N}	55	40	3	2	22 ± 6	Milky	Stable	178 ± 16
LCT_{-O}	50	45	3	2	87 ± 15	Milky	Unstable	634 ± 203

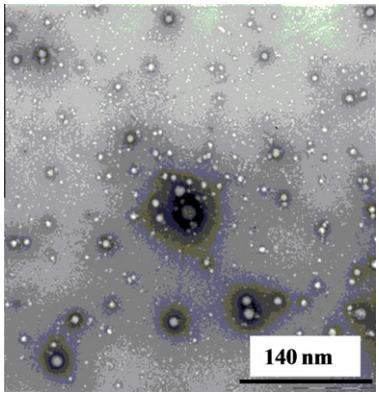

Fig. 2. Transmission electron microscope image of harvested chylomicrons at 80 kV.

resulted in the precipitation of phenothiazines upon addition of simulated intestinal fluid (SIF) and required at least three minutes to fully disperse.

3.3. Characterization of harvested chylomicrons

Chylomicrons from human plasma were prepared by density gradient ultracentrifugation. Quantification of the harvested chylomicrons density was performed by refractometry. Due to their low density (<0.95 g/ml), chylomicrons are confined to the top-gradient fraction. Density calculations showed that before centrifugation the density of the top-gradient fraction was 1.006 g/ml and after centrifugation the density of the top-gradient fraction was 0.55 g/ml. The density of the harvested chylomicrons therefore correlates favorably with results reported by other investigators [5].

The vesicle sizes of harvested chylomicrons were determined by energy filter transmission electron microscope (EFTEM) (Fig. 2). Vesicle sizes with a diameter of 130 ± 60 nm were detected. The image in Fig. 2 shows individual round vesicles suggesting that aggregation did not take place. Furthermore, electron microscopy showed a ~1.5-fold increased in vesicle size of harvested chylomicrons after incubation with LCT_{-N} SNEDDS. For further examination, vesicle size analysis of harvested chylomicrons was performed using dynamic light scattering. The intensity-weighted size distributions revealed two classes of vesicles (Fig. 3). Their mean diameters of chylomicrons were 60–90 nm and 120–150 nm, whereas chylomicrons incubated with LCT_{-N} SNEDDS displayed sizes of 60–90 nm and 160–265 nm. The numbers of vesicles associated with the two peaks were evaluated by examining the scattering properties of the vesicles demonstrating that the majority were small ones. This assessment also suggested that the mean size in the first peak is significantly smaller than the typical size of chylomicrons. This was consistent with the finding that the harvested chylomicrons not only contained apolipoprotein B-48 but also a similar amount of apolipoprotein B-100, which is associated with lipoproteins of smaller size. The larger vesicles of the second peak presumably represent harvested chylomicrons. The first peak of vesicle size distributions represented by apolipoprotein B-48 and apolipoprotein B-100 is not significantly influenced by LCT_{-N} SNEDDS. In contrast, a ~2.5-fold increase in the second peak, probably representing harvested chylomicrons, of vesicle size distributions was observed after 6-h incubation with LCT_{-N} SNEDDS (Fig. 3A/B).

Moreover, all lipoprotein classes display a net negative charge and studies have shown that this charge is due to both apolipoprotein composition of the lipoprotein and its content of anionic lipids [14]. Accordingly, the zeta potential of harvested chylomicrons was analyzed showing a surface potential of −7.9 mV.

3.4. Uptake of molecules by harvested CM from SNEDDS formulations

The association of drugs with chylomicrons is a complex process, which involves the lipophilic core as well as surface apoproteins. The plasma derived harvested chylomicron *ex vivo* model based on LCT-SNEDDS (LCT_{-N}), MCT-SNEDDS (MCT_{-F}) and SCT-SNEDDS (SCT_{-O}) formulations is able to predict the degree of chylomicron association. Strong interaction with chylomicrons may be attributed to the physicochemical properties of phenothiazines. Phenothiazines (PTZ) are useful organic compounds containing a nitrogen (N) atom that easily looses one electron to form a cation radical because of high negative electron density [15]. Because the Mulliken atomic charge (with hydrogen summed into heavy

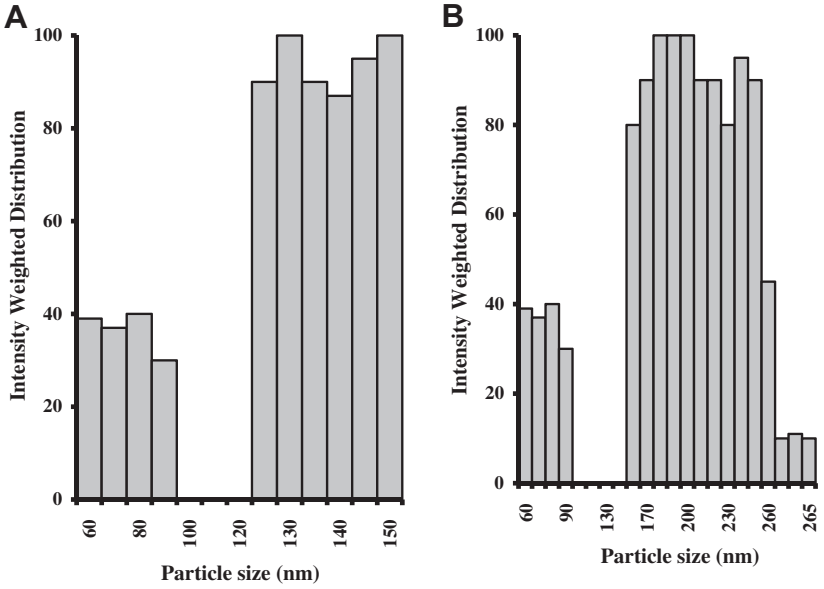

Fig. 3. Particle size distributions of (A) plasma derived isolated chylomicrons and (B) plasma derived isolated chylomicrons after incubation with the long chain triglyceride self-nanoemulsifying drug delivery system LCT_{-N} SNEDDS. Indicated values are means ± SD of at least three experiments.

atoms) of N is −0.412953 [16], an attractive electrostatic interaction can occur between the N atom in PTZs and the polar head of chylomicron phospholipids. Therefore, PTZs are positioned in the membrane of chylomicrons with the N atom oriented toward the polar head of chylomicron phospholipids (Fig. 4). Furthermore, covalent bond formation between the electronegative nitrogen atom of PTZ and nitrogen atoms of apoprotein substructures of chylomicrons ultimately causes a high degree of association. PTZ can form additional hydrogen bonds with triglycerides (TG) and cholesteryl ester substructures and can probably therefore diffuse to a higher extent into chylomicrons.

The association of plasma derived harvested chylomicrons with lipophilic phenothiazines was tested by either using the pure drug solution or the relevant SNEDDS formulation based on short (SCT_{-O}), medium (MCT_{-F}), and long chain triglycerides (LCT_{-N}) (Fig. 5). The uptake of thioridazine was more efficient than the uptake of chlorpromazine. Furthermore, the results show that the affinity of lipophilic drugs to plasma derived harvested chylomicrons was significantly influenced by the type of the SNEDDS formulation. SNEDDS based on long chain triglycerides (LCT_{-N}) were the most effective systems when compared with the pure drug solution (S.S.) or with SNEDDS formulations based on medium (MCT_{-F}) and short chain triglycerides (SCT_{-O}). For thioridazine a 5.6-fold and for chlorpromazine a 3.7-fold higher association with harvested chylomicrons could be observed using a SNEDDS formulation based on long chain triglycerides (LCT_{-N}). Thioridazine showed an almost 2-fold higher affinity to chylomicrons than chlorpromazine although both compounds have similar structures and similar calculated log *P* values. However, thioridazine displays a 2.4-fold higher solubility in long chain triglyceride components (cf. Fig. 1), which could explain the higher drug uptake of isolated chylomicrons.

Refractometry revealed that the density of harvested chylomicrons was 1.8-fold increased after incubation with the LCT-SNEDDS (LCT_{-N}) formulation when compared to the density of harvested chylomicrons before incubation, whereas no significant difference in the density of harvested chylomicrons was observed after incubation with MCT-SNEDDS (MCT_{-F}) and SCT-SNEDDS (SCT_{-O}) formulations (data not shown). However, zeta potential studies showed no significant difference in the surface potential of harvested chylomicrons after incubation with LCT-SNEDDS (LCT_{-N}), MCT-SNEDDS (MCT_{-F}), or SCT-SNEDDS (SCT_{-O}) formulations (cf. Table 1). In addition, the intensity-weighted size distributions of harvested chylomicrons revealed two classes of vesicles (cf. Fig. 3). Furthermore, transmission electron microscope confirmed the above described observation by demonstrating an increase in vesicle size of harvested chylomicrons after incubation with LCT_{-N} SNEDDS at 80 kV (cf. Fig. 6A) and at 80 kV after 70 electron loss energy (cf. Fig. 6B). Fast and high absorption could be the reason for the bulky size of chylomicrons observed after LCT_{-K} incubation (cf. Table 4). Previously, it has been observed that chylomicrons become large after administration of a corn oil bolus to lymph-cannulated rats [17]. Because chylomicrons can function as highly lipophilic model membranes, higher diffusibility or fusibility (cf. Fig. 4) of LCT_{-N} being more lipophilic when compared to MCT_{-F} and SCT_{-O} could be possible. Higher diffusion of LCT_{-N} SNEDDS based formulation into chylomicrons could be the reason for the increased association of the drugs with the harvested chylomicrons. Alternatively, the intensity-weighted vesicle size distribution of harvested chylomicrons was not altered following incubation with MCT_{-F} SNEDDS and SCT_{-O} SNEDDS formulations.

4. Presumptive additional features

SNEDDS as an appropriate lipid source may enhance lymphatic transport of lipophilic compounds by stimulating the production of chylomicrons [1]. Lipids are hydrolyzed in the stomach and small intestine to 2-monoglycerides (MG) and fatty acids (FA), then

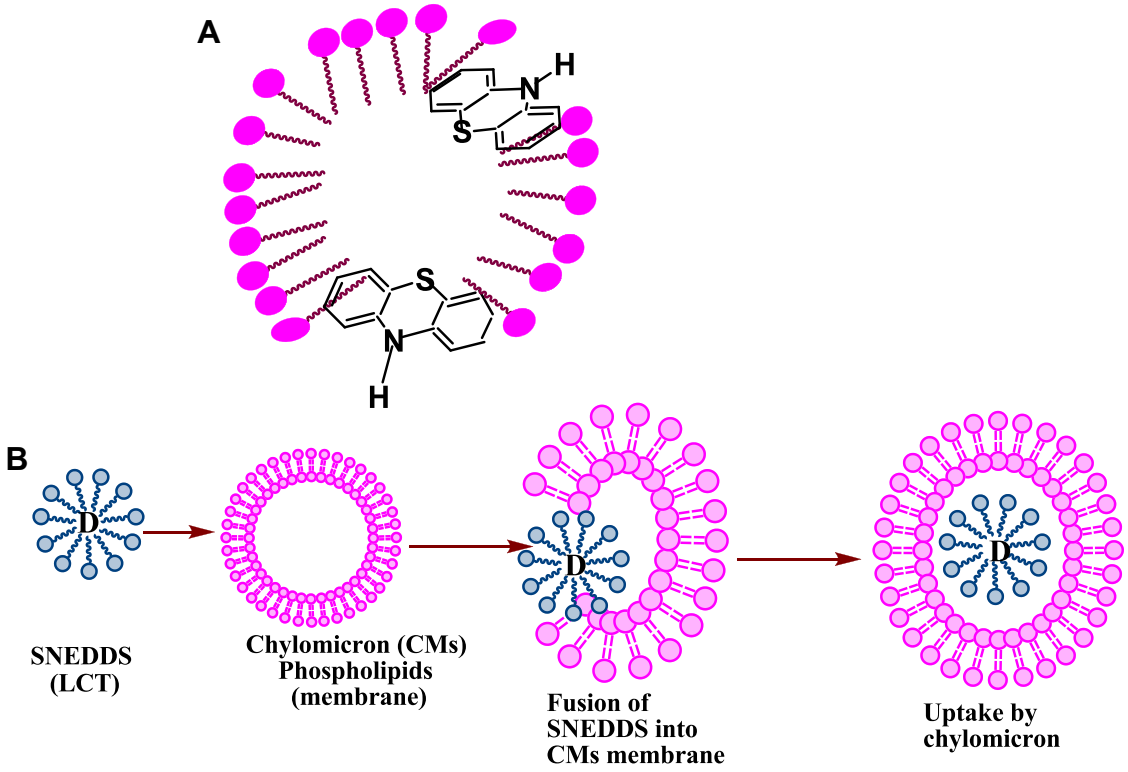

Fig. 4. (A) Electrostatic attractive interaction between the N atom of phenothiazines and the polar head of chylomicron phospholipids. (B) Model of LCT-SNEDDS (LCT_{-N}) diffusion across the biomembrane of chylomicrons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

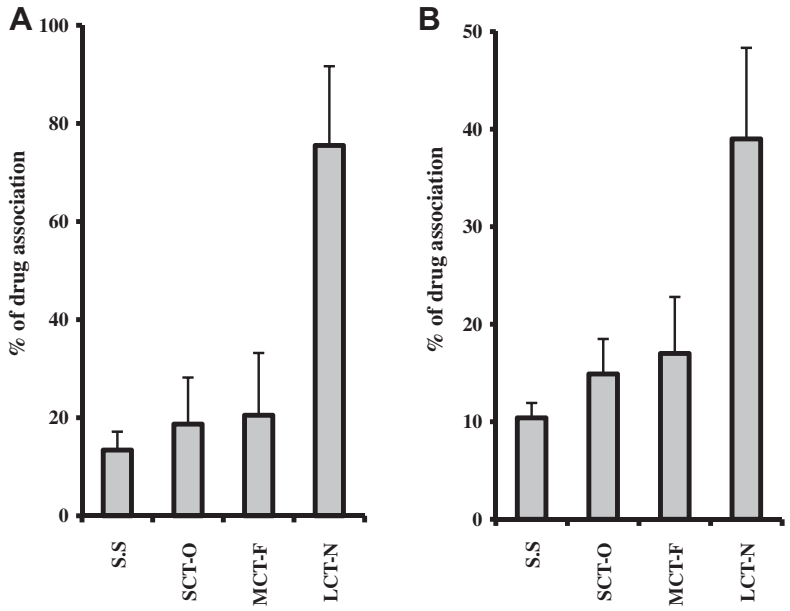

Fig. 5. Uptake of (A) thioridazine and (B) chlorpromazine by plasma derived harvested chylomicrons from different self-nanoemulsifying drug delivery systems (SNEDDS). Uptake from the pure drug (SS, stock solution) was measured as a control. Indicated values are means ± SD of at least three experiments.

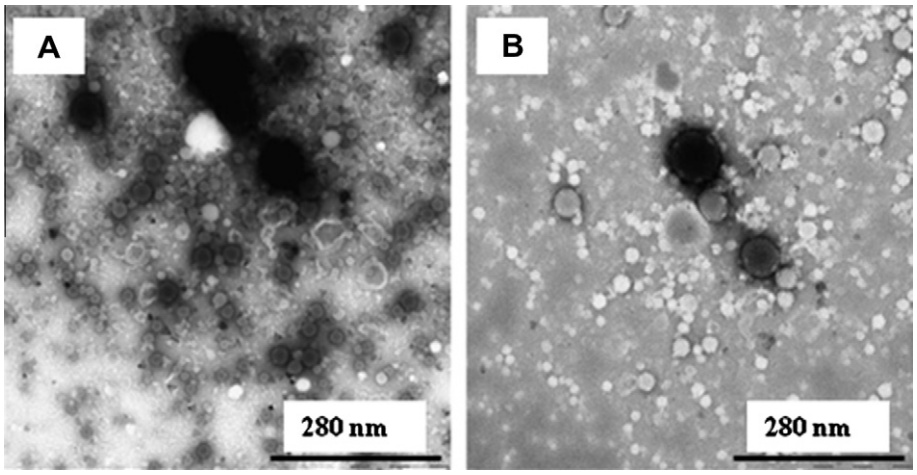

Fig. 6. Transmission electron microscope image of (A) harvested chylomicrons after incubation with LCT_{-N} SNEDDS formulation at 80 kV and (B) harvested chylomicrons after incubation with LCT_{-N} SNEDDS formulation with 70 electron loss energy at 80 kV.

reabsorbed into the enterocyte, re-esterified into triglycerides (TG), and then incorporated into intestinal chylomicrons (Fig. 7). Following exocytosis from the basolateral membrane, the newly secreted chylomicron particles first enter the intercellular space before migrating via the lacteals into the circulation [18]. A mechanistic study of tight junction opening by self-microemulsifying drug delivery systems (SMEDDS) containing the solubilizer Labrasol demonstrated changes in subcellular localization of the tight junction proteins F-actin and ZO-1 [19]. Medium chain mono- and diglycerides have been shown to modulate tight junction function through a phospholipase C dependent pathway [20]. Therefore, we hypothesize that the SNEDDS containing medium chain mono- and diglycerides like Captex 355 can lead to a loss of tight junction integrity and might enter the intercellular space by opening tight junctions *in vivo*. So, intercellular space can provide the possible uptake window for SNEDDS by chylomicrons. The appearance of chylomicrons in the intercellular spaces is followed by their passage into the lamina propria through gaps or discontinuities of the basement membrane [21]. Another possibility for lymphatic transport of lipid-based nanocarriers is specific uptake by membranous epithelial cells (M cells) of Peyer's patches [22]. It is assumed that the SNEDDS can be absorbed transcellularly through M cells in the gut associated lymphoid tissue (GALT). Subsequently, SNEDDS may be transported into the intestinal lamina propria by this transport mechanism and may be delivered to the periphery by lymphatic and/or vascular endothelium. The intestinal lymphatic tissue contains specialized M cells, which are supposed to facilitate transcytotic transport across the intestine. Small particles in the intestinal lumen confine to the apical side of M cells and can be internalized by M cells through transcytosis. The SNEDDS in the present studies are assumed to be absorbed via this mechanism. As reported earlier, there is a particle size-dependent exclusion phenomenon in the gastrointestinal mucosal tissue with 100 nm size particles showing significantly greater tissue uptake than larger particles [23]. Therefore, SNEDDS may be absorbed through the lymphatic system and then transferred into systemic

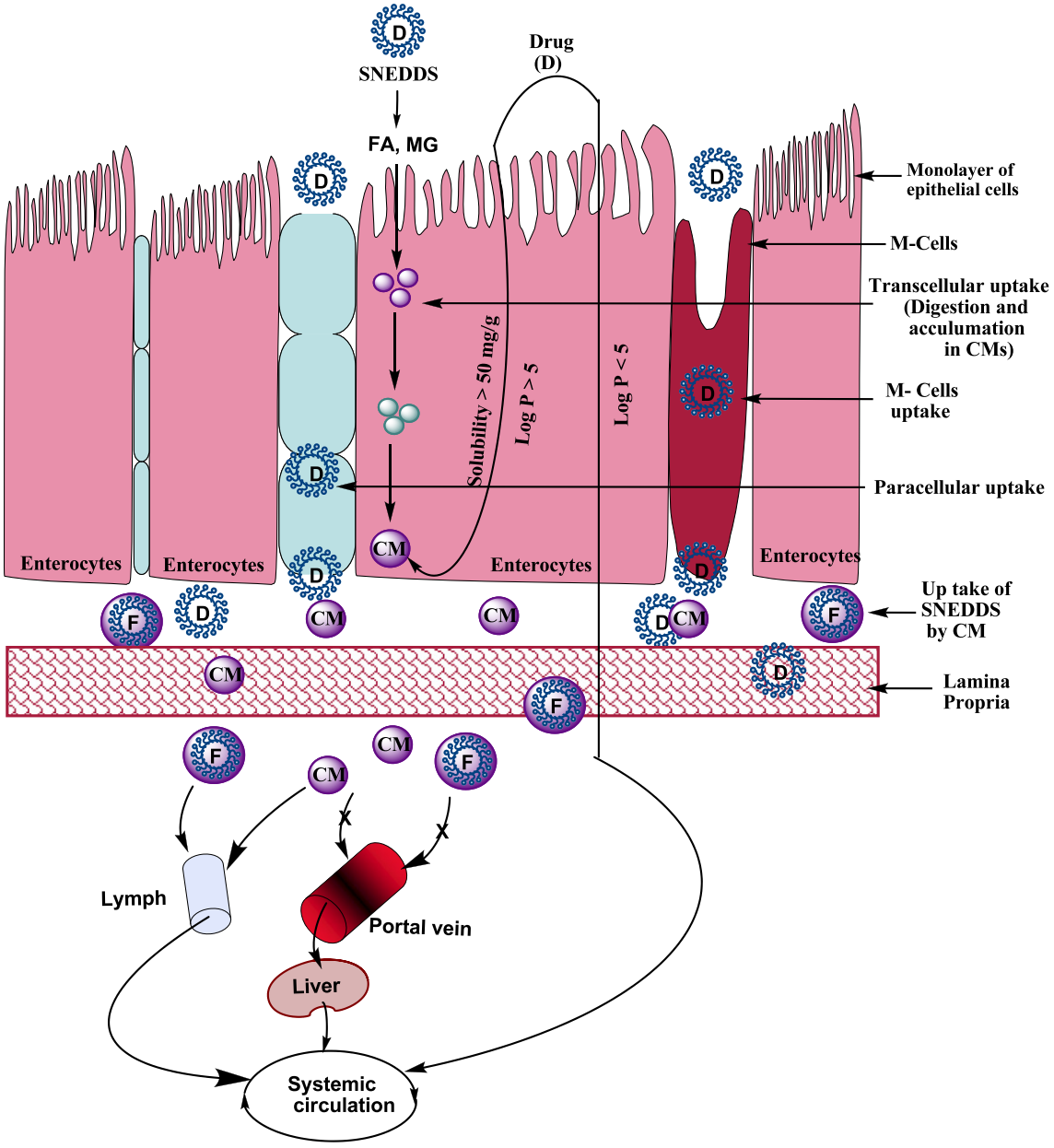

Fig. 7. Schematic diagram illustrating the mechanism of intestinal drug transport from self-nanoemulsifying drug delivery systems (SNEDDS). Three possible pathways of SNEDDS (D) uptake by chylomicrons (CM) via diffusion are as follows: (1) transcellular uptake with digestions and accumulation into CMs, (2) paracellular uptake by opening of tight junctions, (3) M cell uptake at membranous epithelial cells of the Peyer's patches. SNEDDS incorporated chylomicrons (F) might be absorbed through the lymphatic system and then transferred into systemic circulation. The incorporation of the SNEDDS into the chylomicrons (F) can lead to a bypass of the hepatic first-pass metabolism and therefore avoid hepatotoxicity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

circulation with chylomicron associations as shown in Fig. 7. It is likely that the long chain triglyceride SNEDDS formulation will also lead to maximize intestinal lymphatic chlorpromazine and thioridazine transport. Both chlorpromazine and thioridazine undergo extensive first-pass metabolism mainly by hydroxylation, *N*-dealkylation, *N*-oxidation, and *S*-oxidation. Clinical experience to date indicates that chlorpromazine therapy is associated with very rare but severe idiosyncratic hepatotoxicity. Hepatic injury has also been observed with the closely related antipsychotic drug thioridazine. High binding affinity of phenothiazines with chylomicron formulation based on long chain triglycerides indicates that the incorporation of phenothiazines into chylomicrons is an essential step to bypass the hepatic first-pass metabolism and hepatotoxicity prevention (Fig. 7). We hypothesize that a high degree of SNEDDS association with chylomicrons can be utilized to improve the oral bioavailability of highly lipid-soluble phenothiazines by using the lymphatic route transfer to systemic circulation. The lymphatic transport potential can also be utilized to bypass the hepatic first-pass metabolism as well as the known hepatotoxicity of phenothiazines.

5. Conclusion

An optimized SCT, MCT, and LCT self-nanoemulsifying drug delivery system (SNEDDS) containing phenothiazines was developed with particles size less than 200 nm. The present study demonstrates that incorporation of phenothiazines into SNEDDS leads to enhanced uptake by plasma derived chylomicrons, compared to naked phenothiazines in solution. The findings also provide

evidence for the importance of the fatty acid chain length in SNEDDS formulations. Therefore, this chylomicron *ex vivo* model is suitable to predict the intestinal lymphatic transport potential of phenothiazines. Enhanced uptake of phenothiazines by chylomicrons appears to be an important prerequisite for efficient intestinal lymphatic transport and thus for the substantial improvement in oral bioavailability of lipophilic drugs.

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