Pharm Res (2015) 32:2595–2608
DOI 10.1007/s11095-015-1646-x

RESEARCH PAPER

Improvement of Oral Bioavailability of N-251, a Novel Antimalarial Drug, by Increasing Lymphatic Transport with Long-Chain Fatty Acid-Based Self-Nanoemulsifying Drug Delivery System

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Received: 18 September 2014 / Accepted: 2 February 2015 / Published online: 27 February 2015

ABSTRACT

Purpose The objective of this study was to improve the absorption behavior of N-251, a novel antimalarial drug, by preparing an appropriate self-nanoemulsifying drug delivery system (SNEDDS).

Methods Two different types of SNEDDS formulations, medium-chain fatty acid-based SNEDDS (MC-SNEDDS) and long-chain fatty acid-based SNEDDS (LC-SNEDDS), were prepared based on pseudo-ternary phase diagram, and examined for their *in vivo* oral absorption behavior in rats.

Results Oral dosing of MC-SNEDDS formulations significantly improved the bioavailability (BA) of N-251 compared with N-251 powders. However, its high hepatic extraction limited the BA of N-251 to only 0.49 for MC-SNEDDS B, the best formulation of MC-SNEDDS. LC-SNEDDS formulations, especially LC-SNEDDS F provided the highest BA, 0.65, and successfully attenuated the inter-individual difference in the absorption behavior. Furthermore, it was confirmed that lymphatic transport of N-251 for LC-SNEDDS F was significantly increased up to around 3.19 times larger than that for MC-SNEDDS B. Simulation study suggested that 20 to 39% of N-251 uptaken by the small intestine would be delivered to lymphatic system after oral administration of LC-SNEDDS F.

Conclusions SNEDDS formulations significantly improved the absorption behavior of N-251 and long-chain fatty acid-based lipid further improved it by avoiding the hepatic first-pass elimination.

KEY WORDS antimalarial drug · bioavailability · hepatic first-pass elimination · long-chain fatty acid · lymphatic transport · medium-chain fatty acid · self-nanoemulsifying drug delivery system (SNEDDS)

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INTRODUCTION

Oral drug products constitute over 60% of marketed medicines because of their convenience, and many new drug candidates are still being developed as an oral dosage. On the other hand, many poorly water-soluble compounds have been being picked up as new drug candidates. Therefore, poor solubility in water is one of the most serious problems in the subsequent processes of drug development to improve the low bioavailability and large variability in the absorption kinetics after oral administration due to poor solubility (1–3).

Several approaches (4), such as salt formation, nanomization and solid dispersion would be useful for improving the solubility of poorly water-soluble compounds, but we have focused on a lipid-based formulation, in particular, a self-nano- or micro-emulsifying drug delivery system (SNEDDS or SMEDDS). Although very few lipid-based formulations have been marketed, the utilization of lipid excipients has considerable potential (5) and the interest in lipid-based formulations has been growing very recently (6). SNEDDS and/or SMEDDS can solubilize poorly water-soluble compounds in isotropic mixtures of an oil, surfactant and co-surfactant (7). After oral dosing of the mixtures, an oil-in-water nano- or micro-emulsion can be formed with gentle agitation by gastrointestinal motility and with dilution by drinking water and gastrointestinal fluid. A very large surface area due to the small particle size of the nano- or micro-emulsion can lead to rapid lipid digestion and drug release, resulting in rapid drug absorption from the small intestine (8,9). Therefore, SNEDDS and/or SMEDDS would improve the rate and extent of oral bioavailability and reduce the variability in absorption behavior for poorly water-soluble drugs that exhibit dissolution rate-limited absorption (7). Furthermore, the chemical and/or enzymatic stability of drugs might also be expected since drugs are incorporated into nano- or micro-emulsion droplets in the gastrointestinal lumen.

Malaria is one of the most serious diseases in the world and new antimalarial drugs have been extensively desired since *plasmodium falciparum* (*p. falciparum*), the major parasite for the malaria disease, has acquired the resistance against artemisinine, a world-widely used antimalarial drug (10–12). N-251, 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (Fig. 1), is a novel and promising antimalarial drug synthesized by our group (13). Its antimalarial activity against *p. falciparum* is very similar to that of artemisinin, and its toxicity against *p. falciparum* is around 350 fold greater than that against mouse mammary tumor FM3A cells (13), indicating its highly selective toxicity against malaria parasites. Preclinical safety was also confirmed by the study performed under Good Laboratory Practice guidelines (14). The most promising property of N-251 is to cure completely 1% parasites-infected mice without any recurrence (13), while artemisinin is known to be unable to completely kill the parasite in the blood (12). However, since N-251 is a poorly-water soluble drug, its dissolution and subsequent absorption properties must be improved to exert the efficient antimalarial effect after oral administration.

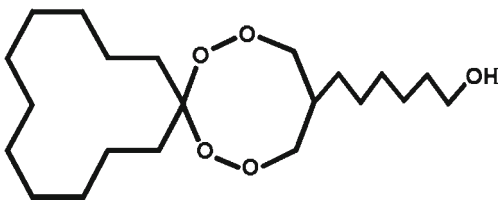

Fig. 1 Chemical structure of N-251.

In the present study, we tried to improve the *in vivo* absorption kinetics of N-251 by SNEDDS formulations. Although medium-chain fatty acid-based SNEDDS (MC-SNEDDS) was prepared first, the improvement of bioavailability by MC-SNEDDS was not enough due to the high hepatic first-pass elimination of N-251. Therefore, long-chain fatty acid-based SNEDDS (LC-SNEDDS) were prepared second for aiming to enhance the bioavailability by circumventing the hepatic first-pass elimination since lipids with long-chain fatty acids promote the transport of drugs into the lymphatic system more effectively than those with short- and/or medium-chain fatty acids (15–17). *In vivo* oral absorption of N-251 and the lymphatic transport after absorption were evaluated for several SNEDDS formulations. Furthermore, we tried to simulate the quantitative contribution of absorption *via* lymphatic system to bioavailability of N-251 after oral administration.

MATERIALS AND METHODS

Materials

N-251 was synthesized as described previously (13). Cremophor EL, Cremophor RH40 were purchased from Sigma Chemical Co. (St. Louis, MO). PEG400, Carbitol, corn oil, peanut oil, soy bean oil, olive oil and sesame oil were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Lauroglycol 90 and Capryol 90 were kindly supplied by CBC Co., Ltd (Tokyo, Japan). Rheodol MO-60 was kindly supplied by Kao Corporation (Tokyo, Japan). All other chemicals and reagents were analytical grade commercial products.

Animals

Male Wistar rats weighing 180–330 g (Charles River Laboratories Japan, Yokohama, Japan), maintained at 25°C and 55% humidity, were allowed free access to standard laboratory chow (Clea Japan, Tokyo) and water. They were fasted for 24 h prior to and during the experiment, but were allowed free access to water. Our investigations were performed after approval by our local ethics committee at Okayama University and in accordance with the "Principles of Laboratory Animal Care (NIH publication # 85–23)".

Determination of Solubility

Excess N-251 was added to 1 mL of each oil, surfactant or co-surfactant, incubated at 37°C and shaken at 150 min^{-1} for 48 h, which was fixed based on our preliminary experiments providing the saturation solubility of N-251. The supernatant

obtained after centrifugation (12,000 rpm for 20 min) was dissolved in the mobile phase for LC/MS/MS analysis and used for the determination of N-251 by LC/MS/MS.

Preparation and Characterization of SNEDDS

SNEDDS were prepared with various ratios of oil, surfactant, co-surfactant and N-251. Practically, N-251 was dissolved in the mixtures of selected components by gently mixing with a stirring bar at room temperature. Sixty eight milligrams of N-251 was contained in 1 g SNEDDS.

To select appropriate SNEDDS formulations, pseudo-ternary phase diagrams of oil, surfactant, co-surfactant and water were formed by the water titration method (18,19). Briefly, mixtures of oil, surfactant and co-surfactant at a given ratio (*w*/*w*) were diluted with water in a drop-by-drop manner (20). Each mixture was gently stirred by a stirring bar and phase clarity was visually observed to judge if the nanooemulsion formed. SNEDDS formulations giving a relatively large area of nanoemulsion formation and providing nanoemulsion in the wide range of dilution ratio by water were chosen as those used for further studies.

For the selected SNEDDS formulations, the size distribution of droplet of nanoemulsion was analyzed following the dynamic light scattering method (Zetasizer Nano, Malvern Instruments Ltd., Worcestershire, UK) after 1 g SNEDDS was diluted in 6.4 mL water, which is the condition for the *in vivo* oral administration study, as described below.

In Vivo Oral and Intravenous Administration Studies

One day before drug administration, the jugular vein of a rat was cannulated with vinyl tubing (ca 20 cm) (SV-45, i.d. 0.58 mm, o.d. 0.96 mm, Natsume, Tokyo, Japan) connected with SR tube (1.5 cm) (i.d. 0.5 mm, o.d. 1 mm Shin-Etsu Polymer Co., Ltd, Tokyo, Japan) under ether anesthesia. In the case of oral administration studies, N-251 powders were intragastrically administered in a gelatin mini-capsule at a dose of 68 mg/kg, and then immediately 6.4 mL/kg of water was ingested. SNEDDS containing N-251 was intragastrically administered at a dose of 68 mg N-251/g SNEDDS/kg and then immediately 6.4 mL/kg water was ingested. In the case of intravenous administration study, N-251 was dissolved into saline containing 10% Cremophor EL and 10% ethanol, and the resultant solution was administered into the tail vein at 3 mg/mL/kg. The dosed rats were allowed to move freely and were given free access to water after 4 h of administration. Blood samples were periodically taken from the cannulated jugular vein. Plasma obtained by centrifugation was deproteinized by acetonitrile and the resulting supernatant was added into the equivolume of highly purified water. The resulting samples were applied to a solid phase extraction column (OASIS MCX 1 cc (30 mg) extraction cartridges, Waters, Milford, MA) equilibrated with 1 mL methanol and 1 mL highly purified water. Then, the column was washed with 1 mL of 20% ethanol and eluted with 1 mL acetonitrile. The effluents were evaporated and the resultant residue was reconstituted by adequate volume of acetonitrile. Obtained samples were introduced into LC/MS/MS for the analysis of N-251.

Isolated Liver Single-Pass Perfusion Study

The liver single-pass perfusion study was performed following the methods reported by Mortimore with some modifications (21). Briefly, the bile duct and portal vein were cannulated with polyethylene tubes, SP10: i.d. 0.28 mm, o.d. 0.61 mm (Natsume Seisakusho Co., Ltd., Tokyo, Japan) and SP120: i.d. 1.57 mm, o.d. 2.08 mm (Natsume Seisakusho Co., Ltd.), respectively, under urethane anesthesia. Then, the perfusate was delivered to the liver by a peristaltic pump (RP-VT, FURUE Science, Tokyo, Japan) at ca 10 mL/min at 37°C. Then, the vena cava just above the right kidney was ligated and then was cut-off just below the vein. Then, the inferior vena cava was cannulated through the right atrium with a polyethylene tubing (PE260: i.d. 1.77 mm, o.d. 2.80 mm; Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). The perfusate consisted of 10% bovine erythrocytes, 4.6% bovine serum albumin, 5.5 mM glucose and 0.8 mM L-glutamine in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) equilibrated with 95% oxygen-5% carbon dioxide, where N-251 was dissolved at 10.0 μg/mL (final concentration) in advance. Bile flow was over 100 μL/20 min, indicating the maintenance of viability of perfused liver (22). Perfusate was collected every 5 min for 1 h. "Plasma" perfusates obtained by centrifugation were treated following the method for rat plasma described in the "*In Vivo* Oral and Intravenous Administration Study", and introduced into LC/MS/MS for the analysis of N-251.

Lymphatic Transport After Oral Administration

To examine the lymphatic transport of N-251, its lymph node concentrations were determined after oral administration of SNEDDS to rats. *In vivo* oral administration was performed following the same procedure described in the "*In Vivo* Oral and Intravenous Administration Study". Then, mesenteric lymph nodes were periodically taken from the rats, and homogenized with three times volume of purified water by homogenizer (EYELA ZN-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The resulting samples were deproteinized and extracted with acetonitrile. The resulting supernatant was treated by following the same methods as described in the "*In Vivo* Oral and Intravenous Administration Study" and introduced into LC/MS/MS for the analysis of N-251.

Analytical Method

The concentration of N-251 was determined by the LC/MS/MS system (LC; Agilent 1200, Agilent Technologies, Tokyo, Japan –MS/MX; API4000TM, AB SCIEX, Tokyo, Japan). A sample of 10 μL was injected to the system equipped with TSKgel Super-octyl column (2.3 μm; i.d. 2.0×100 mm, Tosoh Co., Tokyo, Japan) and the mobile phase, acetonitrile:10 mM ammonium acetate (80:20), was delivered at 0.2 mL/min.

For quantification of N-251, the ESI interface in the positive MRM mode was chosen and a set of parameters used was as follows: ion source spray voltage (IS), 5500 V; atomization temperature (TEM), 300; atomization gas pressure (GAS1), 50 psi (nitrogen); heated auxiliary gas (GAS2), 80 psi (nitrogen); air curtain gas pressure (CUR), 40 psi (nitrogen); collision flow ratio (CAD), 5. The conditions were optimized by directly injecting the standard solutions. A parent ion of N-251 was its NH_4^+-adduct ion (m/z, 390) and a monitoring ion's m/z was 183. Multiple standard curves were developed for the concentration ranges from 6.7 to 6667 ng/mL and the correlation coefficient for standard curves was over 0.986.

Pharmacokinetic Analysis

Area under the plasma concentration–time curve (AUC) after oral administration of N-251 was calculated by following the trapezoidal rule. The highest concentration observed was employed as C_{max} and the time for C_{max} was defined as T_{max}. The absolute bioavailability, F, was calculated by utilizing the AUC value after intravenous administration. Hepatic availability, F_h, in the isolated liver perfusion study was calculated by the following equation:

$$F_h = C_{out}/C_{in} \quad (1)$$

where C_{in} and C_{out} mean concentrations of N-251 in the inflow "plasma" perfusate and outflow "plasma" perfusate at steady state, respectively.

Statistical Analysis

Results are expressed as the mean±S.E. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance in the differences of the means was evaluated using Student's *t*-test or Dunnett's test for single or multiple comparisons of experimental groups, respectively.

RESULTS AND DISCUSSION

It is well known that poorly water-soluble drugs often cause low oral bioavailability and large inter- and/or intra-individual differences in absorption kinetics (1–3). We have already reported that SMEDDS formulation successfully improved the bioavailability and reduced the variability of absorption behavior of griseofulvin, a poorly water-soluble drug (23). N-251 is more lipophilic than griseofulvin (clogP=2.88) (3), since the value of clogP was estimated to 6.67 by ChemBio3D Ultra 11.0.1 (Cambridge Soft, Cambridge, MA). The solubility of N-251 in water is 7.19±0.99 μg/mL (n=6), which is similar to that of griseofulvin (5–6 μg/mL) (2). Molecular weight is also very similar between the two compounds (N-251, 372; griseofulvin, 352.8). Since these similarities suggested that SNEDDS formulation would improve the absorption behavior of N-251, we tried to prepare and examine SNEDDS formulations of N-251 in the present study.

Solubility of N-251 in Various Vehicles

To select components to prepare SNEDDS formulations for N-251, its solubility in several oils, surfactants and co-surfactants was investigated (Table I). Among the oils examined, oils containing mainly medium-chain fatty acids (Capryol 90 and Lauroglycol 90) showed much higher solubility of N-251 than the long-chain fatty acid-based oils, suggesting that higher HLB would be adequate for solubilizing N-251. Although the reason for the tendency of gradual increase in solubility from soybean oil to ethyl oleate in long chain fatty acid-based oils was not clarified yet, it might be related to the amount of oleic acid contained in the oils. The ratio of oleic acid decreases from 100% (ethyl oleate) to ca 20% (soybean oil), while the ratio of linoleic acid increases from 0% to 50–60% (24). In the case of surfactants, Tween 80 with high HLB (ca 15) and Rheodol MO-60 with low HLB (ca 3) showed relatively high solubility while Cremophors used very often for preparation of SNEDDS formulation showed relatively low solubility of N-251. Carbitol indicated the highest solubility among the co-surfactants examined in the present study. Although Carbitol is an excipient for transdermal formulation, its possible use has been often examined for oral dosage form (25,26).

Preparation of MC-SNEDDS Formulation

Based on the results of solubility, it was thought that medium-chain fatty acid-based oil would be suitable for N-251, and then Capryol 90 and Lauroglycol 90 were chosen as the oil components, and Tween 80, Cremophor EL and Cremophor RH40 were examined as the surfactant. Carbitol was fixed as the co-surfactant for preparation.

Pseudo-ternary phase diagrams were developed to select a better oil component and to find the optimum ratios of oil, surfactant and co-surfactant. As a result, formulations utilizing Lauroglycol 90 did not provide any substantial region forming nanoemulsion (data not shown). Furthermore, formulations

Table I Solubility of N-251 in Various Vehicles

Oil	Solubility (mg/mL)	Natural oil	Solubility (mg/mL)	Surfactant	Solubility (mg/mL)	Co-surfactant	Solubility (mg/mL)
Capryol 90	356.98 ± 20.31	Olive oil	92.90 ± 2.36	Cremophor EL	65.41 ± 2.66	PEG 400	76.02 ± 7.36
Lauroglycol 90	250.69 ± 10.06	Peanut oil	82.10 ± 7.60	Cremophor RH40	56.26 ± 1.39	Carbitol	465.95 ± 13.45
Captex 200	138.14 ± 5.96	Sesame oil	80.80 ± 0.31	Tween 80	104.92 ± 2.67	Propylene glycol	24.00 ± 1.72
Ethyl oleate	100.46 ± 0.81	Corn oil	81.20 ± 1.80	Rheodol MO-60	168.59 ± 6.75		
		Soybean oil	78.40 ± 2.05				

Results are expressed as the mean ± S.E. of 3 to 5 experiments

containing Tween 80 as the surfactant provided only very small region forming nanoemulsion (data not shown). Since it was suggested that the higher self-microemulsifying ability of glycerides of medium-chain fatty acid could be attributed to their aqueous solubility being higher than that of glycerides of long-chain fatty acids (27), it is reasonable that Capryol 90 composed of shorter chain fatty acid than Lauroglycol 90 had a larger region of nanoemulsion formation compared with Lauroglycol 90. However, the reason why Tween 80 was not suitable for N-251 preparation is not disclosed yet.

Among many trials, three formulations composed of Capryol 90, Cremophor EL or Cremophor RH40, and Carbitol, were found to be suitable for N-251 because they provided relatively larger region forming nanoemulsion, and MC-SNEDDS A, B and C were chosen as promising formulations for *in vivo* use because they provided nanoemulsion in the wide range of dilution ratio by water (Fig. 2). Table II summarizes the mean droplet sizes and polydispersity indexes after titration with water at the ratio of SNEDDS to water = 1 : 6.4 (*w*/*w*), which is the ratio of SNEDDS to water in an *in vivo* oral absorption study. The obtained results suggested that all the three preparations would provide nanoemulsions with fine particles of monodispersity after oral administration. In the present study, we employed surfactants with HLB of 12–14 (Cremophor EL) and 14–16 (Cremophor RH40), and a co-surfactant, Carbitol, to prepare MC-SNEDDS formulations, which are conditions categorized into Type III proposed by Pouton (28). Pouton also suggested that the formulation containing more hydrophilic components was superior in self-microemulsifying ability and provided smaller droplets than those containing more lipophilic components (28), which was also the case for MC-SNEDDS formulations for N-251.

Improvement of Oral Absorption Kinetics by MC-SNEDDS Formulations

In vivo oral absorption studies were performed for MC-SNEDDS A, B and C formulations. Plasma concentration–time profile of N-251 after oral dosing of SNEDDS A, B or C is shown with those for powder and PEG400 solution (Fig. 3). Pharmacokinetic parameters obtained are summarized in Table III. Significantly lower T_{max}, higher C_{max} and shorter MRT clearly indicated that the absorption of N-251 was much faster from MC-SNEDDS B and C than powder. Significantly larger values of AUC and F evidenced that a larger amount of N-251 was absorbed from SNEDDS B and C than powder. On the other hand, MC-SNEDDS A tended to improve the absorption of N-251, but the improving effect was smaller than those of MC-SNEDDS B and C, and almost equivalent to that of PEG400 solution. Oral administration of nanoemulsion prepared from MC-SNEDDS A in advance significantly improved AUC of N-251 (8.76 ± 0.57 μg/mL·hr, $n=5$, $p<0.01$ vs powder), while no significant change in AUC was found in the cases of MC-SNEDDS B (7.87 ± 1.25 μg/mL·hr, $n=4$, N.S. vs SNEDDS B) and C (6.38 ± 0.66 μg/mL·hr, $n=5$, N.S. vs SNEDDS C). These results suggest that the formation of nanoemulsion from SNEDDS B and C was so rapid that the process of self-nanoemulsification could not affect the absorption kinetics of N-251 after oral dosing of the SNEDDS formulation, while, in the case of MC-SNEDDS A, the formation of nanoemulsion after oral dosing would be insufficient or dilution with gastrointestinal fluid would make the nanoemulsion unstable, which might have led to some precipitation of N-251. However, the details of the difference in the *in vivo* performance among the three preparations remain to be elucidated.

Evaluation of Hepatic Availability of N-251

Although MC-SNEDDS B and C successfully improved the oral absorption of N-251, its bioavailability was only around 50% (Table III). Considering the high lipophilicity of N-251 and no precipitation of N-251 in gastrointestinal tract observed by naked eyes, it was thought that the fraction absorbed would be pretty high. Then, we examined the hepatic first-pass elimination as a possible reason for the bioavailability lower than expected.

Liver perfusion studies were performed twice for N-251 at 10 μg/mL, which was the highest concentration dissolved in the perfusate used in this study. Hepatic availability, F_h,

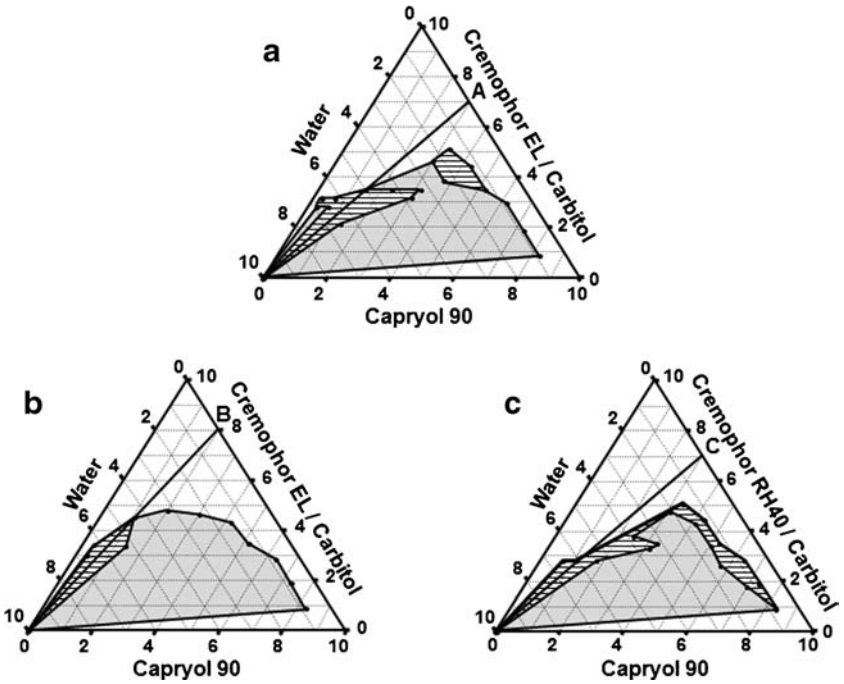

Fig. 2 Pseudo-ternary phase diagrams indicating efficient self-nanoemulsification regions. The lined area represents nanoemulsion formation region and the gray area represents macroemulsion formation region. (**a**) oil, Capryol 90; surfactant/cosurfactant, Cremophor EL/Carbitol = 1:1 (*w/w*); (**b**) oil, Capryol 90; surfactant/cosurfactant, Cremophor EL/Carbitol = 1:2 (*w/w*); (**c**) oil, Capryol 90; surfactant/cosurfactant, Cremophor RH40/Carbitol = 1:1 (*w/w*). Keys: ▤, nanoemulsion formation region; ▭, macroemulsion formation region.

obtained as the ratio of C_{out} to C_{in} at steady state was 0.16 and 0.12 for trials 1 and 2, respectively. This result clearly indicates that N-251 should be highly extracted by the liver. The mechanisms for this elimination have not been elucidated yet and studies for clarification are under way. Biliary excretion of N-251 as unchanged was almost negligible since the biliary excretion rate was ca 2.32 ng/min, which was less than 0.1% of hepatic elimination rate of N-251. F_h values obtained were very low compared with the bioavailability obtained in the oral absorption study (Table III), indicating that hepatic elimination should be saturated in the case of oral absorption studies while it was impossible to perform the liver perfusion study at higher concentration of N-251 due to the low solubility of N-251.

Table II Compositions and Particle Sizes of MC-SNEDDS Formulations

Ingredients	MC-SNEDDS		
	A	B	C
N-251 (mg)	68	68	68
Capryol 90 (g)	0.30	0.20	0.30
Cremophor EL (g)	0.35	0.27	–
Cremophor RH40 (g)	–	–	0.35
Carbitol (g)	0.35	0.53	0.35
Mean particle size (nm)	31.8 ± 0.33	33.5 ± 0.58	30.9 ± 0.54
Polydispersity index	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.03

Mean particle sizes and polydispersity indexes are expressed as the mean ± S.E. of 3 experiments

Preparation of LC-SNEDDS Formulation

Since it was found that the hepatic first-pass elimination should be limiting the bioavailability of N-251 which was lower than expected, we thought that the hepatic first-pass elimination must be attenuated to enhance the bioavailability of N-251 more. Long-chain fatty acids and their glycerides are well known to be transported to the lymphatic system after absorbed from the small intestine (29). It was also reported that lipids with long-chain fatty acids promote the transport of drugs into the lymphatic system more effectively than those with short- and/or medium-chain fatty acids (15–17).

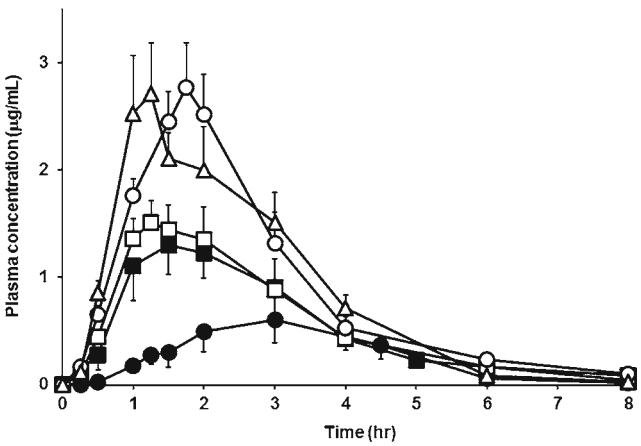

Fig. 3 Plasma concentration - time profiles of N-251 after oral administration as MC-SNEDDS formulations into rats. N-251 was orally administered at 68 mg/kg. Results are expressed as the mean with S.E. of 6 to 9 experiments. Keys: ●, Powder; ■, PEG400; △, MC-SNEDDS A; ○, MC-SNEDDS B; □, MC-SNEDDS C.

Table III Pharmacokinetic Parameters of N-251 After Oral Administration as Powder and MC-SNEDDS into Rats

Formulations	Pharmacokinetic parameters			
	T_{max} (hr)	C_{max} (μg/mL)	AUC (μg/mL·hr)	F
Powder	2.75 ± 0.48	0.69 ± 0.18	2.06 ± 0.69	0.14 ± 0.05 (1.00)
PEG400	2.17 ± 0.38	1.58 ± 0.28	4.16 ± 0.61	0.28 ± 0.04 (2.00)
SNEDDS A	1.56 ± 0.23*	1.83 ± 0.29	4.54 ± 0.83	0.30 ± 0.06 (2.20)
SNEDDS B	1.67 ± 0.06*	2.88 ± 0.36**	7.42 ± 0.83**	0.49 ± 0.06** (3.62)
SNEDDS C	1.29 ± 0.07**	2.93 ± 0.48**	7.05 ± 1.00**	0.47 ± 0.07** (3.42)

T_{max} and C_{max} were observed values. AUC was calculated from 0 to 8 h by trapezoidal rule. F, bioavailability, was expressed with the ratio to that of powder in parentheses. AUC of N-251 after intravenous administration was 0.66 ± 0.02 μg/mL·h (Dose = 3 mg/kg, $n = 8$). Dose of N-251 was 68 mg/kg. Results are expressed as the mean with S.E. of 6 to 9 experiments. **, $p < 0.01$; *, $p < 0.05$, compared with powder

Furthermore, SNEDDS/SMEDDS formulations composed of lipids with long-chain fatty acids were reported to result in the lymphatic transport of larger amount of drugs than those with medium-chain fatty acids (30,31). Therefore, long-chain fatty acid-based lipids were alternatively used to prepare SNEDDS formulations for N-251 to avoid the hepatic first-pass elimination.

Based on the results of N-251 solubility (Table I), olive oil, Rheodol MO-60 with very low value of HLB and Carbitol were chosen as an oil, surfactant and co-surfactant components, respectively. However, it was difficult to find out formulations to provide suitable SNEDDS since olive oil mainly composed of oleic acid as a long-chain fatty acid (24), of which the solubilizing ability and self-emulsifying ability are lower than those of medium-chain fatty acid-based lipids (27,32), was employed. Since oil in water (*o/w*) nano- and/or micro-emulsions are usually formed by using emulsifiers with HLB values ranged between 8 – 18 and the usage of a low and high HLB surfactant leads to the formation of a stable nano- and/or micro-emulsion (32), we tried to utilize Cremophor RH40 with high HLB in addition to Rheodol MO60 with low HLB. Ethanol was also needed as a co-surfactant. After making many pseudo-ternary phase diagrams, three formulations were found to provide relatively large region forming nanoemulsion of N-251. Then, LC-SNEDDS D, E and F were chosen as promising formulations for *in vivo* use because they provided nanoemulsion in the wide range of dilution ratio by water (Fig. 4 and Table IV). Being different from MC-SNEDDS formulations, gel and gel-like emulsion formation regions were found in all the three formulations of LC-SNEDDS (Fig. 4), but nanoemulsions providing fine particles with monodispersity were obtained by the dilution with water at the ratio of SNEDDS to water = 1 : 6.4 (*w/w*) (Table IV).

Further Improvement of Oral Absorption Kinetics by LC-SNEDDS Formulations

Then, we examined the *in vivo* performance of LC-SNEDDS D, E and F formulations. Figure 5 and Table V show the plasma concentration – time profiles of N-251 and obtained pharmacokinetic parameters, respectively. Olive oil solution, examined for the comparison, was found to give the AUC value of N-251 almost equivalent to that for MC-SNEDDS B, although the absorption rate was slower than that for MC-SNEDDS B. On the other hand, all the three preparations of LC-SNEDDS tended to show the further improvement of N-251 bioavailability, particularly, LC-SNEDDS E and F increased the value by 34% compared with MC-SNEDDS B, the best formulation of MC-SNEDDS (Fig. 3 and Table III). Compared with olive oil solution, was shown the clear tendency that N-251 was absorbed faster and greater from the LC-SNEDDS formulations (Fig. 5 and Table V). The higher C_{max} and earlier T_{max} for LC-SNEDDS formulations than those for olive oil solution would be attributed to the more efficient and rapid formation of nanoemulsion and to the subsequent faster absorption of N-251 for LC-SNEDDS.

Then, the inter-individual variation in absorption behavior was evaluated by calculating the coefficients of variation (CV) of T_{max}, C_{max} and AUC for all the preparations examined in the present study (Fig. 6). It should be noted that oral dosing of powders resulted in highly variable absorption behavior as shown by large CV values of C_{max} and AUC. On the other hand, LC-SNEDDS F gave almost the lowest values of CV for all the three parameters, although MC-SNEDDS B and C provided the CV values of T_{max} lower than that for LC-SNEDDS F. The result clearly indicates that LC-SNEDDS F formulation markedly attenuated the variation in absorption behavior compared with powder. It was also found that the C_{max} values of N-251 were the least variable after oral dosing of LC-SNEDDS F formulation among all the preparations examined. Results from the *in vivo* absorption study clearly indicated that the LC-SNEDDS F most successfully improved both the rate and extent of bioavailability of N-251 with the most stable absorption kinetics. We also separately

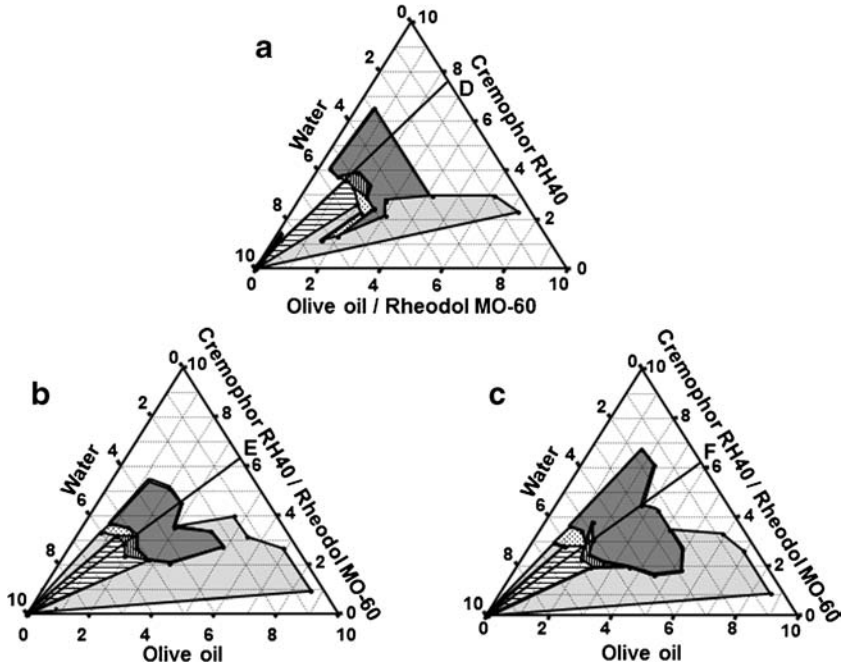

Fig. 4 Pseudo-ternary phase diagrams indicating efficient self-nanoemulsification regions. (**a**) oil/surfactant, Olive oil/Rheodol MO-60 = 1:1 (*w/w*); surfactant, Cremophor RH40; Ethanol, a co-surfactant, was contained at 15% *w/w*. (**b**) oil, Olive oil; surfactant, Cremophor RH40/Rheodol MO-60 = 2:1 (*w/w*); Ethanol, a co-surfactant, was contained at 10% w/w. (**c**) oil, Olive oil; surfactant, Cremophor RH40/Rheodol MO-60 = 2:1 (*w/w*); Carbitol, a co-surfactant, was contained at 10% w/w. Keys: ▤, nanoemulsion region; ▭, macroemulsion region; ▥, gel-like nanoemulsion region; ▨, gel-like macroemulsion region; ▬, gel region; ■, precipitation region.

confirmed that only 3.40 ± 1.68% (n = 4) of dose remained in the gastrointestinal tract at 8 h after oral dosing of LC-SNEDDS F, suggesting that N-251 should be almost completely absorbed. The enhanced permeability by SNEDDS preparations might be one of the reasons for the almost complete absorption of N-251. Although the effect of transport *via* paracellular route would be quite limited because of high lipophilicity of N-251, the effect of SNEDDS formulation on the several mechanisms including tight junction, membrane fluidity and transporters such as P-gp would be one of our next issues to be examined.

Table IV Composition of LC-SNEDDS Formulations and Mean Particle Size

Ingredients	LC-SNEDDS		
	D	E	F
N-251 (mg)	68	68	68
Olive oil (g)	0.13	0.27	0.27
Cremophor RH40 (g)	0.59	0.42	0.42
Rheodol MO-60 (g)	0.13	0.21	0.21
Ethanol (g)	0.15	0.10	–
Carbitol (g)	–	–	0.10
Mean particle size (nm)	23.0 ± 0.90	32.3 ± 0.35	31.2 ± 0.09
Polydispersity index	0.18 ± 0.06	0.18 ± 0.02	0.10 ± 0.01

Mean particle sizes and polydispersity indexes are expressed as the mean ± S.E. of 3 experiments

Effect of Lymphatic Transport on Improved Bioavailability by LC-SNEDDS

Since LC-SNEDDS preparations improved the bioavailability of N-251 compared with MC-SNEDDS, the lymphatic

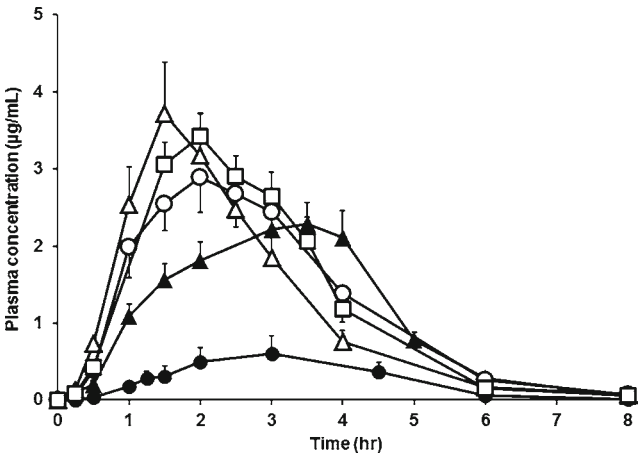

Fig. 5 Plasma concentration - time profiles of N-251 after oral administration as LC-SNEDDS formulations into rats. N-251 was orally administered at 68 mg/kg. Results are expressed as the mean with S.E. of 6 to 9 experiments. Keys: ●, Powder; ▲, Olive oil; △, LC-SNEDDS D; ○, LC-SNEDDS E; □, LC-SNEDDS F. Data of powder were cited from Fig. 3 for comparison with LC-SNEDDS preparations.

Table V Pharmacokinetic Parameters of N-251 After Oral Administration as Olive Oil or LC-SNEDDS into Rats

Formulations	Pharmacokinetic parameters			
	T_{max} (hr)	C_{max} (μg/mL)	AUC (μg/mL·hr)	F
Olive oil	3.11 ± 0.36	2.48 ± 0.25**	8.37 ± 0.73**	0.56 ± 0.05** (4.05)
LC-SNEDDS D	1.64 ± 0.14††	3.86 ± 0.60**,†	9.20 ± 1.05**	0.61 ± 0.07** (4.46)
LC-SNEDDS E	2.57 ± 0.38	3.41 ± 0.39**	9.80 ± 0.85**	0.65 ± 0.06** (4.76)
LC-SNEDDS F	2.28 ± 0.17	3.75 ± 0.21**,†	9.77 ± 0.78**	0.65 ± 0.05** (4.74)

T_{max} and C_{max} were observed values. AUC was calculated from 0 to 8 h by trapezoidal rule. F, bioavailability, was expressed with the ratio to that of powder in parentheses. AUC of N-251 after intravenous administration was 0.66 ± 0.02 μg/mL·h (Dose = 3 mg/kg, $n = 8$). Dose of N-251 was 68 mg/kg. Results are expressed as the mean ± S.E. of 6 to 9 experiments. **, $p < 0.01$, compared with powder (Table III). ††, $p < 0.01$; †, $p < 0.05$, compared with Olive oil

transport of N-251 after oral administration was evaluated as a possible mechanism for the improvement of bioavailability by LC-SNEDDS. Figure 7 shows the mesenteric lymph node concentration – time profiles of N-251 after oral administration of LC-SNEDDS F and MC-SNEDDS B. Significantly higher maximum concentration (LC-SNEDDS F, 42.3 μg/g; MC-SNEDDS B, 14.9 μg/g) and larger value of AUC (LC-SNEDDS F, 181.5 μg/g·hr; MC-SNEDDS B, 56.9 μg/g·hr) were observed for LC-SNEDDS F than MC-SNEDDS B. The results clearly indicate that LC-SNEDDS composed of long-chain fatty acid-based lipids significantly promotes the lymphatic transport of N-251 after the uptake from the small intestine, compared with MC-SNEDDS composed of medium-chain fatty acid-based lipids. Charman and Stella have suggested that drugs with logP value in excess of 5 and solubility in triglyceride of long-chain fatty acid in excess of 50 mg/mL are likely to be transported to the lymphatic system (33). Since N-251 meets the pre-requisites for preferential lymphatic transport described above, it would be transported into the lymphatic system and actually its substantial lymphatic transport was observed even after oral dosing as MC-SNEDDS B (Fig. 7). Caliph *et al.* (17) clearly indicated that the lymphatic transport of halofantrine, a lipophilic drug, increased with the increase in chain length of the lipid of vehicle used for oral dosing. Figure 7 clearly indicates that it was also the case for N-251.

T_{max} values in plasma concentration – time profiles observed relatively later for LC-SNEDDS than MC-SNEDDS (Figs. 3 and 5, Tables III and V) would be partly attributed to the larger amounts of N-251 reached the systemic circulation *via* lymphatic system for LC-SNEDDS. Since the flow rate of intestinal lymph is approximately 1/500 of that of portal blood (36), it would need more time for N-251 molecules *via* intestinal lymph to reach the systemic circulation than those *via* portal vein after absorbed.

As for the improvement of bioavailability, it was reported that the bioavailability of several drugs such as vitamin D3, probucol and halofantrine was significantly higher after oral dosing as a long-chain triglyceride solution than that after dosing as a medium-chain triglyceride solution, but vitamin E, griseofulvin and progesterone were reported to show the opposite way (35). On the other hand, there was no significant

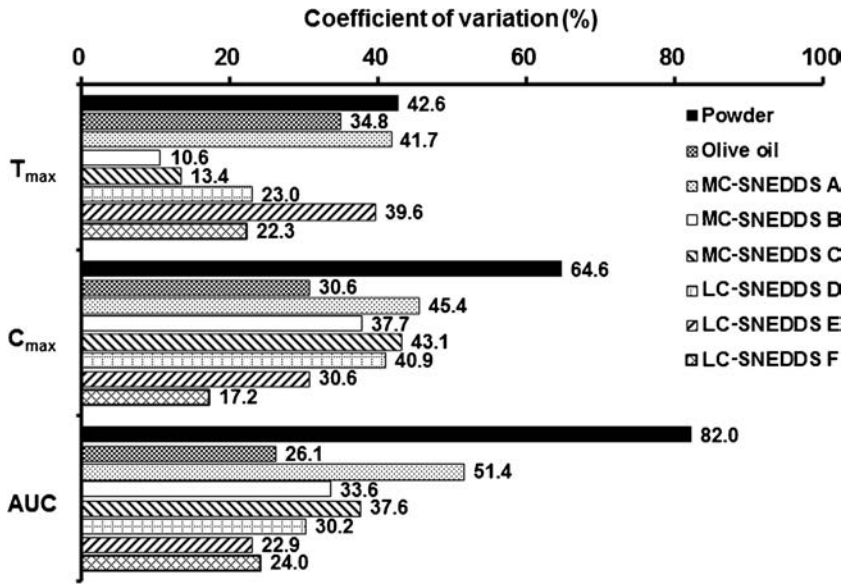

Fig. 6 Decrease in variance of absorption behavior by SNEDDS formulations. Coefficients of variation were calculated from the results obtained in the *in vivo* oral absorption studies (Figs. 3 and 5, Tables III and V).

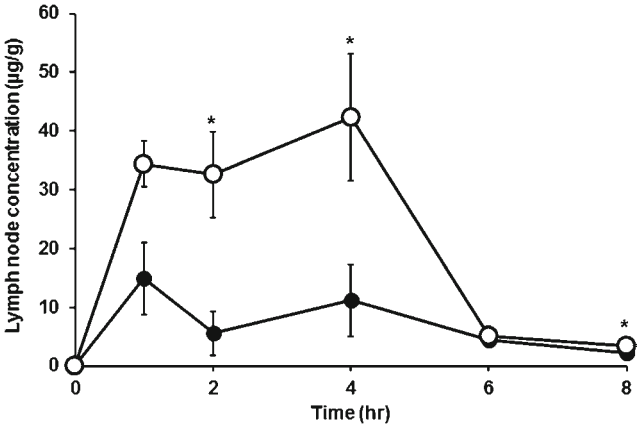

Fig. 7 Lymph node concentration - time profiles of N-251 after oral administration as MC-SNEDDS B and LC-SNEDDS F formulations into rats. N-251 was orally administered at 68 mg/kg. Results are expressed as the mean with S.E. of 3 to 6 experiments. Keys: ●, MC-SNEDDS B; ○, LC-SNEDDS F. *, $p < 0.05$, compared with MC-SNEDDS B.

difference in bioavailability of seocalcitol and dexamethasone between long- and medium-chain triglyceride formulations (35). Therefore, it seems that determining factors for the improvement of bioavailability would be dependent on drugs and vehicles examined. In the case of N-251, the larger contribution of lymphatic absorption from LC-SNEDDS would be responsible for the larger bioavailability of N-251 compared with MC-SNEDDS. Considering its high hepatic extraction, the avoidance of hepatic first-pass elimination by lymphatic transport would be a significant impact on the bioavailability of N-251 after oral dosing. Caliph *et al.* has also suggested that systemic pharmacokinetics of lipophilic drugs might be influenced by lipoprotein concentrations in plasma enhanced by oral dosing of long-chain fatty acid-based vehicle (17,36), but the possibility remains to be clarified for N-251.

Non-Linearity in Bioavailability of N-251 After Oral Dosing

Considering the values of bioavailability obtained by the *in vivo* absorption study (Tables III and V) and the values of hepatic availability obtained by liver perfusion study, the hepatic first-pass elimination must have been saturated in the *in vivo* absorption study. Because of the low water-solubility of N-251, it was not possible to perform the liver perfusion study by employing the concentration higher than 10 μg/mL of N-251. To confirm the saturation of hepatic extraction, then, *in vivo* oral absorption studies were performed at additional two doses (8 and 20 mg/kg) lower than 65 mg/kg for LC-SNEDDS F formulation. Bioavailability values calculated for 8 and 20 mg/kg were 0.31±0.03 and 0.34±0.05, respectively, which were significantly lower than that for 68 mg/kg, 0.65 ±0.05 (Table V) ($p<0.01$), suggesting that some process during the absorption of N-251 should be saturable. Considering the very low value of hepatic availability observed in the liver perfusion study, the hepatic first-pass elimination would have been saturated at high dose (68 mg/kg).

Simulation of Quantitative Contribution of Lymphatic Transport to Bioavailability of N-251

Considering the non-linear hepatic first-pass elimination and lymphatic transport observed (Fig. 7), we tried to quantitatively estimate the contribution of lymphatic transport to bioavailability of N-251. Bioavailability (F) of drugs absorbed *via* portal vein after oral administration is usually expressed as the following equation:

$$F = Fa \times Fg \times Fh \quad (2)$$

where Fa, Fg and Fh mean fraction of dose absorbed, gastrointestinal availability and hepatic availability, respectively. The Eq. (2) is based on the general concept of oral absorption as follows: Drugs are absorbed into the intestinal epithelial cells at Fa, and the absorbed drugs (Fa) are transported to the portal vein at Fg as unchanged. Finally, drugs entered the liver (Fa x Fg) can reach the systemic circulation at Fh. On the other hand, drug molecules absorbed *via* lymphatic system avoid the hepatic extraction. Therefore, the bioavailability of drugs absorbed *via* lymphatic system is expressed as the following equation by removing Fh from the Eq. (2):

$$F = Fa \times Fg \quad (3)$$

Since the route of lymphatic or portal vein transport of drugs is determined after drugs are uptaken into the intestinal epithelial cells, the bioavailability of drugs at least partly transported into lymphatic system can be described by the following Eq. (4), given that the ratios of lymphatic transport and portal vein transport of drugs absorbed into the intestinal epithelial cells are "a" and "b" (a+b=1):

$$F = a \times Fa \times Fg + b \times Fa \times Fg \times Fh \quad (4)$$

Since the ratio of lymphatic transport of drugs was reported to be dependent on the nature of oils used (15–17,30,31,37), the ratios were also independently defined as a^F and a^B for LC-SNEDDS F and MC-SNEDDS B, respectively. In the case of N-251, Fa can be assumed to be unity because N-251 was considered to be almost completely absorbed as described above. Since long-chain fatty acid-based lipids were suggested to inhibit the metabolism of drugs in the enterocytes (34,38), Fg^F and Fg^B were defined independently for LC-SNEDDS F and MC-SNEDDS B, respectively.

However, both Fg and "a" were assumed to be linear in terms of dose of drugs for the sake of ease.

LC-SNEDDS F – Low Dose (8 mg/kg)

Then, F of N-251 can be expressed when administered as LC-SNEDDS F at low doses by the following Eq. (5):

$$F^{F}_{low} = a^{F} \times Fg^{F} + b^{F} \times Fg^{F} \times Fh_{low} \quad (5)$$

Since F^{F}_{low} is 0.31 (Dose 8 mg/kg) and Fh_{low} is 0.14, Fg^{F} can be described by the Eq. (6):

$$Fg^{F} = \frac{0.31}{a^{F} + b^{F} \times 0.14} \quad (6)$$

LC-SNEDDS F – High Dose (68 mg/kg)

F of N-251 at high dose (LC-SNEDDS F) can be described by the following Eq. (7):

$$F^{F}_{high} = a^{F} \times Fg^{F} + b^{F} \times Fg^{F} \times Fh^{F}_{high} \quad (7)$$

where F^{F}_{high} is 0.65 (Fig. 5 and Table V; Dose 68 mg/kg). Then, Fh^{F}_{high} can be expressed by the Eq. (8):

$$Fh^{F}_{high} = \frac{0.65 - a^{F} \times Fg^{F}}{b^{F} \times Fg^{F}} \quad (8)$$

MC-SNEDDS B – High Dose (68 mg/kg)

Next, the bioavailability of N-251 can be described by the Eq. (9) when it was orally administered as MC-SNEDDS B at 68 mg/kg.

$$F^{B}_{high} = a^{B} \times Fg^{B} + b^{B} \times Fg^{B} \times Fh^{B}_{high} \quad (9)$$

where superscript B means parameters for MC-SNEDDS B. F^{B}_{high} is 0.49 (Fig. 3 and Table III; Dose 68 mg/kg). Then, Fh^{B}_{high} can be shown by the Eq. (10):

$$Fh^{B}_{high} = \frac{0.49 - a^{B} \times Fg^{B}}{b^{B} \times Fg^{B}} \quad (10)$$

Based on the AUC values of lymph node concentrations of N-251 (Fig. 7), the ratio of lymphatic transport for LC-SNEDDS F, a^{F}, can be expressed by using the ratio of lymphatic transport for MC-SNEDDS B, a^{B}, as follows:

$$a^{F} = a^{B} \times \frac{AUC^{F}_{lymph}}{AUC^{B}_{lymph}} = a^{B} \times \frac{181.5}{59.9} = a^{B} \times 3.19 \quad (11)$$

Taken the equations described above together, Fg^{F} and Fh^{F}_{high} can be described as a function of a^{F} as follows:

$$Fg^{F} = \frac{0.31}{a^{F} + (1 - a^{F}) \times 0.14} \quad (12)$$

$$Fh^{F}_{high} = \frac{0.65 - a^{F} \times Fg^{F}}{(1 - a^{F}) \times Fg^{F}} \quad (13)$$

Utilizing Eqs. (12) and (13), the simulation was performed to estimate the probable contribution of lymphatic transport to the bioavailability of N-251 after oral administration as LC-SNEDDS F (Fig. 8). Since F^{F}_{high} is 0.65 (Table V), Fg^{F} should range from 0.65 to unity, which resulted in the value of a^{F} ranging from 0.20 to 0.39. This result means that a^{B} should range from 0.06 to 0.12 at the same time. Subsequently, it was suggested that Fh^{F}_{high} should be from 0.56 to 0.99.

In the case of MC-SNEDDS B, Fh^{B}_{high} can be described by the Eq. (14):

$$Fh^{B}_{high} = \frac{0.49 - \frac{a^{F}}{3.19} \times Fg^{B}}{\left(1 - \frac{a^{F}}{3.19}\right) \times Fg^{B}} \quad (14)$$

where Fg^{B} is an independent variable and Fh^{B}_{high} was calculated as a function of a^{F} by using arbitral values of Fg^{B} between 0.49 and unity (Fig. 8). Since the amount of N-251 transported to the liver is considered to be larger for MC-SNEDDS B than that for LC-SNEDDS F based on the result shown in Fig. 7, Fh^{B}_{high} would be equal to or smaller than Fh^{F}_{high}, which narrowed probable values of Fh^{B}_{high} to be between 0.49 and 0.84.

Figure 9 summarizes the results of simulation performed above. Based on the observed data and several hypotheses, the contributions of lymphatic transport to the bioavailability of N-251 were suggested to be 30–38% and 6–12% for LC-SNEDDS F and MC-SNEDDS B, respectively. This result confirmed that the lymphatic transport of N-251 enhanced by LC-SNEDDS F resulted in the improved bioavailability.

Since the bioavailability of N-251 was successfully improved by LC-SNEDDS F formulation in the present study, we will investigate the details of absorption mechanisms including the release kinetics of N-251 from nanoemulsion and

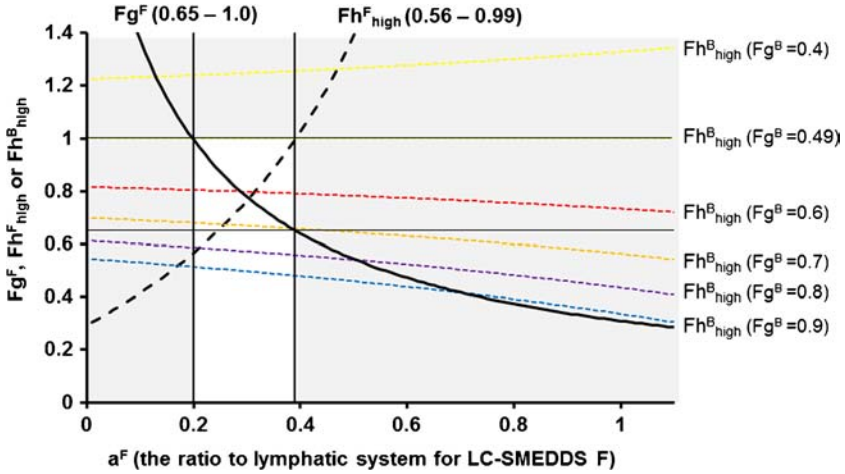

Fig. 8 Simulation for estimating absorption behavior of N-251 orally administered as SNEDDS formulations. Simulation was performed based on the results of *in vivo* absorption studies (Figs. 3, 5 and 7) and isolated liver perfusion study by utilizing Eqs. (11)–(13). Fg^F and Fg^B mean the gastrointestinal availability for LC-SNEDDS F and MC-SNEDDS B, respectively. Fh^F_{high} and Fh^B_{high} are hepatic availability at high dose for LC-SNEDDS F and MC-SNEDDS B, respectively. a^F represents the ratio of lymphatic transport of N-251 absorbed into the intestinal epithelial cells after oral dosing of LC-SNEDDS F formulation.

the effect of oil species and their amounts on the bioavailability of N-251 in our next project.

In conclusions, LC-SNEDDS F composed of long-chain fatty acid-based lipids was able to increase the oral

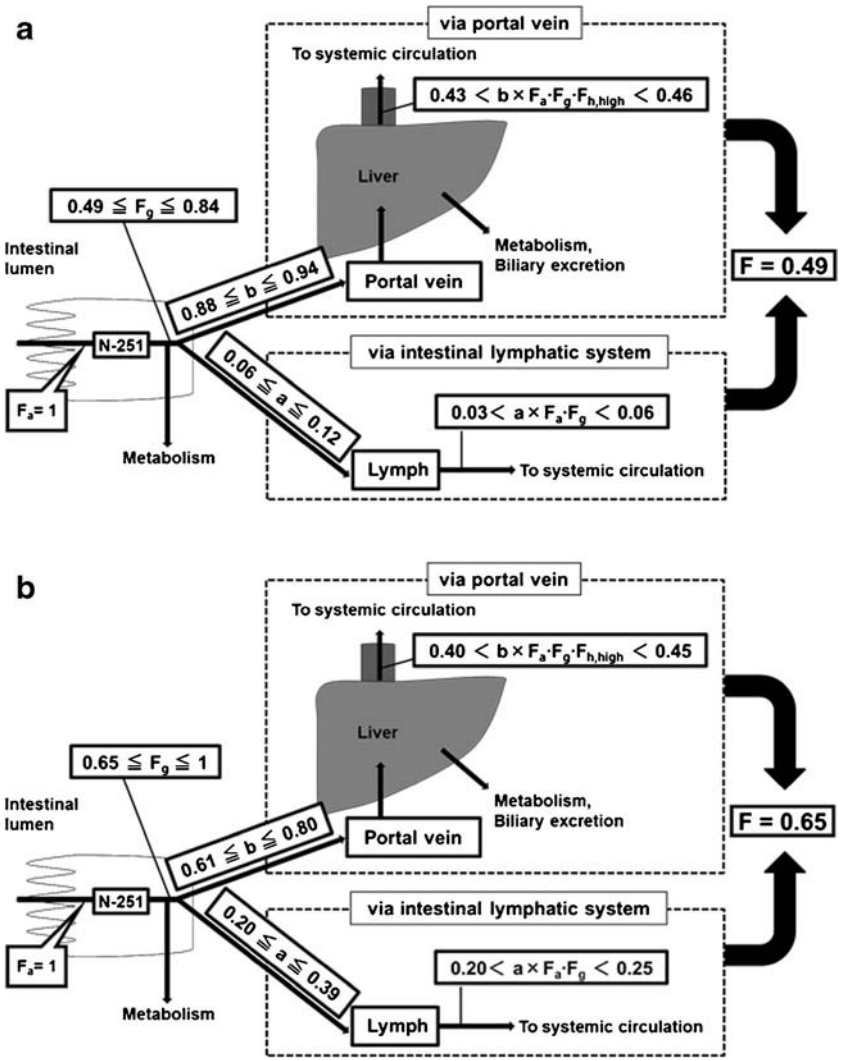

Fig. 9 Schematic summary of N-251 absorption after oral administration as MC-SNEDDS B (**a**) and LC-SNEDDS F (**b**) formulations. "*a*" and "*b*" indicate the ratios of lymphatic transport and portal vein transport of drugs absorbed into the intestinal epithelial cells, respectively. Fa and Fg mean fraction of dose absorbed and gastrointestinal availability, respectively. Fh represents hepatic availability at high dose of N-251.

bioavailability of N-251, a novel antimalarial and poorly water-soluble drug. The improvement of bioavailability by LC-SNEDDS F would be attributed to the lymphatic transport of larger amount of N-251, of which the quantitative contribution to the improved bioavailability was successfully estimated by the simulation study.

ACKNOWLEDGMENTS AND DISCLOSURES

This study was partially supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (Project No. 04–09 and 09–21, PI; Y.W.), Grant-in-Aid for Scientific Research (B) (25305008, H.-S.K.), and Grant-in-Aid for Scientific Research (C) (22590099, H.-S.K.) from the Ministry of Education, Culture, Sports, Science and Technologies, Japan.

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