Design Development and *In vivo* Evaluation of Solid Lipid Nanoparticles of Raloxifene Hydrochloride for Enhancement of Oral Bioavailability

Navin Chandra Pant[1*], Vijay Juyal[1]
Department of Pharmaceutical Sciences, Bhimtal Campus, Kumaun University, Nainital
***Corresponding author:** navpant@gmail.com*

Abstract

Raloxifene hydrochloride (RLX-HCL) is an orally selective estrogen receptor modulator (SERM) with poor bioavailability of nearly 2% due to its poor aqueous solubility and extensivefirst pass metabolism. In order to improve the oral bioavailability of raloxifene, raloxifene loaded solid lipid nanoparticles (SLNs) have been developed using Compritol 888 ATO as lipid carrier and poloxamer 407 as surfactant. The RLX-loaded SLNs were developed using micro-emulsion method. According to the BBD design, a total 17 formulations (SLN1-SLN17) were developed and characterized. The optimized RLX-SLNs formulation showed sustained release *in vitroly*. Pharmacokinetics of raloxifene loaded solid lipid nanoparticles after oral administration to Wistar rats was studied. The RLX-SLNs significantly ($p < 0.05$) enhanced oral bioavailability 3 -fold as compared to RLX-free suspension in female Wistar rats. The RLX-SLNs can potentially enhance the oral bioavailability of RLX.

Keywords: SLN; Raloxifene; Osteoporosis; DoE; Everted gut sac; Lymphatic transport.

DOI Number: 10.14704/nq.2022.20.9.NQ44388 **Neuro Quantology 2022; 20(9):3380-3392**

Introduction

Osteoporosis is a disease condition with a greater risk of fracture as a cause of decrease in bone mineral density and bone quality [1]. There are many reported causes for osteoporosis, but menopauseis the most common and potential cause. Post-menopause, levels of the estrogenfall which leads to disturbed density of bone minerals with susceptibility to bone fractures [2].It affects women worldwide [3,4]. Raloxifene hydrochloride (RLX-HCL) is a popular selective estrogen receptor modulator [5], which is used to prevent and treat postmenopausal osteoporosis. It has proven effective to preserve bone density, preventing the incidence of vertebral fractures in postmenopausal women [5,6]. It is also used for the treatment of breast cancer [5,7]. According to the Biopharmaceutics classification system and the Biopharmaceutics drug disposition classification system, RLX is Class II drug [8]. RLX is highly permeable and absorbed 60% by the oral route; however, its bioavailability is only 2% in humans as a cause of extensive pre-systemic intestinal glucuronidation and permeability glycoprotein (Pgp) efflux [9,10]. The bioavailability of RLX in animals varies with species from 0–39% [11].

The oral route is the most preferred route for the administration of drugs as it offers thegreatest patient compliance. It accounts for more than a 50% share of the global drug delivery market [12].The various approaches for the oral bioavailability enhancement of RLX, such asdry suspensions of cyclodextrin inclusion complexes [13], mesoporous carbon nanospheres [14], proliposome powders [15], solid dispersions [16,17] and self-micro emulsifying drug delivery system solids [18] have been explored previously. Lipid nanocarriers can be suitable nanocarriers for RLX owing to their capability to mainly deliver poorly soluble and lipophilicdrugs [19]. Lipid nanocarriers have also been shown to decrease inter-individual variability [20]. The orally administered lipid nanoparticles enter into systemic circulation by different uptake mechanisms such as receptor-mediated endocytosis, non-specific transcellular transport, paracellular transport and M-cell-mediated transport [21]. The enhanced oral bioavailability of drugs loaded in solid lipid nanoparticles (SLNs)

is due to their small particle size, increased surface area and surface properties (like charge) that improve their bioadhesion to the gut wall, leading to prolonged residence time in the gastrointestinal tract (GIT). Moreover, the production of the mono and diacylglycerols during the digestion of triglyceridesby lipases in the GIT induces the secretion of bile salts that together with mono and diacylglycerols form micelles. These micelles further help to enhance the oral absorption of drugs. Moreover, SLNs can also facilitate lymphatic transport by initiating the formation of lipoprotein and intestinal lipid flux giving the bulk bioavailability of drugs [22]. The composition of SLNs and its excipients such as the amount of surfactant can significantly change the particle size, which also affects the lymphatic transport. The direct uptake of lipid nanocarriers through M-cells and lymphatic transport also plays the major role in the enhancement of their bioavailability [23].

Even though several research works are being carried out to enhance the solubility and bioavailability of raloxifene by formulating it as tablets [24], microemulsions [25], microspheres [26], transdermal patch [27], and polymeric nanoparticles, still it is required to design the formulation having combined advantages like sustained release and avoiding first pass metabolism of RLX-HCL. Therefore, the aim of the present work is to improve the bioavailability of RLX-HCL by formulating it as SLNs. RLX-HCL loaded SLNs were preparedby oil-in-water (o/w) micro-emulsion containing Compritol 888 ATO as internal phase,poloxamer 407 as surfactant, distilled filtered water as continuous phase[28],. Surfactant concentration and sonication time were taken as optimizing parameters, and the SLNs prepared were evaluated *in vitro* by its particle size, zeta potential, entrapment efficiency, surface morphology and drug release behaviour[29],. Stability studies were conducted for the best formulation for 6 months. Bioavailability studies in male Wistar rats were performed, and pharmacokinetic parameters were assessed using pharmacokinetic software for SLNs and compared with that of pure, marketed tablet and RLX-HCL suspension[30].

Materials and Methods

Materials

The raloxifene hydrochloride was obtained as a gift sample from Archerchem Healthcare Pvt India. Poloxamer 407 was purchased from SD fine chemicals, Mumbai, India. Compritol 888 ATO was obtained as a gift sample from Archerchem Healthcare Pvt India. Methanol and Acetonitrile were procured form Merck Pvt. Ltd., Mumbai, India.. The Potassium hydrogen phosphate, Hydrochloric acid was purchased from Loba Chemie Pvt. Ltd, Mumbai, India. All the other chemicals used were of analytical grade and the solvents were of high- performance liquid chromatography (HPLC) grade. Freshly collected Milli-Qwater (Millipore, Billerica, MA, USA) was used in the formulation preparation.

Preparation of solid lipid nanoparticles

RLX-loaded nanoparticles were prepared from a warm oil-in-water (o/w) micro- emulsion containing Compritol 888 ATO as internal phase, poloxamer 407 as surfactant, distilled filtered water as continuous phase. In the first step Raloxifene was added to the meltedCompritol 888 ATO; successively an aqueous solution of poloxamer 407 were added obtaining a clear micro-emulsion at 70º C (±1ºC). SLNs were obtained by dispersing the warm o/w micro-emulsion in cold water (2–3ºC) under mechanical stirring at a ratio 1:10 (micro-emulsion-water, v/v). The colloidal milk suspension of SLN was centrifuged at 35000 rpm (Centrifuge XL-90 Bachman Coulter USA equipped with rotor 70.1 Ti) for 1 hr at 4ºC. Nanoparticles were then suspended in water, freeze-dried by connecting a round-bottomed flask to a lyophilized (VirTis Bench top, SP scientific, Warminster USA), and refrigerated for successive characterization.

Formulation Design

Different batches of RLX-Loaded Solid Lipid nanoparticles were prepared based on factorial designs. Particle size and % entrapment efficiency were selected as responses. The statistical analysis of the factorial design formulations was performed using Design-Expert® software (Version 10, Stat-Ease Inc.). Seventeen batches were prepared as suggested by the software using surface-response factorial design with three levels, two factors and two responses. Different combinations used to prepare SLN using factorial designs are presented in table 2.

Optimization data analysis and model-validation

ANOVA was used to establish the statistical validation of the polynomial equations generated by Design Expert® Software. Fitting a multiple linear regression model to a 32 Factorial design give a predictor equation incorporating interactive and polynomial term to evaluate the responses:

$$Y=b0+b1X1+b2X2+b12X1X2+b11X12+b22X22 \quad (1)$$

Where Y is the measured response associated with each factor level combination; b0 is an intercept representing the arithmetic average of all quantitative outcomes of nine runs; bi (b1, b2, b11, b12 and b22) are regression coefficients computed from the observed experimental values of Y and X1, X2 and X3 are the coded levels of independent variables. The terms X1 X2, X3 represent the interaction terms. Three-dimensional response surface plotsresulting from equations were obtained by the Design Expert® software.

Characterization of Solid lipid Nanoparticles

***In-Vitro* Drug Release**

In-vitro diffusion study of lyophilised powder of optimised batch was carried out by Franz diffusion cell having 2.0 cm diameter and 12.5ml capacity. Dialysis membrane (HiMedia) having molecular weight cut off range 12000 – 14000 kDa was used as diffusion membrane. Pieces of dialysis membrane were soaked in phosphate buffer pH 7.4 for 24 h prior to experiment. Diffusion cell was filled with saline phosphate buffer, pH 7.4 and dialysis membrane was mounted on cell. The temperature was maintained at 37oC ± 0.5^0C. After a pre-incubation time of 20 minutes, the lyophilised powder equivalent to mg of RLX was dispersedin 10ml of saline phosphate buffer pH 7.4 and was placed in the donor chamber. Samples were periodically withdrawn from the receptor compartment for 24 hours having 1, 2, 4, 6, 12, 24 hours of time interval and replaced with the same amount of fresh phosphate buffer and assayedby UV–visible spectrophotometer at 286 nm.

Statistical analysis

Statistical data analysis was performed using the student t-test with $p < 0.05$ as the minimal level of significance. The results were expressed as mean standard deviation (SD) obtained from three separate experiments (n = 3). Mathematical fit functions were evaluated by ANOVA analysis.

***In-vivo* pharmacokinetic studies in rats**

In the experiment, female Wister rats weighing 180–220 g were employed. The Institutional Animal Ethics Committee approved the experimental protocol. Experimental animals were starved for 12 hours prior to treatment and remained fasted for another 4 hours afterward. Following that, rats were fed rat chow diet ad libitum. Freshly made solid lipid nanoparticles (SLNs) formulations were used in all of the trials.

Pharmacokinetic studies of free Raloxifene hydrochloride, orally administered Raloxifene tablet of equivalent dose, and Raloxifene-SLNs were conducted after oral (15 mg/kg) and intravenous (IV, 4 mg/kg) administration of free Raloxifene hydrochloride, orally administered Raloxifene tablet of equivalent dose, and Raloxifene -SLNs. The mice were placed into three groups, each with six animals, for both oral and IV pharmacokinetic studies.Groups 1 and 2 received an optimized Raloxifene-SLNs formulation including free Raloxifene hydrochloride (suspended in 0.5 percent w/v methyl cellulose, molecular weight 14,000 Da, viscosity 15 cps). Evista, a Raloxifene tablet, was given to Group 3 via oral administration (Elly LillY). Similarly, in an IV pharmacokinetic trial, groups 1 and 2 were given free Raloxifene hydrochloride solution (dissolved in PEG 400: water pre-mix (1:1)), while the treatment group was given Raloxifene -SLNs formulation, and group 3 was given Raloxifene tablet Evista (Elly Lilly).

At pre-dose (0, 1, 2, 4, 6, 8, 12, 24, and 48 h) post-dose for oral studies and at pre-dose (0, 1, 2, 4, 6, 8, and 12 h post-dose for IV studies), blood samples (0.15ml) were collected fromthe orbital sinus into microfuge tubes containing anti-coagulant (3.8 percent w/v sodium citrate). The tubes were immediately centrifuged (MIRKO 120, Hettich) at 4000 rpm for 15 minutes to separate plasma, then stored at -80 °C until HPLC analysis. The maximum plasma concentration (Cmax) and the time to attain that concentration (Tmax) were calculated directlyfrom the plasma concentration versus time graph curve. The trapezoid rule was used to compute the areas

under the curves (AUC0-48 and AUC0-inf). The slope of the log plasma concentration versus time plot was used to compute the elimination rate constant (Kel), and theelimination half-life (t1/2) was estimated using the formula t1/2 = 0.693/Kel.

HPLC estimation of Raloxifene in rat plasma

Shimadzu LC-20 low pressure gradient solvent delivery segment (Shimadzu, Kyoto, Japan) with DGU-20-A512 degassing unit, SPD-M20A Prominence Diode array UV/VIS detector spanning a range of 190-800 nm, SIL- 20AHT auto-sampler, CTO-10AS VP Columnoven, and LC workstation data processor The chromatographic separation of RLX from plasmawas accomplished using a thermo-fisher syncronis RP-column C-18 (15mm x 4.6mm, 5m poresize) with a loop size of 201. The sample was run for 15 minutes with a mobile phase containing phosphate buffer (pH 6.1) and acetonitrile (66:34, v/v) at 30°C, 1.0/min flow rate, and UV detection at 285 nm. Ethyl acetate was used as the extracting solvent in a liquid/liquid extraction process. In a glass tube, 0.450 ml of rat plasma was accurately measured. 50 l IS (diclofenac sodium dissolved in methanol and water, concentration: 2 g/ml) was added to thisand thoroughly mixed. A volume of 4 mL ethyl acetate was added to the plasma and vortex mixed for 45 seconds before centrifugation at 4500 rpm for 25 minutes. The organic layer in the supernatant was isolated into a separate test tube and slowly evaporated to dryness using astream of nitrogen gas at temperatures below 50°C. The dried layer was reconstituted with 300 ml acetonitrile, filtered through a 0.22m membrane, and HPLC analysis was performed. The range of calibration was 50ng/ml to 2000ng/ml.

Uptake of Raloxifene-SLN into rat everted gut sac

For intestinal uptake of lipid-based nanoparticles, several endocytic uptake pathways have been identified in the literature. We used rat intestinal segments to conduct everted gut sac investigations to better understand the uptake process of Raloxifene-SLNs.

Female Wistar rats (n = 3) were fasted for 12 hours but given free access to water ad libitum before the experiment. The entire small intestine segment was removed by cutting across the higher end of the duodenum and the lower end of the ileum after anesthesia (urethane, 1.25 g/kg, i.p.). Manual stripping was used to separate the mesentery. Different segments of the small intestine were recognized after washing with normal saline (0.9 percent w/v NaCl). 8-10cm of hair was quickly pulled and gently everted over a glass rod. The everted intestine was then slipped off the glass rod and placed at 37 °C in a flat dish containing Krebs Henseleit bicarbonate (KHB) buffer oxygenated with O2/CO2 (95%/5%). The open end of the intestine was clamped and secured with a silk suture, while the other end was filled with buffer at 37 °C using a 0.5 ml syringe. After carefully slipping the intestinal sac off the needle, the loose ligature at the proximal end was tightened. At 37 °C, the sacs were placed in individual incubation chambers containing either free raloxifene hydrochloride or Raloxifene-SLNs (effective concentration of raloxifene hydrochloride: 4.0 g/ml) produced in oxygenated KHB buffer.

Everted gut sacs were incubated at 4 °C or in the presence of certain endocytic inhibitors such chlorpromazine (CPZ) (10 g/ml) and nystatin (NYT) (25 g/ml) at 37 °C to determine theuptake process. After a 30-minute incubation period, the contents of the intestinal sacs were carefully removed, wiped onto filter paper, and collected. Sacs were rinsed three times with KHB buffer, with the rinsing combined with the original content for examination. A validated HPLC method was used to examine the samples.

Lymphatic transport of Raloxifene-SLN in rats

Solid Lipid nanoparticles are commonly recognized to be taken up by enterocytes and moved through the lymphatic system to eventually reach the systemic circulation. Intestinal transport tests were carried out in the presence and absence of cycloheximide, a lymph transport inhibitor (CXI). CXI is known to limit enterocyte chylomicron secretion and thereby lymphatic drug transport without causing damage to other active and passive absorption pathways [12- 16]. Six female Wistar rats weighing 180 to 220 g were randomly allocated into two groups forthis study. By intra peritoneal route, each group received saline (control group) or CXI solution in saline (3 mg/kg) (treatment group). Both groups of rats received Raloxifene-SLNs formulation (15 mg/kg) via oral gavage one hour after dosing. Pre-dose (0, 0.5, 1, 2, 4, 6, and

8 h post-dose) blood samples (0.15 ml) were taken from rats and samples were processed and analysed as previously described.

Results and Discussion

Formulation characterization

The RLX-loaded SLNs were developed using micro-emulsion method. According to the BBD design, a total 17 formulations (SLN1-SLN17) were developed and characterized for size, ZP, PDI, morphology and %EE (Table-1). The developed RLX-loaded SLNs have the size and %EE in the range of 165.63±2.62 nm to 315.33±4.87 nm and 75.21±2.32% to 95.32±2.11% (Table-1).

Table 1: RLX-loaded SLNs and obtained responses (Mean± SD)

Formulation Code	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A: Lipid	B: P407	C: Sonication	Size	EE
	(mg)	(%v/v)	(min)	(nm)	(%)
SLN1	500	7	7.5	228.12±4.32	76.52±1.56
SLN2	1500	5	5	238.33±5.15	95.32±2.11
SLN3	1000	5	7.5	165.63±2.62	93.51±1.98
SLN4	500	5	5	185.32±2.56	69.21±1.56
SLN5	1000	7	10	257.65±3.55	76.87±2.32
SLN6	1000	7	5	249.21±4.21	88.43±2.65
SLN7	1000	3	10	280.43±5.76	74.21±1.76
SLN8	1000	5	7.5	168.21±3.11	91.43±3.11
SLN9	1000	3	5	284.21±4.34	75.21±2.32
SLN10	500	3	7.5	255.55±3.87	58.54±1.12
SLN11	1000	5	7.5	169.43±2.22	90.56±2.43
SLN12	1500	5	10	245.32±3.65	86.43±1.66
SLN13	500	5	10	285.32±4.78	75.98±2.31
SLN14	1500	3	7.5	315.33±4.87	91.21±3.21
SLN15	1000	5	7.5	167.87±3.21	91.34±2.13
SLN16	1000	5	7.5	168.43±2.87	91.11±2.34
SLN17	1500	7	7.5	280.23±4.13	78.32±1.67

Optimized formulation and drug release

Depending upon the optimized combination obtained from QbD design, a fresh batch of RLX-loaded SLNs was developed to validate the outcome. The fresh prepared nano- formulation has a size of 168.97 nm and %EE of 93.14%. Drug release of RLX-loaded SLNswas compared with that of pure RLX Fig. 3 (A). Free RLX was completely dissolved within 4 hr. However, the RLX-loaded SLNs showed a typical biphasic drug release pattern. An initial rapid release (burst release) within first 1-2 hrs followed by a slow-release phase for up to 72 hr. The initial rapid release of the drug was due to the RLX present on the surface of the particle. In addition, the P407 which cover the surface of the SLNs can also entrapped some of the drugwhich released at the initial time point. After this, the drug diffused slowly from the lipid matrix(Fig. 3).

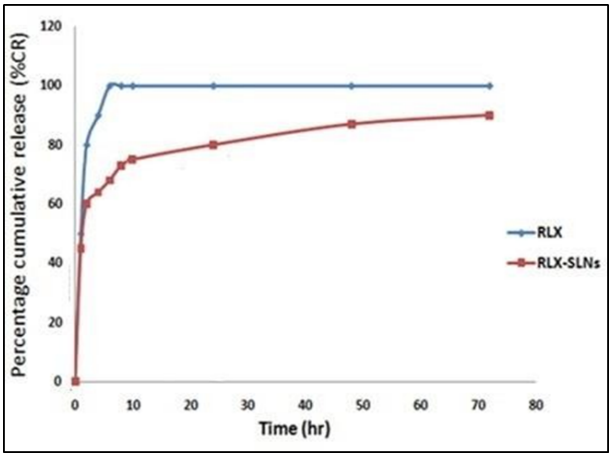

Fig. 3: *In-vitro* drug release profile for RLX-SLNs and free drug

In-Vivo Pharmacokinetic studies in rats

A non-compartmental model was used to examine the pharmacokinetic parameters of Raloxifene from the tested formulations, and the following values were recorded: Cmax, Tmax, AUC0-48, AUC0-inf, and t1/2. All of the formulations' pharmacokinetic properties are listed in Table 2, and the related plasma concentration versus time graphs are shown in Fig. 8.

Table 2: Pharmacokinetic parameters for raloxifene after oral administration of free raloxifene hydrochloride suspension RLX-SLNs and Oral tablet suspension to rats (15 mg/kg, n = 6)

Formulation code	Cmax (mg/ml)	Tmax (h)	AUC_{0-t} (µg/h/ml)	MRT_{0-t} (h)	$t_{1/2}$ (h)	Frel
Free Raloxifene hydrochloride	158.26±26.038	2.89±3.25	1.83±0.59	10.21±1.24	9.87±1.3	
Optimized RLX-SLNs	336.82±38.96***	4.53±2.17**	6.954±1.21***	16.54±3.2**	5.19±2.34**	3.8
Oral tablet suspension	145.24± 29.18	1.64± 1.12	4.026±0.47	9.56±1.28	4.21±0.59	
Each value represents the mean ± SD (n = 6). (**P < 0.05; ***P < 0.01)						

Cmax and Tmax were reported to be 158.26±26.038, 336.82±38.96 mg/ml and 2.89±3.25, 4.53±2.17h respectively, for the formulations free raloxifene hydrochloride and Raloxifene- SLNs before and after administration. The marketed formulation's oral suspension, on the other hand, had Cmax and Tmax values of 145.24±29.18 ng and 1.64±1.12 h respectively. There was a significant difference in Cmax and Tmax values between free raloxifene hydrochloride, Raloxifene- SLNs, and oral suspension (P≤0.05). Fig. 8 shows the plasma concentration versus time profile. A substantial increase (P≤0.05) in AUC0-t Raloxifene-SLNs was observed when compared to free raloxifene hydrochloride. In addition, when compared to free raloxifene hydrochloride and oral tablet suspension, the solid lipid nanoparticle formulation provides a longer-lasting release of Raloxifene. The Raloxifene- SLN's AUC0-t and MRT0-t were found to be 6.954±1.211g.hr/ml and 16.54±3.2, respectively. The AUC0-t and MRT0-t for oral tablet suspensions of Raloxifene and free raloxifene hydrochloride were (4.026±0.47, 1.83±0.59 g.hr/ml and 9.56±1.28, 10.21±1.24hr, respectively), which were considerably (p≤0.05) lower than the Raloxifene-SLNs. The increased AUC in solid lipid nanoparticles could be attributed to the drug concentration remaining within the pharmacologically efficacious region for a longer period of time. When compared to other formulations, the nano particle formulation has a significantly larger AUC0– t value (p≤0.05), indicating that it has a higher bioavailability for Raloxifene when delivered orally. Because of the close interaction of raloxifene hydrochloride with lipid, it appears that putting it into solid lipid nanoparticles aided in circumventing significant gut wall processing;absorption through the oral lymphatic region ensured an increase in bioavailability. Furthermore, raloxifene

hydrochloride from SLN formulations had a substantially longer meanresidence time (MRT) than free raloxifene hydrochloride and oral tablet suspension (P≤0.05). In comparison to free raloxifene hydrochloride and oral tablet suspension, oral bioavailability of raloxifene hydrochloride SLNs increased by 3.83 and 1.727 times in SLN formulation.

The t1/2 of Raloxifene-SLNs, free raloxifene hydrochloride, and oral tablet suspension were 5.19±2.34, 9.87±1.3, and 4.21±0.59 hours, respectively. AUC0-t was utilized to assess the improved formulation's greater bioavailability (stated in mean, n=6) in pharmacokinetic experiments. Our findings were consistent with those of other studies. The greater bioavailability of these solid lipid nanoparticle carriers demonstrates their efficacy in delivering poorly soluble medicines like raloxifene. Free raloxifene hydrochloride and oral tablet suspension were shown to be significantly less (p≤0.005) than Raloxifene-SLNs in all pharmacokinetic parameters. As a result, it's possible to conclude that raloxifene hydrochloride and oral tablet suspension aren't suitable for Raloxifene transdermal delivery.

Table 3 demonstrates that SLNs had a significantly lower rate of elimination (Ke) and clearance (P≤0.05) than free raloxifene hydrochloride and oral tablet solution in an IV pharmacokinetic investigation. Reduced Ke and clearance can be linked to an increase in MRT for raloxifene hydrochloride in SLNs. These in-vivo tests support our hypothesis that employing a greater carbon chain lipid with an emulsifier-stabilizer improves the bioavailability of raloxifene hydrochloride (P407). Raloxifene hydrochloride's BA is improvedby the protective effect of lipids along with slow clearance.

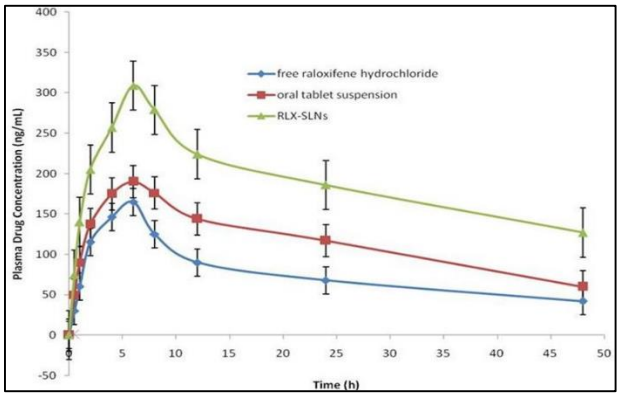

Fig. 8: Plasma concentrations vs. time profile of Raloxifene in wistar rats after oral administration of free raloxifene suspension, Raloxifene-SLNs and oral tablet suspension (15 mg/kg) (n=6)

Table 3: Pharmacokinetic parameters after intravenous administration of free raloxifenehydrochloride solution, oral tablet solution and Raloxifene-SLNs to rats (2.4 mg/kg, n = 3)

Formulation code	K_e(per hr)	V_d(L/kg)	Clearance(L/kg/h)	$t_{1/2}$ (h)
FreeRaloxifene hydrochloride	0.6432±0.1238	0.0243±0.0035	0.0107±0.0129	1.077±0.0014
Optimized Raloxifene-SLNs	0.1822±0.0196*	0.0083±0.0017**	0.0006±0.0021**	3.8035±0.1281***
Oral tablet suspension	1.2462±0.0298	0.0483±0.0112	0.0372±0.0217	0.5560±0.028
Each value represents the mean ± SD (n = 6). (**P < 0.05; ***P < 0.01)				

Uptake of RLX-SLN into rat intestinal sacs

Using a rat everted gut sac model, we studied uptake of Raloxifene-SLNs at two distinct temperatures: 37°C (Control) and 4°C. Energy-dependent active processes like endocytosis have been shown to be hindered at lower temperatures (4°C) [30]. At 4°C, there was a significant (P≤ 0.001) drop in Raloxifene-SLNs relative apparent permeability (Papp), with uptake reduced to 78 percent of that at similar doses. These findings show that the majority of SLNs are taken up via energy-dependent endocytic mechanisms, with the rest taken up by physical adhesion or free drug diffusion.

For oral uptake of nano-medicine, many energy-dependent endocytic mechanisms have been documented. Clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-

caveolae independent endocytosis are all significant activities (importantly, macro pinocytosis). While receptor-mediated endocytosis is handled by clathrin and caveolae, macro pinocytosis is a non-specific actin-dependent transport mechanism that is not mediated by receptors. Large (0.5 to 2 m) vesicular structures called macro pinosomes grow at the cell surface during macro pinocytosis; particles less than 2 m can be absorbed into enterocytes by this process. We did not examine SLN uptake entirely by macro pinocytosis because it is a non-specific mechanism for endocytic uptake.

We investigated cellular uptake mechanisms for SLNs by treating cells with various chemical inhibitors of clathrin-mediated endocytosis and caveolae-mediated endocytosis.Chlorpromazine, a cationic, amphiphilic drug, has been shown to block clathrin-mediated endocytosis by inducing clathrin to accumulate in late endosomes and decreasing its availability on the enterocyte surface [26]. In our experiment, the permeability of Raloxifene-SLNs was reduced by 48 percent (P≤0.01) (Fig. 9) when compared to control. We conclude that a clathrin-mediated pathway contributes to the uptake of Raloxifene-SLNs based on the findings.

Caveolae are lipid rafts that are high in cholesterol and sphingolipids. They're flask-shaped invaginations on the plasma membrane that can swallow cargo molecules or carriers that bind to their surface. Caveolae-mediated cholesterol sequestration is inhibited by the antifungal medication nystatin. According to our findings, nystatin administration reduced apparent permeability of SLNs by 30% (P≤0.05) at equivalent doses when compared to control (Fig. 9).

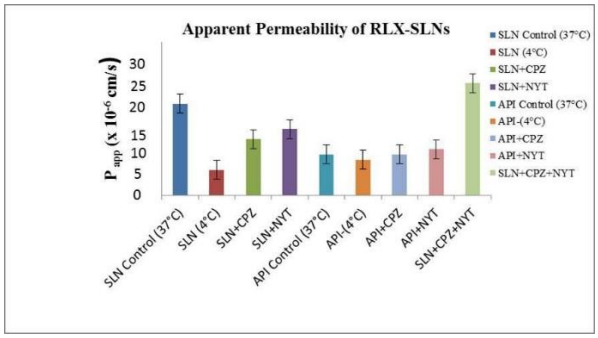

Fig. 9: Apparent permeability (Papp) Of Raloxifene-SLNs in rat everted gut sac

The uptake of Raloxifene-SLNs from the rat gut appears to include both clathrin and caveolae-mediated endocytosis mechanisms. The permeability of Raloxifene-SLNs was also found to be 2 times (P≤0.001) higher than that of free raloxifene hydrochloride. We found no significant differences in free raloxifene hydrochloride transport among treatment settings. This shows that the active endocytic absorption of free raloxifene hydrochloride does not occur. Free raloxifene hydrochloride is most likely absorbed through a passive diffusion method.

Lymphatic transport of Raloxifene-SLNs in rats

Drugs loaded into solid lipid carriers can access the lymphatic transport system via two mechanisms: (1) M-cells and gut-associated lymphoid tissue, which consists of lymphoidal follicles forming Peyer's patches; and (2) M-cells and gut-associated lymphoid tissue, which consists of lymphoidal follicles forming Peyer's patches. (2) Using triglyceride-rich lipoproteins to stimulate chylomicron synthesis and transit [26]. These triglyceride-rich lipoproteins preferentially enter lymphatic capillaries, whose cells are organised in an overlapping pattern with gaps, in the latter situation. As a result, they circumvent the portal hepatic circulation and enter the lymphatic system.

To further understand the involvement of chylomicrons and lymphatic transport in Raloxifene-SLNs, we gave rat's cycloheximide, a lymphatic transport inhibitor, and tracked their oral pharmacokinetics. The lymphatic transport pathway has been shown to be inhibited by cycloheximide without affecting other active or passive absorption channels. The plasma concentration of CXI treated rats (treatment) was considerably (p≤0.05) lower than the control group rats, according to the results of a lymphatic transport investigation (Fig. 10). The peak plasma concentration (Cmax) of raloxifene hydrochloride was reduced by 36% and the AUC0- t was reduced by 30% in the therapy group. This could be explained by CXI's suppression of SLN lymphatic transport. The role of the lymphatic transport system in the oral absorption of Raloxifene-SLN is demonstrated in this work.

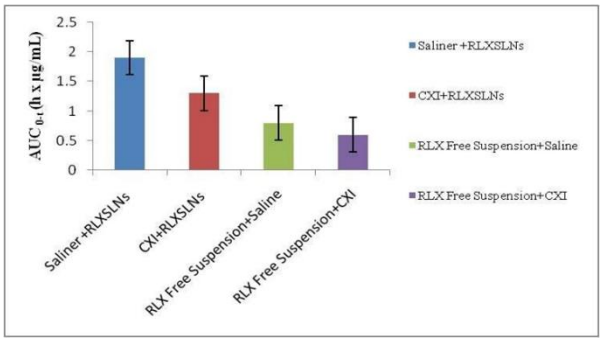

Fig. 10: Area under curve values after oral administration of Raloxifene-SLNs, Raloxifene free suspension with CXI or saline (p≤0.05) (n=6)

Anti-osteoporotic activity of SLNs

Effect on body weight

At the end of the study there was significant increase in the body weight in OVX groupwhen compared with sham control group ($p < 0.001$), whereas treatment with RLX- SLNs, oral tablet suspension, and free RLX showed significant increase in the body weight when compared with OVX group ($p < 0.001$).

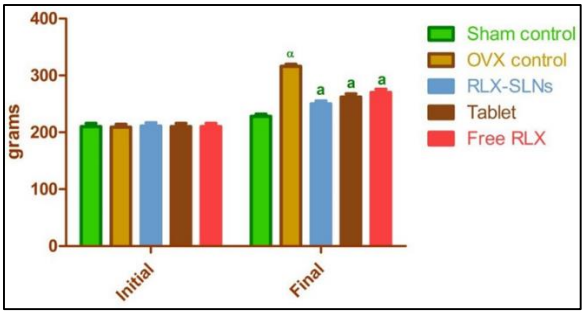

Fig.11: Effect on body weight

OVX: Ovariectomized; RLX: Raloxifene; SLN: Solid lipid nanoparticles. 3Data were expressed as mean ± SEM. αP < 0.001, when compared with sham control group, aP< 0.001, compared to OVX group. Difference between the groups was analysed by one-way analysis ofvariance (ANOVA) followed by tukey's test.

Physical parameters of the femur

Effect on femur length, femur weight and hardness of lumbar vertebra

Ovariectomy caused significant decrease in the length of the femur when compared with the sham operated group ($p < 0.05$), treatment with RLX- SLNs caused slight increase in the lengthof the femur bone ($p < 0.05$). The weight of femur was also decreased in OVX group when compared to sham control group ($p < 0.001$), which upon 42 days of treatment with RLX- SLNs was increased significantly ($p < 0.01$), whereas treatment with oral tablet suspension andfree RLX failed to show any significant increment in the bone length and femur weight. Thus,RLX-SLNs seemed to have a protective effect on bone loss. The hardness of the lumbar vertebra had decreased in OVX group in comparison to the sham control group ($p < 0.001$), however treatment with RLX- SLNs, oral tablet suspension and free RLX showed a significant increase in the hardness of the lumbar vertebra when compared to OVX group ($p < 0.001$).

Table 12: Effect on femur length, femur weight and lumbar hardness in sham operated and ovariectomized rats.

Sr. No.	Treatmentgroups	Femur length(cm)	Femur weight(gm)	Lumbar hardness (N)
1.	Sham control	3.5 ± 0.024	0.55 ± 0.0059	319 ± 3.5
2.	OVX control	2.8 ± 0.16	0.40 ± 0.0075	169 ± 4.9
3.	RLX-SLNs	3.1 ± 0.057	0.50 ± 0.020	293 ± 7.2
4.	Tablet	2.9 ± 0.11	0.47 ± 0.020	281 ± 10
5.	Free RLX	2.9 ± 0.17	0.46 ± 0.021	268 ± 3.8

Effect on weight of ash, % of ash, and ash calcium of femoral bone

Significant decrease in the weight of ash was observed in the OVX group when compared to sham control group ($p < 0.001$), Treatment with RLX-SLNs, oral tablet suspension, and Free RLX showed significant increment in the weight of ash ($p < 0.001$, $p < 0.001$, $p < 0.01$), whencompared with OVX group. Significant decrease in the percentage of ash was observed in the OVX group when compared to sham control group ($p < 0.001$), Treatment with RLX-SLNs, oral tablet

suspension, and Free RLX showed significant increment in the weight of ash ($p < 0.001$, $p < 0.01$, $p < 0.05$), when compared with OVX group.

Ash calcium of femoral vein was significant decreased in the OVX group when compared to sham control group ($p < 0.001$), Treatment with RLX-SLNs, oral tablet suspension, and Free RLX showed significant increment in the weight of ash ($p < 0.001$, $p < 0.01$, $p < 0.05$), when compared with OVX group.

Table 13: Effect on weight of ash, percentage of ash, and ash calcium of femoral bone insham operated and ovariectomized rats.

Sr. No.	Treatmentgroups	Weight of ash(gm)	% of ash	Ash calcium(mg/dL)
1.	Sham control	0.27 ± 0.0075	49 ± 0.74	6.1 ± 0.45
2.	OVX control	0.18 ± 0.0043$^{\alpha}$	32 ± 0.90$^{\alpha}$	2.4 ± 0.14$^{\alpha}$
3.	RLX-SLNs	0.25 ± 0.0064^{a}	43 ± 0.95^{a}	5.1 ± 0.25^{a}
4.	Tablet	0.24 ± 0.0044^{a}	39 ± 0.85^{b}	4.5 ± 0.19^{b}
5.	Free RLX	0.22 ± 0.0068^{b}	37 ± 1.1^{c}	3.8 ± 0.13^{c}

OVX: Ovariectomized; RLX: Raloxifene; SLN: Solid lipid nanoparticles.

Data were expressed as mean ± SEM. $^{\alpha}P < 0.001$, when compared with sham control group, $^{a}P < 0.001$, $^{b}P < 0.01$, and $^{c}P < 0.05$ compared to OVX group. Difference between the groups wasanalyzed by one-way analysis of variance (ANOVA) followed by tukey's test.

Levels of serum calcium, phosphorous and alkaline phosphatase in sham operated and OVX rats

Serum calcium levels were significantly decreased in the OVX group when compared to shamcontrol group ($p < 0.001$), treatment with RLX-SLNs, oral tablet suspension, and free RLX showed significant increment in the calcium levels ($p < 0.001$, $p < 0.01$, $p < 0.05$), when compared with OVX group. Serum phosphorus levels were significantly decreased in the OVX group when compared to sham control group ($p < 0.001$), Treatment with RLX-SLNs, oral tablet suspension, and free RLX showed significant increment in the calcium levels ($p < 0.001$, $p < 0.001$, $p < 0.01$) whencompared with OVX group. Serum alkaline phosphate levels were significantly increased in the OVX group when compared to sham control group ($p < 0.001$), Treatment with RLX-SLNs and oral tablet suspension showed significant decrease in the ALP levels ($p < 0.001$, $p < 0.01$), when compared with OVX group. However, Free RLX group failed to show significant decrease in the levels of serum ALP when compared to OVX group.

Table 14: Effect on levels of serum calcium, phosphorous, and alkaline phosphatase anduterine in sham operated and ovariectomized rats

Sr. No.	Treatment groups	Serum levels		Alkaline phosphatase (IU/L)
		Calcium (mg/dL)	Phosphorous(mg/dL)	
1.	Sham control	7.6 ± 0.50	7.3 ± 0.24	204 ± 5.9
2.	OVX control	2.3 ± 0.35$^{\alpha}$	3.2 ± 0.12$^{\alpha}$	265 ± 4.4$^{\alpha}$
3.	RLX-SLNs	5.5 ± 0.26^{a}	6.4 ± 0.23^{a}	232 ± 4.7^{b}
4.	Tablet	4.9 ± 0.13^{b}	5.0 ± 0.22^{a}	241 ± 5.3^{c}
5.	Free RLX	4.0 ± 0.35^{c}	4.6 ± 0.26^{b}	250 ± 4.2

OVX: Ovariectomized; RLX: Raloxifene; SLN: Solid lipid nanoparticles.

Data were expressed as mean ± SEM. $^{\alpha}P < 0.001$, when compared with sham control group, $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$ compared to OVX group. Difference between the groupswas analyzed by one-way analysis of variance (ANOVA) followed by tukey's test.

Effect on uterine weight

Decreased relative weight of uterus was observed in the OVX group when compared with sham control group ($p < 0.001$), treatment with RLX-SLNs showed significant increment in the uterine weigh ($p < 0.01$) when compared with OVX group, however, treatment with oraltablet suspension and free RLX did not show any significant change in the uterine weight when compared with OVX group.

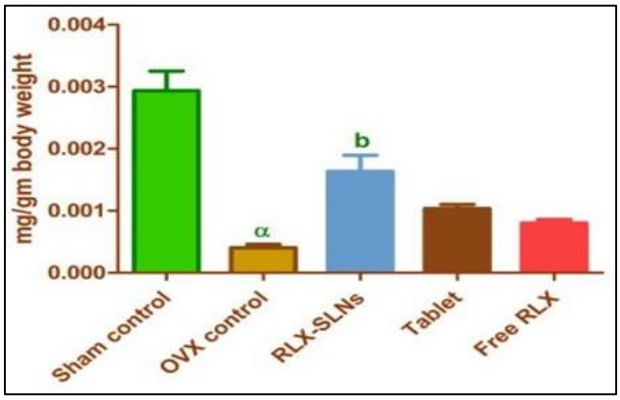

Fig. 12: Effect on uterine weight

OVX: Ovariectomized; RLX: Raloxifene; SLN: Solid lipid nanoparticles.

Data were expressed as mean ± SEM. $^{\alpha}P < 0.001$, when compared with sham control group, $^{b}P < 0.01$ compared to OVX group. Difference between the groups was analyzed by one-way analysis of variance (ANOVA) followed by tukey's test.

Effect on serum TNF-α levels

TNF-α levels were significantly increased in the OVX group when compared with sham control group ($p < 0.001$), treatment with RLX-SLNs, oral tablet suspension and free RLX showed significant decrease in the TNF- α levels when compared with control group ($p < 0.001$, $p < 0.001$, and $p < 0.01$).

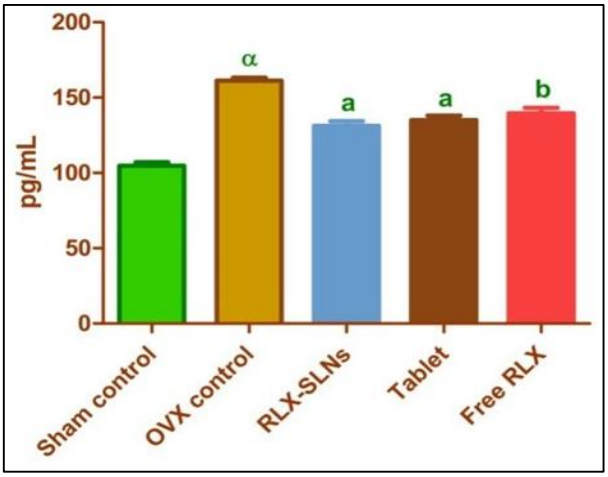

Fig. 13: Effect on serum TNF-α levels

OVX: Ovariectomized; RLX: Raloxifene; SLN: Solid lipid nanoparticles.

Data were expressed as mean ± SEM. $^{\alpha}P < 0.001$, when compared with sham control group, $^{a}P < 0.001$ and $^{b}P < 0.01$ compared to OVX group. Difference between the groups was analyzedby one-way analysis of variance (ANOVA) followed by tukey's test.

Conclusion

The poor water solubility of a drug not only affects its oral bioavailability and overall therapeutic effects, but also poses a significant challenge for formulation development. Over the past decade, SLNs have emerged as a potential system that can effectively overcome these limitations. The high biocompatibility, biodegradability, protective properties of drugs in the gastrointestinal tract and sustained release properties make SLN an ideal candidate to address problems of low oral bioavailability. In the present study, SLNs based on Compritol 888 ATOwere successfully produced by the emulsion process. The effect of the variables on the properties of the SLNs loaded with RLX was analysed using BBD. Finally, the optimized formulation was evaluated for in vitro release and in vivo performance. Cmax and Tmax were reported to be 158.26±26.038, and 2.89±3.25, respectively for optimized SLN RLX formulation. The Raloxifene- SLN's AUC_{0-t} and MRT_{0-t} were found to be 6.954±1.211g.hr/ml and 16.54±3.2, respectively. The increased AUC in solid lipid nanoparticles could be attributed to the drug concentration remaining within the pharmacologically efficacious region for a longer period of time. Longer AUC values indicate higher

bioavailability for Raloxifene when delivered orally. Also analysed for Anti-osteoporotic activity of SLNs. Finally, the designed SLNs loaded with RLX proved to be stable. In conclusion, SLN RLX may be an effective strategy to reduce the dose of the drug and increase its oral bioavailability.

References

1) Prevention and management of osteoporosis. World Health Organ Tech. Rep. Ser. 2003, 921, 1–164.

2) Francis, R.M. An Atlas of Osteoporosis: The Encyclopedia of Visual Medicine Series.J. R. Soc. Med. 1993, 86,682.

3) Kanis, J. Assessment of Osteoporosis at the Primary Health Care Level; Technical Report; World Health Organization Collaborating Centre for Metabolic Bone Diseases:Geneva, Switzerland, 2008; pp. 6–9.

4) Johnell, O.; Kanis, J.A. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporosis Int. 2006, 17, 1726–1733.

5) Thiebaud, D.; Secrest, R.J. Selective estrogen receptor modulators: Mechanism of action and clinical experience. Focus on raloxifene. Reprod. Fertil. Dev. 2001, 13, 331–336.

6) Lufkin, E.G.; Whitaker, M.D.; Nickelsen, T.; Argueta, R.; Caplan, R.H.; Knickerbocker, R.K.; Riggs, B.L.Treatment of established postmenopausal osteoporosis with raloxifene: A randomized trial. J. Bone Miner.Res. 1998, 13, 1747– 1754.

7) Pinsky, P.F.; Miller, E.A.; Heckman-Stoddard, B.M.; Minasian, L. Breast Cancer Characteristics and Survival among Users versus Nonusers of Raloxifene. Cancer Prev. Res. 2020, 13, 83–90.

8) Amidon, G.L.; Lennernas, H.; Shah, V.P.; Crison, J.R. A theoretical basis for a biopharmaceutical drug classification: The correlation of in vitro drug product dissolution and in vivo bioavailability. Pharm. Res.1995, 12, 413–420.

9) Gluck, O.; Maricic, M. Skeletal and nonskeletal effects of raloxifene. Curr. Osteoporos. Rep. 2003, 1, 123–128.

10) TrdanLusin, T.; Mrhar, A.; Stieger, B.; Kullak-Ublick, G.A.; Marc, J.; Ostanek, B.; Zavratnik, A.; Kristl, A.; Berginc, K.; Delic, K.; et al. Influence of hepatic and intestinal efflux transporters and their genetic variants on the pharmacokinetics and pharmacodynamics of raloxifene in osteoporosis treatment. Transl. Res. 2012, 160, 298–308.

11) Ravi, P.R.; Aditya, N.; Kathuria, H.; Malekar, S.; Vats, R. Lipid nanoparticles for oral delivery of raloxifene: Optimization, stability, in vivo evaluation and uptake mechanism. Eur. J. Pharm. Biopharm. 2014, 87, 114–124.

12) L. V. Avioli, "SERM drugs for the prevention of osteoporosis," Trends in Endocrinology and Metabolism, vol. 10, no. 8, pp. 317–319, 1999.

13) N. Furusyo, E. Ogawa, M. Sudoh et al., "Raloxifene hydrochloride is an adjuvant antiviral treatment of postmenopausal women with chronic hepatitis C: a randomized trial," Journal of Hepatology, vol. 57, no. 6, pp. 1186–1192, 2012.

14) M. Hibner, J. F. Magrina, S. R. Lefler, J. L. Cornella, A. R. Pizarro, and J. C. Loftus, "Effects of raloxifene hydrochloride on endometrial cancer cells in vitro," GynecologicOncology, vol. 93, no. 3, pp. 642–646, 2004.

15) O. Gluck and M. Maricic, "Skeletal and nonskeletal effects of raloxifene," Current Osteoporosis Reports, vol. 1, no. 3, pp. 123–128, 2003.

16) R. K. Jha, S. Tiwari, and B. Mishra, "Bioadhesive microspheres for bioavailability enhancement of raloxifene hydrochloride: formulation and pharmacokinetic evaluation," AAPS PharmSciTech, vol. 12, pp. 650–657, 2011.

17) R.-K. Chang and A. H. Shojaei, "Effect of a lipoidic excipient on the absorption profileof compound UK 81252 in dogs after oral administration," Journal of Pharmacy and Pharmaceutical Sciences, vol. 7, no. 1, pp. 8–12, 2004.

18) V. Jannin, J. Musakhanian, and D. Marchaud, "Approaches for the development of solid and semi-solid lipid-based formulations,"

Advanced Drug Delivery Reviews, vol.60, no. 6, pp. 734–746, 2008.

19) M. K. Rawat, A. Jain, A. Mishra, M. S. Muthu, and S. Singh, "Development of repaglinide loaded solid lipid nanocarrier: selection of fabrication method," Current Drug Delivery, vol. 7, no. 1, pp. 44–50, 2010.

20) S. Ghoshal, J. Witta, J. Zhong, W. de Villiers, and E. Eckhardt, "Chylomicrons promote intestinal absorption of lipopolysaccharides," Journal of Lipid Research, vol. 50, no. 1,pp. 90–97, 2009.

21) M. J. Cano-Cebrián, T. Zornoza, L. Granero, and A. Polache, "Intestinal absorption enhancement via the paracellular route by fatty acids, chitosans and others: a target for drug delivery," Current Drug Delivery, vol. 2, no. 1, pp. 9–22, 2005.

22) C. M. O'Driscoll, "Lipid-based formulations for intestinal lymphatic delivery," European Journal of Pharmaceutical Sciences, vol. 15, no. 5, pp. 405–415, 2002.

23) M. Stuchlik and S. Zak, "Lipid-based vehicle for oral drug delivery," Biomedical Papers of the Medical Faculty of the University Palacky, vol. 145, no. 2, pp. 17–26, 2001.

24) D. G. Fatouros, D. M. Karpf, F. S. Nielsen, and A. Mullertz, "Clinical studies with oral lipid based formulations of poorly soluble compounds," Therapeutics and Clinical Risk Management, vol. 3, no. 4, pp. 591–604, 2007.

25) M. Uner and G. Yener, "Importance of Solid Lipid Nanoparticles (SLN) in various administration routes and future perspectives," International Journal of Nanomedicine, vol. 2, no. 3, pp. 289–300, 2007.

26) A. zurMühlen, C. Schwarz, and W. Mehnert, "Solid Lipid Nanoparticles (SLN) for controlled drug delivery—drug release and release mechanism," European Journal of Pharmaceutics and Biopharmaceutics, vol. 45, no. 2, pp. 149–155, 1998.

27) Lu, B.; Xiong, S.B.; Yang, H.; Yin, X.D.; Chao, R.B. Solid lipid nanoparticles of mitoxantrone for local injection against breast cancer and its lymph node metastases. Eur. J. Pharm. Sci. 2006, 28, 86–95.

28) Severino, P.; Andreani, T.; Macedo, A.S.; Fangueiro, J.F.; Santana, M.H.; Silva, A.M.; Souto, E.B. Current State-of-Art and New Trends on Lipid Nanoparticles (SLN and NLC) for Oral Drug Delivery. J. Drug Deliv. 2012, 2012, 750891.

29) Yang, S.C.; Lu, L.F.; Cai, Y.; Zhu, J.B.; Liang, B.W.; Yang, C.Z. Body distribution inmice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on brain. J. Control. Release 1999, 59, 299–307.