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RESEARCH PAPER

# Improved Oral Bioavailability of BCS Class 2 Compounds by Self Nano-Emulsifying Drug Delivery Systems (SNEDDS): The Underlying Mechanisms for Amiodarone and Talinolol

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## ABSTRACT

***Purpose*** Superior bioavailability of BCS Class 2 compounds incorporated into SNEDDS was previously reported. This study aims to elucidate the underlying mechanisms accountable for this phenomenon.

***Methods*** SNEDDS of amiodarone (AM) and talinolol were developed. Pharmacokinetic parameters were assessed *in vivo*. Effect on intestinal permeability, P-gp efflux and toxicity was evaluated *in vitro* (Caco-2) and *ex vivo* (Ussing). Solubilization was assessed *in vitro* (Dynamic Lipolysis Model). Effect on intraenterocyte metabolism was evaluated using CYP3A4 microsomes.

***Results*** Oral administration of AM-SNEDDS and talinolol-SNEDDS resulted in higher and less variable AUC and Cmax. *In vitro*, higher talinolol-SNEDDS Papp indicated Pgp inhibition. Lipolysis of AM-SNEDDS resulted in higher AM concentration in the fraction available for absorption. Incubation of AM-SNEDDS with CYP3A4 indicated CYP inhibition. SNEDDS didn't alter mannitol Papp and TEER. SNEDDS effect was transient.

***Conclusions*** Multiple mechanisms are accountable for improved bioavailability and reduced variability of Class-2 compounds by SNEDDS: increased solubilization, reduced intraenterocyte metabolism and reduced P-gp efflux. SNEDDS effect is reversible and doesn't cause intestinal tissue or cell damage. These comprehensive findings can be used for intelligent selection of drugs for which oral bioavailability will improve upon incorporation into SNEDDS, based on recognition of the drug's absorption barriers and the ability of SNEDDS to overcome them.

**KEY WORDS** first pass metabolism · intestinal absorption · intestinal solubilization · intra-enterocyte metabolism · lipophilic drugs · P-gp efflux · self emulsifying drug delivery systems

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## ABBREVIATIONS

| | |
|---|---|
| AM | Amiodarone |
| AUC | Area under the plasma drug concentration vs. time curve |
| BCS | Biopharmaceutics Classification System |
| BDDCS | Biopharmaceutics Drug Disposition System Classification |
| $Cl_{tot}$ | Total body clearance |
| $C_{max}$ | Maximal concentration of drug in plasma |
| CsA | Cyclosporine A |
| GFJ | Grapefruit Juice |
| GI | Gastrointestinal |
| LDH | Lactate Dehydrogenase |
| P-gp | P-glycoprotein |
| PK | Pharmacokinetics |
| SNEDDS | Self Nano-Emulsifying Drug Delivery System |
| TEER | Transepithelial Electrical Resistance |
| $T_{max}$ | Time to reach maximal concentration of drug in plasma |
| $V_{ss}$ | Volume of distribution at steady state |

## INTRODUCTION

Many of the newly discovered drug candidates, as well as existing drugs, exhibit limited water solubility and are associated with poor oral absorption and highly variable bioavailability (1). Most of these compounds have high intestinal permeability and therefore categorized as Class 2 of the Biopharmaceutical Classification System (BCS) proposed by Amidon *et al.* (2).

The use of lipid and surfactant based formulations is one of the most popular up to date approaches to overcome the limited and unpredicted oral bioavailability of poorly water soluble compounds (3). SNEDDS is an isotropic mixture of an active compound in a combination of lipids, surfactants

and co-solvent that upon gentle agitation in aqueous phase, such as the upper GI lumen content, spontaneously forms drug encapsulated O/W microemulsion with a particle diameter of 50 nm or less (4). The drug encapsulated O/W emulsion formed can be either a true thermodynamically stable micro-emulsion or a kinetically stable nano-emulsion (5).

SNEDDS of cyclosporine A (CsA) previously developed by our group (6) resulted in increased bioavailability in humans. Other SNEDDS were shown to increase bioavailability of additional poorly water soluble compounds, such as ontazolast (7), halofantrine (8) and danazol (9), compared to solid dosage forms. In some cases, SNEDDS utilization even enabled decreased influence of food effect (10) and bile secretion on oral bioavailability, and reduced the intra and inter patient variability in plasma concentrations profile and bioavailability(11).

Traditionally, the increased oral bioavailability achieved by incorporation of the active compound into SNEDDS was attributed to the increased solubility of the compound in the GI milieu, implying that the absorption enhancement could be explained by the increase in effective luminal drug concentration(3,12).

However, it should be emphasized, that aside from low water solubility, there are several additional common barriers for oral absorption of BCS Class 2 molecules. In their Biopharmaceutics Drug Disposition Classification System (BDDCS), Wu and Benet (13) showed that GI efflux transporter effects predominate for Class 2 compounds. The high permeability of the compounds limited by their low solubility yields low drug concentration upon its entrance to the enterocyte, and thus prevents the saturation of the efflux transporters and metabolic enzymes. Moreover, transporter-enzyme interplay in the enterocyte is extremely important for Class 2 compounds that are substrates for CYP3A enzymes and P-gp efflux transporter.Such interplay, which results in overall low systemic bioavailability following oral administration, occurs at the enterocyte level, prior to absorption to the portal circulation and hepatic first pass metabolism. It can be termed as intestinal first pass metabolism, a phenomenon which contribution to low oral bioavailability was previously not fully recognized.

While in cases of griseofulvin (14,15), vitamin A (16) and other solubility limited compounds it is reasonable to attribute the increased bioavailability following incorporation of the drug into SNEDDS to improved solubilization, in cases of extensive pre-systemic (and especially intestinal) metabolism substrates, such as CsA (6,17), tacrolimus (18), simvastatin *etc.*, enhancement of solubility is probably not the only mechanism behind the improved bioavailability. For example, CsA, a Class 2 P-gp and CYP3A substrate did not have a permeability difficulty and was absorbed at least 86% when solubilized in the previously commercially available lipid based formulation (Sandimmune®), but underwent a marked intraenterocyte first pass extraction of 60% or more (19). Thus, the newer S(N)EDDS CsA formulations, like the one developed by our group (6) and others (17), characterized by higher and more predictive bioavailability, probably affected not only the solubilization of the compound, but additional absorption barriers.

Moreover, several recent studies demonstrated that the solubilization capacity of SNEDDS decreases following lipolysis process, mainly due to the fact that the nano-vesicle structures do not remain intact following lipolysis, but rather become a part of mixed micelles formed by bile salts, phospholipids and other GI composites (20). Furthermore, some of the excipients that compose SNEDDS undergo digestion that leads to significant loss of solubilization capacity (21).

Hence, despite numerous reports of improved bioavailability of BCS Class 2 compound following incorporation into SNEDDS, the exact mechanism behind this phenomenon at the intestinal absorption level remains a "black box" and its systematic investigation and revelation is the rationale of current study. The purposed mechanisms that could contribute to increased oral bioavailability of Class 2 molecules and that were assessed in current study occur at different stages of orally administered drug absorption process: 1. Enhanced solubilization in the GI milieu; 2. Increased trans and paracellular permeability 3. Inhibition of the intra-enterocyte P-gp efflux pumps; and 4. Inhibition of intra-enterocyte metabolism by CYP 3A enzymes. The aim of our study was to methodically assess each of the purposed absorption barriers and to determine whether the use of SNEDDS affects them. For that purpose we selected strategy of utilization of multiple *in vivo*, *ex vivo* and *in vitro* models that can isolate and separately evaluate each of the mechanisms under investigation.

In addition, cell and tissue toxicity potential of SNEDDS was assessed, since several studies attributed the increased bioavailability following SNEDDS utilization to its toxic effect on the tissue and cell membrane integrity, which resulted in increased intestinal transport of the drug (22,23).

Amiodarone (AM) - an antiarrhythmic BCS Class 2 (24) compound with complex pharmacokinetic profile and narrow therapeutic range (25) was selected as a model compound to be incorporated into SNEDDS. In addition to low water solubility, it is characterized by an erratic and unpredictable oral absorption that is mainly mediated by intestinal wall metabolism *via* CYP3A4 and gastrointestinal excretion mediated by P-gp (26). Taken together, these characteristics make AM suitable model molecule for assessment of the SNEDDS effects on absorption barriers and bioavailability.

Talinolol is a β1 selective adrenergic blocker which biotransformation in humans is negligible (less than 1%) (27). Furthermore, PK/PD model developed by Tubic *et al.* indicated talinolol to undergo nonlinear drug absorption due to P-gp mediated intestinal secretion (28). Thus, using talinolol as model drug assessment of SNEDDS effects on P-gp mediated efflux pumps activity can be investigated

without an interference with metabolism by CYP 3A4 enzymes *in vivo* and *in vitro*.

Additionally, testosterone, a known CYP 3A substrate (29) was used for *in vitro* assessment of SNEDDS effects on CYP3A4 separately.

## MATERIALS AND METHODS

### Chemicals

All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich. Tween 20, Span 80, Cremophor RH40 and Lecithin (Epikuron 200) were obtained from Dexcel® Pharma (Or-Akiva, Israel). All solvents were HPLC grade and obtained from JT Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA). Amiodacore® ampoules (Sanofi Aventis, France) were purchased from the Hadassah Medical Center pharmacy. Talinolol was kindly provided by Degussa, Essen, Germany.

### Animals

Male Wistar rats weighing 300–350 g were used for the *in vivo* PK and *ex vivo* intestinal permeability studies. The project adhered to the principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985).

### SNEDDS Preparation

AM SNEDDS was prepared by pre-concentrate preparation method. The final SNEDDS composition was based on preliminary formulation optimization studies and selected according to optimal solubilization capacity of the active ingredient and smallest particle size obtained upon dilution in aqueous phase. Initially, two kinds of mixtures were prepared. The first is a mixture of amphiphilic co-solvent (ethyl lactate) and phospholipid (lecithin) at the ratio of 8:1, respectively. The second is a mixture of a triglyceride (trilaurin), polyoxyl 40-hydroxy castor oil, Tween 20, and Span 80 at the ratio of 1:1:1:1. Both mixtures were gently stirred and heated to 40°C till homogenous solutions were formed. Both mixtures were added at the ratio of 1:1 w/w to a clean scintillation tube containing AM powder, forming the AM SNEDDS pre-concentrate containing 3% AM. This pre-concentrate was gently stirred and heated to 40°C till homogenous solution was formed. Upon gentle agitation in aqueous phase, this pre-concentrate spontaneously forms drug encapsulated O/W nano-emulsion. Amiodacre® ampoules content (50 mg/ml amiodarone HCl, 20.2 mg/mL benzyl alcohol and 100 mg/mL Tween 80 in water) was used as control AM throughout all *in vivo* and *in vitro* studies.

Talinolol SNEDDS was prepared by the same method as AM SNEDDS described above, except for the different triglyceride that was used. For the preparation of the a talinolol SNEDDS tricaprin instead of trilaurin was used, to obtain optimal clear appearance and particle size bellow 50 nm upon dilution in aqueous phase. The concentration of talinolol in the pre-concentrate was 1%.

Blank SNEDDS were prepared by the same method, except no active ingredient was added.

### SNEDDS Characterization

#### *Particle Size and ζ Potential*

Particle size and ζ potential were determined using Zetasizer Nano ZS ZEN 3600 (Malvern Instruments Ltd, Malvern, UK). Prior to particle size and ζ potential determination 200 μl of the pre-concentrate were vortex-mixed in 1,800 μl distilled water at 37°C for 30s. The measurements were taken using Folded Capillary Cells (Malvern Instruments Ltd, Malvern, UK). Before the measurement was taken the cell was flushed through with ethanol followed by de-ionised water to facilitate wetting and cleaning of the cell. Amiodarone and talinolol SNEDDS were administered to animals following 1:10 dilution of the pre-concentrate in water. Thus, the characterizations were performed following similar dilution.

#### *SNEDDS Drug Load Assessment*

AM SNEDDS loading was assessed following 1:10 dilutions of the pre-concentrate prepared as described above. The obtained solution (400 μL) was placed into Nanosep® centrifugal devices (Pall Life Sciences, Ann Arbor, MI, US) with 30K cut-off membranes and centrifuged for 30 min at 10,000 RPM at 25°C. Additionally, AM solution (300 μg/mL) was placed inside similar centrifugal devices and centrifuged in the same manner to assess the non-specific adsorption of AM to the test-tube and membrane surface. Duplicates were used for each concentration. The original solutions and the filtrates obtained were analyzed for AM concentrations as described in "Analytical Methods" section.

The non-specific adsorption (NSA) percent was calculated as:

$$\frac{100 - \text{AM solution conc. after centrifugation} * 100}{\text{AM solution conc. before centrifugation}}$$

AM load percent was calculated as:

$$\frac{100 - \text{AM conc. after centrifugation of AM SNEDDS} * 100}{(\text{AM conc. before centrifugation of AM SNEDDS}) * (100\% - \text{NSA}\%)}$$

### *In Vivo* Studies

All surgical and experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee

of the Hebrew University Hadassah Medical School Jerusalem, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's expectations for animal care and use/ethics committees.

Male Wistar rats (Harlan, Israel), 275–300 g in weight, were used for all surgical procedures. All animals were deprived of food but not water 12 h prior to the experiments. Animals were anesthetized for the period of surgery by intra-peritoneal injection of 1 mL/kg of ketamine-xylazine solution (9:1 respectively), placed on a heated surface and maintained at 37°C (Harvard Apparatus Inc., Holliston, MA). An indwelling cannula was placed in the right jugular vein of each animal for systemic blood sampling, by a method described before (30). The cannula was tunneled beneath the skin and exteriorized at the dorsal part of the neck. After completion of the surgical procedure, the animals were transferred to metabolic cages to recover overnight (12–18 h). During this recovery period, food, but not water, was deprived. Throughout the experiment free access to food was available 4 h post oral administration. Animals were randomly assigned to the different experimental groups.

### *AM Relative Bioavailability Studies*

For bioavailability studies, dispersed AM SNEDDS was freshly prepared 30 min before each experiment, by vortex-mixing of the pre-concentrate in water (1:10 v/v) pre-heated to 37°C for 30 sec. The obtained AM concentration was 3 mg/mL. dispersed AM SNEDDS (12.5 mg/kg) was administered to the animals by oral gavage ($n=6$). The control group received 12.5 mg/kg AM solution prepared from Amiodacore® ampoules content (AM 50 mg/mL) dissolved in water to obtain 2.5 mg/mL concentration ($n=6$).

Systemic blood samples (0.35 mL) were taken at 5 min pre-dose, 0.5,1 2, 4, 8, 12, 24, 36 and 48 h post-dose. To prevent dehydration equal volumes of physiological solution were administered to the rats following each withdrawal of blood sample. Plasma was separated by centrifugation (4,000 g, 7 min, 4°C) and stored at −20°C pending analysis.

### *Talinolol Relative Bioavailability Studies*

For bioavailability studies, dispersed Talinolol SNEDDS was freshly prepared 30 min before each experiment, by vortex-mixing of the pre-concentrate in water (1:10 v/v) pre-heated to 37°C for 30 sec. The obtained talinolol concentration was 3 mg/mL. Dispersed talinolol SNEDDS (4 mg/kg) was administered to the animals by oral gavage ($n=5$). The control group received 4 mg/kg talinolol dissolved in PEG400:water:ethanol 25:60:15 to obtain 1 mg/mL concentration ($n=5$).

Systemic blood samples (0.35 mL) were taken at 5 min pre-dose, 0.5,1 2, 3,4,5,6 and 8, h post-dose. To prevent dehydration equal volumes of physiological solution were administered to the rats following each withdrawal of blood sample. Plasma was separated by centrifugation (4,000 g, 7 min, 4°C) and stored at −20°C pending analysis.

## *Ex Vivo* Permeability Study

Permeability experiments were performed in a modified Ussing chamber system (Physiological Instruments, Inc., San Diego, CA). Animals were euthanized in $CO_2$ prior to the experiment. Following a midline incision to the rat, 25 cm of small intestine was removed and placed in ice-cold Ringer bicarbonate buffer (NaCl 6.54 g, KCl 0.37 g, $CaCl_2\_2H_2O$ 0.18g, $MgCl_2\_6H_2O$ 0.24 g, $NaHCO_3$ 2.1 g, $Na_2HPO_4$ 0.23 g, $NaH_2PO_4$ 0.05g in 1,000 mL). All buffer solutions were freshly prepared and equilibrated to pH 7.4 and 290 mOsm. The jejunal portion of the small intestine (10–15 cm distal to the pylorus) was used. Peyer patches could be easily identified visually, and sections containing them were not used in these studies. The individual segments were obtained, and underlying muscularis was removed from the serosal side of the tissue before mounting. The exposed tissue surface area was 0.5 $cm^2$ and fluid volume in each half-cell was 3 mL. The system was preheated to 37°C. Modified Ringer buffers were added to the serosal and the mucosal sides (mucosal modified Ringer buffer contained 10 mM mannitol and serosal modified Ringer buffer contained 8 mM D-glucose and 2 mM mannitol). The tissue oxygenation and the solution mixing were performed by bubbling with 95% $O_2$−5% $CO_2$. The system was equilibrated for 30 min, and then the buffers were removed and replaced with 3 mL of serosal buffer on the basolateral side. Test solutions (mucosal buffer containing 100 μg/mL free AM or dispersed AM SNEDDS) added to the mucosal side of the chamber. At predetermined times (0, 30, 60, 90, 120, and 150 min) samples (150 μl) were withdrawn and replaced by blank (non containing compound) buffer to maintain sink conditions. The integrity of the epithelial tissue was monitored by measuring the transepithelial electrical resistance (TEER) throughout the experiment.

## Caco-2 *In Vitro* Studies

### *Cell Lines and Culture Conditions*

Caco-2 cells were obtained from ATCC. Cells were grown in 75 $cm^2$ flasks with approximately $0.5\times10^6$ cells/flask at 37°C in a 5% $CO_2$ atmosphere and at relative humidity of 95%. The culture growth medium consisted of DMEM supplemented with 10% heat-inactivated FBS, 1% NEAA, and 2 mM L-glutamine. The medium was replaced twice weekly.

Cells (passage 60–66) were seeded at density of $25\times10^5$ cells/cm$^2$ on untreated culture inserts of polycarbonate membrane with 0.4 μm pores and surface area of 1.1 cm$^2$. Culture inserts containing Caco-2 monolayer were placed in 12 mm transwell plates. Culture medium was replaced every other day. Transport studies were performed 21–23 days after seeding, when the cells were fully differentiated and TEER values became stable (200–500 Ω·cm$^2$). TEER was measured by Millicell ERS-2 System (Millipore inc. Billerica, MA).

### *In Vitro Permeability Studies*

For determination of SNEDDS ability to increase transcellular intestinal transport, a study was performed using AM and talinolol as a model molecules.

Caco-2 transport studies were initiated by medium removal from both sides of the monolayer followed by replacement with apical and basolateral buffers (31) prewarmed to 37°C. The cells were equilibrated for 30 min at 37°C on shaker (100 cycles/min).

*Talinolol Experimental Protocol.* Apical buffer (0.6 mL) containing 33 μg/mL talinolol or 33 μg/mL dispersed talinolol SNEDDS was added to the apical side of the monolayer in the apical to basolateral (A−B) direction studies, and 1.5 mL of basolateral buffer containing 1% w/v bovine serum albumin was added to the receiver compartment on the basolateral side of the monolayer. In the basolateral to apical (B−A) direction studies, 1.5 mL of basolateral buffer containing 33 μg/mL talinolol, or 33 μg/mL dispersed talinolol SNEDDS was added to the basolateral side of the monolayer, and 0.6 mL of apical buffer containing 1% w/v bovine serum albumin was added to the receiver compartment on the apical side of the monolayer.

*AM Experimental Protocol.* Apical buffer (0.6 mL) containing 100 μg/mL AM or 100 μg/mL dispersed AM SNEDDS was added to the apical side of the monolayer in the apical to basolateral (A−B) direction studies, and 1.5 mL of basolateral buffer containing 1% w/v bovine serum albumin was added to the receiver compartment on the basolateral side of the monolayer. In the basolateral to apical (B−A) direction studies, 1.5 mL of basolateral buffer containing 100 μg/mL AM, or 100 μg/mL dispersed AM SNEDDS was added to the basolateral side of the monolayer, and 0.6 mL of apical buffer containing 1% w/v bovine serum albumin was added to the receiver compartment on the apical side of the monolayer.

At fixed time points (0, 30, 60, 90, 120, and 150 min), 150 μL samples were withdrawn from the receiver side, and similar volumes of blank buffer were added to maintain constant volume. At the first and last time points (0 and 150 min), samples were taken from the donor side as well, in order to confirm mass balance. Samples were immediately assayed for drug content. $C^{14}$-Mannitol, a commonly used marker for passive paracellular permeability, was used for further evaluation of proper carrying out of each study

### *In Vitro paracellular transport studies*

Caco-2 permeability studies were performed with paracellular transport marker $C^{14}$-Mannitol (2 μCi/mL). Each experiment was initiated by placing dispersed blank SNEDDS (0.3%, 1%, and 3% v/v) or buffer each containing $C^{14}$-Mannitol to the apical side of the monolayer. Samples (200 μL) were withdrawn from the basolateral side at the same fixed time points described above, and similar volumes of blank buffer were added following each withdrawal. $C^{14}$-Mannitol concentrations in dispersed blank SNEDDS containing transwells were measured and compared to the $C^{14}$-Mannitol values measured in control transwells. The radioactivity (DPM) was counted by means of a liquid scintillation analyzer (Packard, Tri-carb 2900TR, USA) against a calibration curve of $R^2=0.997$.

### *Transepithelial Electrical Resistance (TEER) Studies*

Caco-2 cells were grown on 12-transwell plate and cultured for 21 days as described above. Before the experiment, Caco-2 cell monolayers were equilibrated in pre-warmed apical and basolateral buffers at 37°C for 30 min. The apical buffer was replaced with 0.6 mL pre-warmed (37°C) apical buffer containing dispersed blank SNEDDS (0.3%, 1%, and 3% v/v). In order to evaluate the effect of the dispersed SNEDDS on the paracellular transport across the cells (*i.e.* the extent to which the tight junctions are opened), TEER values were measured at the above time points and compared to the TEER values measured in control cells containing only buffer.

### *Data Analysis*

Permeability Coefficient (Papp) for each compound was calculated from the linear plot of drug accumulated *versus* time, using the following equation:

$$\text{Papp} = 1/(C_0 \times A) \times dQ/dt$$

where dQ/dt is steady state appearance rate of the drug on the receiver side, $C_0$ is the initial concentration of the drug on the donor side, and A is the exposed tissue surface area (0.5 cm$^2$ tissue area in *ex vivo* studies and 1.1 cm$^2$ in the Caco-2 experiments).

Efflux ratio was calculated by comparing Papp from the apical side of the membrane to basolateral side (A to B) to the transport in the opposite direction.

### *Cellular Toxicity Studies*

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is rapidly released into the culture supernatant when the plasma membrane is damaged. SNEDDS toxicity was assessed over a range of concentrations (0.1% to 10% in medium) in Caco-2 cells grown as described above in 24 wells plate (25×105 cells/cm2) using LDH cytotoxicity detection kit (Colonotech, CA, USA). After the incubation of the cell culture with blank SNEDDS containing medium for 2 h, the plate was centrifuged and the supernatant medium was incubated with the reaction mixture. Absorbance was measured at 490 nm using a PowerWave X340 microplate reader (Bio-Tec instruments Inc., Winooski, VT, USA).

Extent of absorption is proportional to the extent of cell damage. Percent of cell damage is calculated by the following equation:

$$Cytotoxicity(\%) = \frac{Triplicate\ absorbance - Low\ control \times 100}{High\ control - Low\ Control}$$

where low control is spontaneous LDH release measured in untreated cells medium and high control is 100% measured in cells incubated with 1% Triton X-100 containing medium.

### *In Vitro* Dynamic Lipolysis Model Studies

The procedure for the dynamic *in vitro* lipolysis experiment was based on a previous report by Sek *et al.* (32) with the fallowing modifications, in order to achieve maximum pseudophysiological conditions. The experiment medium, which was comprised of 35.5 mL of digestion buffer (50 mM tris maleate, 150 mM NaCl, 5 mM $CaCl_2$, pH=7.4) containing 5 mM taurocholic acid and 1.25 mM phosphatidylcholine (conditions mimicking fasted state GIT), was continuously stirred and maintained at 37°C. AM-SNEDDS or AM (1 mL) were then dispersed in the medium to obtain a final concentration of 22.5 μg/mL, and stirred for 15 min. Fresh pancreatin extract was prepared by adding 1 g of porcine pancreatin powder to 5 mL digestion buffer, stirring for 15 min followed by centrifugation, as previously described in the literature (33). 3.5 mL of the pancreatin extract (1,000 IU/mL) was inserted into the medium to initiate the enzymatic digestion. In order to achieve maximum pseudophysiological conditions, a pH-titrator unit was used (DL-50 Graphix, Mettler Toledo Inc., Columbus, OH, USA) to maintain the pH at 7.0 throughout the experiment. This is important since during the lipolysis process of triglycerides, free fatty acids are liberated and consequently the pH decreases. The experiment was continued for 30 min, in which time the enzymatic digestion process was completed, as indicated by the completion of the pH titration. Subsequently, the medium was ultracentrifuged (L8-55 Ultracentrifuge, SW-41 rotor, Beckman Co., Palo Alto, CA) at 40K rpm for 90 min, and a separation into three phases was received: an aqueous phase (containing bile salts, fatty acids and mono-glycerides), a lipid phase (containing undigested triglycerides and diglycerides) and sediment (containing un-dissolved drug). Following oral administration, dissolution of the drug molecule in the intestinal milieu is a prerequisite for the absorption process. According to parsimony concept we use the assumption that drug molecules solubilized in the aqueous phase of the lipolysis medium are to be available for absorption, in contrast to drug in the sediment which will practically not contribute markedly to the amount absorbed *in vivo*. Hence, drug concentration in the aqueous phase was measured.

### Enzymatic Inhibition Studies

For determination of enzymatic inhibition by SNEDDS, pooled rat CYP3A4 microsomes (BD Biosciences, Woburn, MA, USA) were used. The reaction was initiated by adding ice cold microsomes (0.5 mg/mL final concentration) to preheated phosphate buffer (0.1M, pH 7.4) containing NADPH (0.66 mg/mL) and dispersed AM-SNEDDS (2.15 μl, equivalent to AM 100 μM) or AM (100 μM). At predetermined times (30, 60, 90, and 120 min), 100 μL samples were withdrawn and the reaction was terminated by adding 100 μL ice cold ACN. The concentrations of intact AM were determined by previously described methods.

### Investigation of Duration of SNEDDS Effect on GI Absorption and Enterocyte Recovery

Freshly dispersed as described above blank SNEDDS was administered by oral gavage to the study group. The dispersed blank SNEDDS volume was equal to dispersed AM SNEDDS volume administered in the bioavailability studies. The control group was administered with water at the same time point. After 2 h 12.5 mg/kg AM solution prepared from Amiodacore® ampoules content as described above was administered to both groups. In the investigation of duration of SNEDDS effect on GI absorption of AM, the last sampling point was 12 h, assuming the effect, which was previously shown by us to occur at the absorption phase, will not last beyond this stage.

### Analytical Methods

The amount of AM in plasma and medium was determined using a high performance liquid chromatography (HPLC) system (Waters 2695 Separation Module) with a photodiode array UV detector (Waters 2996), by a method described

before with some modifications (34). Plasma or medium aliquots of 150 mL were mixed with 150 mL of ACN containing the internal standard (risperidone 10 μg/mL) and vortex-mixed for 1 min. Three milliliters of n-hexane were added, followed by 1 min vortex-mixing. After centrifugation at 1,500G for 7 min, the organic layer was transferred to fresh glass test tubes, evaporated to dryness (Vacuum Evaporation System, Labconco, Kansas City, MO) and reconstituted in 130 mL of ACN. The resulting solution (80 μl) was injected into the HPLC system. conditioned as follows: Luna, 3u CN 100A, 150*4.00 mm column (Phenomenex®, Torrance, CA), an isocratic mobile phase, acetonitrile : ammonium formiate buffer (pH 3.5) (75:25 v/v), flow rate of 1.5 mL/min at 55°C. AM and risperidone were detected at 242 and 277 nm, respectively. The range of quantification for AM was 100–0.05 μg/mL.

Talinolol concentrations were analyzed using LC-MS system comprised of Waters pump (600 controller), Waters autosampler (717 plus Auto-sampler) and Waters Micro-mass ZQ mass spectrometer (Waters corporation, Milford, MA) as previously described (35). Buffer samples (150 μL) were mixed with 200 μL NaOH (1M). Talinolol was extracted with 4 mL ethyl acetate and evaporated to dryness by vacuum evaporator. The residue was reconstituted with 70 μL of 20% acetonitrile and 80% water. The volume of injection was 20 μL. The mobile phase consisted of ACN:water (20:80) containing 0.1% (v/v) formic acid. The flow was set to 0.25 mL/min. The separation was achieved by XTerra C18 MS column (3.5 μm, 2.1×100 mm, Waters) that was kept at 35°C. Retention time for talinolol was 6.5 min. The detection mass (m/z) was 364.3. The limit of quantification was 1 ng/mL.

### PK Analysis

Noncompartmental pharmacokinetic analysis was performed using WinNonlin software (version 5.1, Pharsight, Mountain View, CA)

### Statistical Analysis

All values are expressed as mean ± standard deviation (S.D.) if not stated otherwise. To determine statistical significantly differences among the experimental groups, the two-tailed paired Student's *t*-test was used. A $p$ value of less than 0.05 was termed significant.

## RESULTS

### SNEDDS Characterization

SNEDDS administered to animals was prepared in advance by 1:10 (v/v) dilution of the pre-concentrate in water. Thus, we characterized SNEDDS following this dilution. The mean particles diameter, polydispersity index, and an average ζ potential are presented in Table I. We have also tested the size of particles formed upon dispersing the pre-concentrate at different degrees of dilutions 1:10, 1:100 and 1:500. The formulation was found to be robust, as by dispersing the pre-concentrate the particles were uniform in size upon all degrees of the dilutions (data not shown). AM SNEDDS was assessed for drug load. AM-SNEDDS 1:10 dilution in aqueous phase resulted in high drug load of >98% of the drug initially dissolved in the pre-concentrate. The result suggests that the amount of free AM not incorporated into SNEDDS was negligible and thus, all the PK and PD parameters assessed further in this work result from AM-SNEDDS effects, and that the possible existence of free drug in the test solution was ruled out.

### *In Vivo* Studies

The plasma concentration time profiles for AM and dispersed AM SNEDDS following oral administration of 12.5 mg/kg AM to rats are shown in Fig. 1. The corresponding AUC and Cmax parameters obtained in these *in vivo* experiments are listed in Table II.

The bioavailability of dispersed AM SNEDDS was significantly greater in comparison to AM alone. Similar results were obtained for the Cmax values.

Absolute bioavailability (F) values were calculated using the following equation, using the AM $AUC_{iv}$ values from our preliminary IV studies.

$$F = \frac{AUC_{iv} * Dose_{po}}{AUC_{po} * Dose_{iv}}$$

In addition, the absolute CL values were back calculated by multiplying the CL/F obtained for each group by its corresponding F value calculated as described above. No significant difference was found between the CL values of the different study groups and the $CL_{iv}$ value from our preliminary study, implying that SNEDDS administration did not affect AM clearance rate and extent, and that the increased bioavailability was achieved during the absorption phase at the intestinal level.

**Table I** Particle Size, ζ Potential and Polydispersity Index (PDI) of Various SNEDDS Obtained by 1:10 v/v dilution in Aqueous Phase. (Data presented as mean ± SD, $n=3$)

| Compound | Particle Size (d.nm) | ζ Potential (Mv) | PDI |
|---|---|---|---|
| Amiodarone | 10 ± 0.03 | +35 ± 0.11 | 0.48 ± 0.001 |
| Talinolol | 45 ± 0.07 | +6 ± 0.08 | 0.45 ± 0.001 |
| Blank | 52 ± 0.23 | −13 ± 0.04 | 0.38 ± 0.001 |

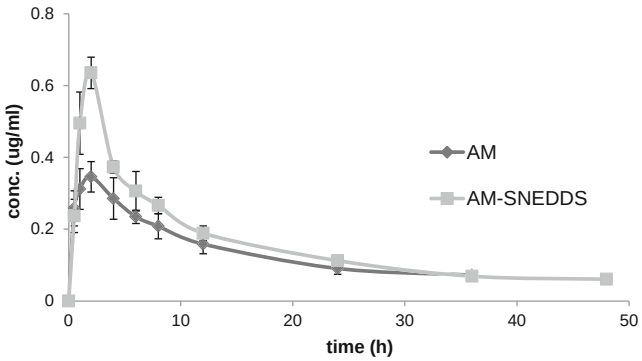


**Fig. 1** Plasma AM concentration *vs.* time plot (mean ± S.E.M.) following PO administration of AM and dispersed AM SNEDDS, 12.5 mg/kg ($n = 6$ for each group).

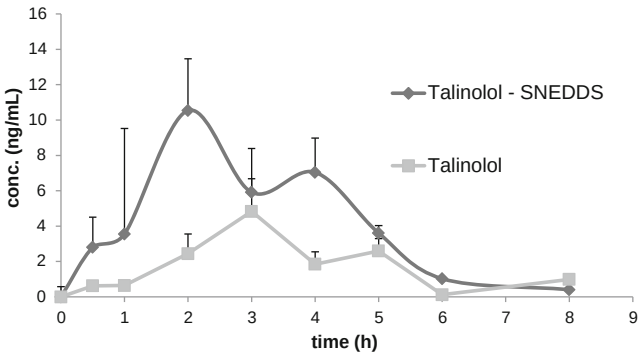


**Fig. 2** Plasma talinolol concentration *vs.* time plot (mean ± SD) following PO administration of talinolol and dispersed talinolol SNEDDS 4 mg/kg ($n = 6$ for each group).

Talinolol concentration *vs.* time plot following oral administration of talinolol alone or dispersed talinolol-SNEDDS (4 mg/kg) is presented in Fig. 2, and the derived PK parameters are presented in Table III. The relative oral bioavailability of dispersed talinolol-SNEDDS was significantly greater in comparison to talinolol alone.

### *In Vitro* Permeability Study

Our *in vitro* permeability studies of talinolol through Caco-2 resulted in significantly higher ($p<0.001$) permeability coefficient values of dispersed talinolol SNEDDS *vs.* talinolol ($5.03\times 10^{-6}$ and $7.66\times10^{-7}$ respectively) in A to B direction (Fig. 3). As expected for this P-gp substrate (36), talinolol Papp values obtained in the B to A direction are higher than the corresponding Papp values obtained in the A to B direction. Interestingly, B to A permeability resulted in statistically significant ($p<0.001$) reduction in Papp values obtained for dispersed talinolol SNEDDS *vs.* talinolol ($5.26\times10^{-6}$ and $1.77\times10^{-5}$respectively).

As for AM, the efflux ratio was calculated by comparing transport from the apical side of the membrane to basolateral side (A to B) to the transport in the opposite direction. The efflux ratio obtained in Caco-2 studies was 1.88 (Fig. 4), confirming AM to be a weak P-gp substrate. On the other hand, AM undergoes intra-enterocyte metabolism by CYP3A4 enzymes. Many groups have reported that one of the drawbacks of Caco-2 cell model is a very weak expression of the intraenterocyte CYP3A4 (37,38) as opposed to Ussing chamber model (39,40). Thus the evaluation of SNEDDS effect in Caco-2 cells using AM as a model compound is not efficient, and further AM SNEDDS transport studies were conducted in the Ussing chamber model.

**Table II** Pharmacokinetic Parameters Derived From Oral Administration of Amiodarone and Dispersed AM SNEDDS 12.5 mg/kg ($n = 6$ for each group)

| | AM | AM SNEDDS |
|---|---|---|
| AUC (hr*ug/mL) | 4.54 ± 2.6 | 9.52 ± 0.47(*) |
| Cmax (ug/mL) | 0.36 ± 0.07 | 0.66 ± 0.08(*) |
| T1/2 (hr) | 11.3 ± 4.26 | 18.0 ± 5.41 |
| V/F (mL/kg) | 34320 ± 5447 | 33774 ± 8638 |
| CL/F (mL/hr/kg) | 2412 ± 1428 | 1315 ± 66.3 |
| F(%) | 23.66 | 49.60(*) |

* Significant difference ($p < 0.05$) from AM corresponding value

### *Ex Vivo* Permeability Study

Figure 4 demonstrates the significantly higher Papp was obtained following dispersed AM SNEDDS *vs.* AM transport studies in the side by side diffusion (Ussing) chamber *ex-vivo* model with mean values of $6.79\pm25\times10-6$ and $2.51\pm1.0\times10-6$ cm/sec respectively ($p<0.05$).

### Toxicity Studies

LDH release was measured as a marker of apical membrane damage. SNEDDS was administered to animals as a 10% v/v dispersed solution, producing together with the gastrointestinal fluids maximal SNEDDS concentration of < 3% v/v in rat intestine. Upon gastric emptying the SNEDDS concentration further decreases. In humans SNEDDS is administered as a pre-concentrate with a glass of water, which collectively with gastric fluids volume will produce maximal SNEDDS concentration of 0.3%. Based on these calculations, we assessed SNEDDS toxicity over a range of concentrations (3% − 0.3% v/v). As shown in Table IV, SNEDDS increased LDH

**Table III** PK Parameters Derived From PO Administration of Talinolol *vs.* Dispersed Talinolol SNEDDS 4 mg/kg ($n = 6$ for each group)

| | AUC (hr*ng/mL) | Cmax (ng/mL) |
|---|---|---|
| talinolol | 11.9 ± 2.71 | 5.55 ± 1.65 |
| talinolol SNEDDS | 36.0 ± 8.09(*) | 15.28 ± 4.7(*) |

*Significant difference ($p < 0.05$) from talinolol corresponding value was found

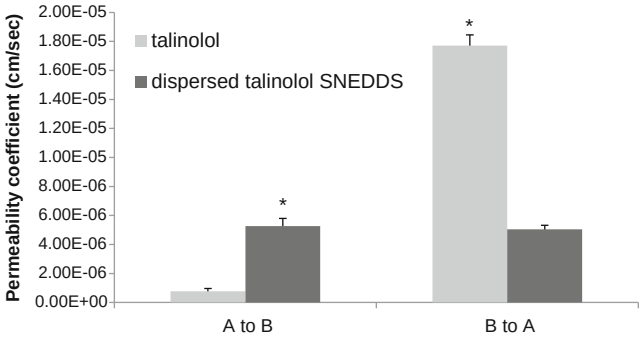


**Fig. 3** Papp (±SEM) values of talinolol *vs.* dispersed talinolol SNEDDS in Caco-2 model in A to B and B to A directions ($n=3$ for each group).

release in concentration dependent manner with highest toxicity obtained at 3% SNEDDS concentration (4.27%) and negligible toxicity (<1%) produce by SNEDDS concentration of 1% and less compared to 100% toxicity standard. These results suggest that no significant damage is caused to the apical cell membrane of the enterocyte by dispersed SNEDDS administration.

Table IV also represents the Papp values of mannitol (a para cellular transport marker) following incubation with dispersed blank SNEDDS (3 concentrations) or control (apical buffer not containing SNEDDS) in Caco-2 model. There was no significant difference in the permeability of mannitol in the presence of dispersed blank SNEEDS (all tested concentrations) compared to control ($p>0.05$).

As for TEER studies, no significant difference in TEER values in Ussing chamber model in the presence of blank SNEDDS *vs.* control was found.

These observations indicate that SNEDDS neither affect para cellular permeability, nor disrupt the enterocyte monolayer integrity in biorelevant concentrations.

### *In Vitro* Dynamic Lipolysis Model Study

Our *in vitro* lipolysis model studies demonstrated superior solubilization of AM-SNEDDS *vs.* AM alone in the aqueous phase of the digestion medium which represents the amount of drug available for absorption following lipolysis process (92.2± 1.33% *vs.* 59.1±9.45% of the initial concentration respectively). Following oral administration, dissolution of the drug molecule in the intestinal milieu is a prerequisite for the absorption process. Consequently, drug molecules that precipitated during the lipolysis process are not expect to be available for absorption in *in vivo* conditions, hence, drug concentration in the solid phase was not measured. However, it had been noted that in case drug precipitation occurs in an amorphous form, rather than in a crystalline form, it can contribute to absorption in case the solubilization capacity of the local environment is large enough to facilitate fast dissolution (41). No detectable lipid phase was observed following centrifugation of AM lipolysate, hence only aqueous phase AM concentrations were determined.

**Table IV** Percent of LDH Release (Relatively to 100% Release Control upon Incubation of Caco-2 Cell With Medium Containing Dispersed Blank SNEDDS. TEER Values (±SD) of Intestinal Tissue in Ussing Chambers in the Presence of Blank SNEEDS *vs.* Buffer. Papp Values (±SD) of Mannitol Across the Caco-2 Monolayers in the Presence of Blank SNEEDS *vs.* Buffer (Various SNEDDS Concentrations in Medium, $n=3$ for Each Study)

| | Control | 0.3% SNEDDS | 1% SNEDDS | 3% SNEDDS |
|---|---|---|---|---|
| LDH release (% of control) | 0 | <1% | <1% | <5% |
| Mannitol Papp (cm/sec) | $4.8\times10^{-7}$ | $2.8\times10^{-7}$ | $6\times10^{-7}$ | $1.4\times10^{-6}$ |
| | $\pm4.9\times10^{-8}$ | $\pm4.2\times10^{-8}$ | $\pm1.2\times10^{-8}$ | $\pm1.4\times10^{-7}$ |
| TEER after 120 min.(Ω) | 73±3.3 | 67±2.3 | 63±6.3 | 69±4.1 |

### Enzymatic Stability Study

To comprehend the effects of SNEDDS on intra-enterocyte metabolic activity, we tested its effect on CYP3A4 mediated AM metabolism in isolated rat CYP 3A4 microsomes (Fig. 5).

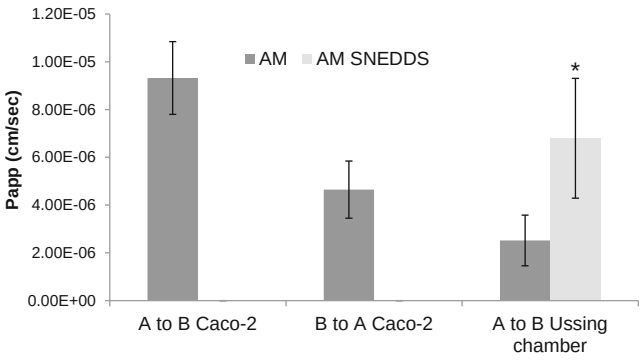


**Fig. 4** Papp (±SEM) values of AM in Caco-2 model and Papp (±SEM) values of AM vs. dispersed AM-SNEDDS in side by side diffusion (Ussing) chamber *ex vivo* model in apical to basolateral (A to B) and basolateral to apical (B to A) directions ($n=3$ for each group).

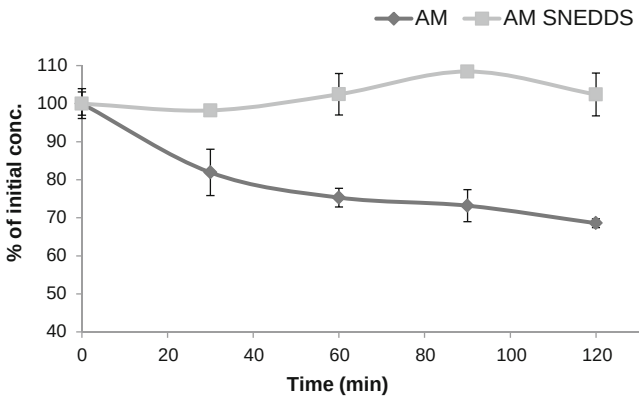


**Fig. 5** Intact AM concentrations remaining following 120 min. incubation of AM SNEDDS *vs.* AM in isolated rat CYP3A4 microsomes. ($n=3$ for each study).

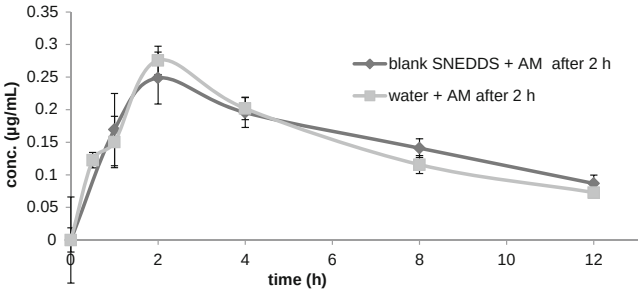


**Fig. 6** Plasma AM concentration–time plot (mean ± S.E.M.) following PO administration of dispersed blank SNEDDS following AM (12.5 mg/kg) administration after 2 h or water following AM (12.5 mg/kg) administration after 2 h ($n=3$ for each group).

Significant difference ($p<0.01$) was found between intact AM concentrations remaining following 120 min. incubation of dispersed AM SNEDDS *vs.* AM (102.4±5.61% and 68.57± 1.17% respectively).

### Duration of SNEDDS Effect on GI Absorption and Enterocyte Recovery Study

The plasma concentration time profiles for AM following oral administration (12.5 mg/kg) 2 h after administration of dispersed blank SNEDDS or water are shown in Fig. 6. No significant difference was found in the obtained AM plasma concentrations between the groups. These results suggest that the effect of SNEDDS on AM bioavailability is reversible and lasts no more than 2 h.

## DISCUSSION

Oral administration of AM SNEDDS resulted in significantly higher AUC and $C_{max}$ values in comparison to AM (Fig. 1). In addition, incorporation of AM into the SNEDDS resulted in reduced variability in the AUC and $C_{max}$ values (Table II), fivefold and threefold reduction respectively. This difference emphasizes the added value of this particular delivery system, that is, reduced plasma fluctuations *i.e.* a more predictive absorption.

It should be emphasized that all the other PK parameters obtained for AM SNEDDS (Table II) were not affected by the delivery system and remained not significantly different from those obtained following IV drug administration in our preliminary studies (data not shown) and the free AM oral administration . This finding supports our hypothesis that the effect of SNEDDS is limited to the enterocyte level and does not involve systemic alterations of PK parameters such as clearance and volume of distribution. It also emphasizes that increased bioavailability of intestinal first pass metabolism transport substrates can be achieved *via* alterations in intestinal absorption barriers without affecting other metabolic pathways.

Table III and Fig. 2 demonstrate the dramatic, three-fold increase in relative talinolol oral bioavailability upon incorporation into SNEDDS (both AUC and $C_{max}$). The double peak phenomenon during talinolol absorption was previously reported (42). The first peak after oral administration is plausibly explained by fast intestinal uptake into the blood. According to Weitschies *et al.*, (42) about half of the absorbed oral dose appears in the blood nearly at the same time as paracetamol - a drug rapidly and completely absorbed from all parts of the intestine, and therefore being a suitable probe drug to evaluate intestinal absorption. The other part obviously follows non-metabolic presystemic processing, leading to delayed bioavailability. Thus the second peak can be explained by the processing *via* a presystemic storage compartment within or behind the intestinal absorption barrier. However, the possibility that a fraction of the dose administered precipitated in the intestinal milieu and later was redissolved causing a delayed absorption cannot be ruled out.

As for the shifts in talinolol $t_{max}$ at both peaks, Weitschies *et al.* (42) demonstrated that when a meal that was served in close proximity to swallowing the fast-disintegrating talinolol capsules, the late peak shifted dramatically to shorter $t_{max}$ values. The acceleration of talinolol absorption by SNEDDS might be caused by a similar mechanism. There may be many interactions of the non-ionized form of talinolol with the enterocyte lipid and peptide processing micro-domains, which may possibly explain its absorption in association with dietary components of the meal, or of SNEDDS in our case. As recently shown, the intestine seems to be able to store alimentary fat for several hours within the jejunal tissue to release it into plasma following later stimuli such as glucose ingestion (43).

As aforementioned, talinolol is a BCS Class 2 compound not subjected to metabolic clearance, while being a strong P-gp efflux pump substrate (27). Thus, the increase in its relative oral bioavailability can be attributed to both increased solubilization in the GI milieu and to inhibition of its intestinal efflux by P-gp pumps, as will be further elaborated.

When evaluating SNEDDS, the size of the droplets formed upon exposure to an aqueous phase is a crucial factor in the *in vivo* performance of the resulting emulsion. Upon exposure to an aqueous phase, our SNEDDS produce droplets size in the nanometer range. Such droplet size leads to formation of visually clear and stable SNEDDS. The large surface area that results from the nano-range particle size enables pancreatic lipase to hydrolyze triglycerides more efficiently forming mixed micelles, which promote solubilization of the lipophilic drug in the aqueous environment of the intestinal lumen (44). Several studies report smaller droplet size measured *in vitro* to have a favorable effect on the bioavailability of the drug incorporated into SNEDDS (44,45). Tarr and Yalkowsky assessed the bioavailability of CsA in rats by administrating the drug in microemulsions forming different particle sizes. They reported enhanced oral absorption of CsA in rats by

reducing the size of the droplets. It is important to notice that in the stated study the formulations were prepared using the same ingredients and differed only by the method by which they were prepared. Thus, it is possible to rule out any other influence on the bioavailability of the drug and to isolate solely the effect of the size of the particles (44,45). Though in this described study a microemulsion was assessed, it demonstrates a very valuable principle which can be of a great importance for the nano-dispersion systems. Findings of a study published in 2004 by Bekerman et. al. corroborated the results described by Tarr and Yalkowsky. This study evaluated several dispersible SNEDDS incorporating CsA *in vivo* (in healthy volunteers). The range of the particle size formed by introducing the SNEDDS into an aqueous phase varied between 25 to 400 nm. Study findings demonstrated an inverse correlation between particle size of a tested particles and the oral bioavailability of the incorporated CsA (6). A similar tendency was observed and reported by several other researches investigating versatile lipid based formulations and lipid based encapsulation systems (46,47).

When examining this phenomenon, the structure of the apical membrane of the enterocyte also has to be taken into consideration. Microvilli are microscopic cellular membrane protrusions that increase the surface area of cells, and form the brush border of the gut wall. The inter-villous space diameter, as reported by Brown (48) ranges between 50 and 250 nm. Thus, nano-sized structures have a prominent advantage in gaining access to additional surface area available for absorption (*i.e.* inter-villous space), while larger structures (SEDDS, liposomes, *etc.*) are not capable of penetrating this space and thus have reduced effective absorption surface.

Yet, since various mechanisms are involved in Class 2 compounds oral absorption process, we cannot attribute the improved bioavailability and reduced variability in plasma concentrations profile solely to nano-range particle size.

ζ potential is another key SNEDDS characteristic, which determines whether the particles within the aqueous phase tend to flocculate. However, in the case of the SNEDDS under investigation, this characteristic is not much of a crucial importance since this formulation is administered as a pre-concentrate which forms nanodispersion only upon exposure to the fluids of the GI milieu. Thus, the long-term stability of the obtained nano-emulsion is irrelevant. Moreover, for formulations designed for oral administration, to achieve the highest physical stability in GI medium, the combination of a steric stabilizer is recommended (*e.g.*, Tween 80 or Poloxamer 188). Steric stabilization is little or less impaired by the presence of electrolytes compared with electrostatic solubilization (high zeta potential) (49). The positive charge of the ζ potential probably results from the positively charged AM ($pK_a$ 6.56 (50)) in the slightly acidic environment of the AM SNEDDS dispersed in water (pH ~ 5.5).

Several studies proposed increased solubilization in the GI milieu as the main mechanism for improved bioavailability of Class 2 compounds achieved by SNEDDS (3,12). *In vitro* dynamic lipolysis model is being increasingly used as a tool to facilitate *in vitro* evaluation of lipid-based drug delivery systems. Previous studies pointed out the correlation between solubilization profiles observed during *in vitro* lipolysis and the oral bioavailability, suggesting that enhancement in absorption could be explained by the increase in effective luminal drug concentration (51,52). Indeed, our *in vitro* lipolysis model studies demonstrated superior solubilization of AM-SNEDDS *vs.* AM in the aqueous phase of the digestion medium which represents the amount of drug available for absorption following lipolysis process. These findings support our *in vivo* results and corroborate results obtained by Fatouros *et al.* (53), which showed a correlation between superior probucol solubilization when incorporated in SNEDDS *in vitro* and increased *in vivo* bioavailability. Yet, improved solubilization may not be the only mechanism contributing to the overall improved bioavailability of compounds that share various additional absorption barriers. Wu *et al.* demonstrated that CsA, a Class 2 P-gp and CYP3A substrate, in the previous commercially available formulation (Sandimmune®) did not have an permeability difficulty and was absorbed at least 86%, but underwent a marked intraenterocyte first pass extraction of 60% or more (19). Thus, the newer SNEDDS cyclosporine formulations, like the one developed by our group (6) and others (17), characterized by higher and more predictive bioavailability, probably affected not only the solubilization of the compound, but additional absorption barriers.

Ussing chamber *ex vivo* model consists of vital intestinal tissue mounted between the donor and acceptor chambers, and thus expresses most of the barriers relevant for *in vivo* absorption of Class 2 compounds: solubilization, unstirred water layer, cell membrane, intra-cellular metabolism, and efflux. Our findings obtained in this model (Fig. 4) showed significantly higher Papp values for dispersed AM SNEDDS in comparison to AM. This difference caused by SNEDDS corroborates our *in vivo* findings, but still does not provide an unequivocal answer regarding the mechanism responsible for increased bioavailability.

Increased transport of BCS class 2 compounds through the enterocyte upon incorporation into SNEDDS was proposed as a possible explanation for improved bioavailability. The reasons proposed for such increased transport are elaborated in the "Introduction" section. Briefly, increased membrane permeability, inhibition of intra-enterocyte metabolism and of the P-gp efflux pumps activity are the main alternatives. We assessed this hypothesis in the Caco-2 cell model, which is widely accepted in studies of drug permeability, transport and absorption enhancing effect on cell membrane (54,55). Permeability of AM through the Caco-2 monolayer was not

significantly different from that of dispersed AM SNEDDS neither in apical to basal (A to B) nor in basal to apical (B to A) direction (data not shown). This observation is opposed to the results obtained in the Ussing model, where the transport of AM through the enterocyte was significantly higher in the case of SNEDDS AM. Such contradictory results can be explained by the different characteristics of these two models. Though both are well established *in vitro* models for evaluation of intestinal transport, they have their strengths and weaknesses in different screening situations.

It is generally accepted that a compound is considered to be a significant P-gp substrate if the efflux ratio of B to A to A to B compartments is >2 in an epithelial cell system (56). In the case of AM, the efflux ratio obtained in Caco-2 studies was 1.88 (Fig. 4) confirming AM to be a weak P-gp substrate.

Caco-2 cells, due to their cancerous origin, have been reported to demonstrate over expression of P-gp compared to the human colon (57). On the other hand, Caco-2 cells lack normal expression levels of important metabolic enzymes such as CYP3A4 (37,38) as opposed to the Ussing chamber model (39,40). Because of the large differences between the expressions of drug metabolizing enzymes *in vivo*, the use of these cells for drug metabolism studies is limited. (40). This fact could cause a lack in detection of the SNEDDS effect on CYP3A4 substrates, such as AM, and in turn lead to underestimation of SNEDDS effect on the overall transport through the enterocyte in this model.

Thus, the evaluation of SNEDDS effects in the Caco-2 model using a CYP3A4 substrate as a model compound is not efficient, and can lead to underestimation of SNEDDS effect on the absorption in this model. Therefore, in order to properly evaluate the underlying barrier for absorption *in-vivo*, it is crucial to use the most suitable model with consideration of the tested compound. In case of AM, the more appropriate model to evaluate the effect of SNEDDS on the intra –enterocyte metabolism and, as a consecutive result, the effect on the permeability and bioavailability is the Ussing chamber model.

Another proposed mechanism for increased bioavailability following incorporation of an active compound into SNEDDS is integration of surfactants into the enterocyte membrane which results in increased membrane fluidity (22,23). Increased membrane fluidity and permeability would result in higher AM transport through the Caco-2 cells in the case of AM SNEDDS. However, such a finding was not observed in our studies. The results of Caco 2 permeability studies indicated that there was no change in AM permeability. These results suggest that our SNEDDS didn't affect enterocyte membrane structure and integrity. It should be noted that our studies assessing the permeability of a model drug AM is an indirect way to investigate SNEDDS effect on membrane structure and integrity. A direct assessment of membrane fluidity by methods proposed, for instance, by Hugger *et al.* (58,59) is beneficial to provide more data for an unequivocal conclusion regarding SNEDDS effect on membrane fluidity.

Another parameter that can be properly evaluated in Caco-2 cells is the effect on P-gp efflux pump activity. Once examining an exclusive P-gp substrate – talinolol (27), we were able to isolate the effect of SNEDDS on P-gp activity. Our studies resulted in significantly higher Papp of dispersed talinolol SNEDDS *vs.* talinolol (Fig. 3) in the A to B direction, suggesting that SNEDDS has the ability to inhibit P-gp efflux and thus to increase the bioavailability of compounds subjected to intra-enterocyte efflux. As expected for a P-gp substrate, Papp value obtained in the B to A direction is higher than the corresponding value obtained in the A to B direction. Interestingly, B to A permeability resulted in significantly reduced Papp values of dispersed talinolol SNEDDS *vs.* talinolol. This finding is distinctive for a P-gp substrate in the presence of a P-gp inhibitor and previously was demonstrated for talinolol B to A transport with and without the presence of verapamil, another known P-gp inhibitor (60). In that case the B to A/A to B ratio was reduced from 7.4 to 3, and in our case from 22.9 to 1. While A to B efflux is increased due to P-gp inhibition, the transport in the opposite direction is decreased as a result of reduced P-gp contribution to drug transport from the enterocyte to the lumen. The overall talinolol transport in the B to A direction in case of free talinolol is a combination of passive diffusion through the enterocyte membrane and active secretion to the luminal side by P-gp pump activity. When P-gp activity is inhibited, like in case of introduction of SNEDDS to the cell medium, only the passive diffusion fraction contributes to the transport and thus the overall transport is reduced. We assume that the A to B and the B to A permeability of talinolol in the presence of SNEDDS (*i.e.* inhibition of P-gp) equalized, since under those conditions both are dependent solely on passive diffusion.

Hence, both the increased talinolol transport in the A to B direction, and the reduced transport in the opposite direction in the presence of SNEDDS, confirm our initial hypothesis regarding the ability of SNEDDS to inhibit P-gp activity in Caco 2 cell line. Moreover, the AUC of an orally administered exclusive P-gp substrate talinolol was increased by 3 fold when it was incorporated into SNEDDS. Our *in vivo* results corroborate with the results of Caco 2 studies and further reinforce the inhibitory effect of SNEDDS on P-gp.

Since no difference in AM transport was obtained in the Caco-2 model, while a significant difference was obtained in the Ussing chamber model, and taking into consideration that one of the major differences between these two models is the expression of drug metabolizing enzymes, as elaborated above, we hypothesized that the higher transport in the Ussing chamber model can be attributed to inhibition of CYP3A4 activity by SNEDDS. Hence, we decided to separately assess

SNEDDS effects on CYP3A4 metabolic activity in isolated rat CYP 3A4 microsomes (Fig. 4). AM is a suitable model compound for such assessment, since its oral bioavailability is largely dependent on the extent of pre-systemic intra-enterocyte metabolism by CYP3A4. A significant difference was found between intact AM concentrations remaining following incubation of AM SNEDDS *vs.* AM, with higher intact AM concentrations remaining following AM SNEDDS incubation. These findings indicate an inhibitory effect of SNEDDS on the metabolic activity of CYP 3A4 microsomes.

Our results corroborate previous studies that reported the ability of different surfactants that are commonly used as SNEDDS ingredients *e.g.* Cremophore EL, Labrasol, Tween 20/80, Span 80 to reduce P-gp efflux pump activity(61–64) and inhibit oxidative metabolism (65). However, it should be emphasized that *in vivo*, the surfactant concentrations obtained in the GI tract upon our SNEDDS oral administration was several times lower than the previously reported concentrations required for increasing the bioavailability of drugs subjected to pre-systemic metabolism. Thus, such delivery system, in which several excipients are co-administered and co-localized in close proximity to the gut wall, has a unique inhibitory effect on oxidative metabolism and P-gp efflux even at low and non-toxic excipient concentrations, that were not demonstrated up until now .

Previously, increased para-cellular transport resulting from damage caused to the structure tight junctions and tissue integrity was proposed as a possible mechanism responsible for increased bioavailability of SNEDDS (66). The TEER measurements throughout the Ussing chamber studies and the mannitol permeability across Caco-2 cells have been used as indicators for tight junctions and tissue integrity in assessment of the effect of SNEDDS on para-cellular transport. Our findings (Table IV) indicate that SNEDDS does not affect para-cellular transport and does not interfere with tight junctions arrangement. This results suggest that the components of SNEDDS in their respective concentrations do not noticeably alter the integrity of Caco-2 cell monolayers. As for SNEDDS cytotoxicity, our LDH release results clearly demonstrate that in biorelevant concentrations SNEDDS does not cause damage to the cell membrane. Hence, the effect of SNEDDS on AM bioavailability is not secondary to their cytotoxicity.

When assessing the duration of SNEDDS effect on AM absorption, we found that two hours following oral blank SNEDDS administration, the AM absorption parameters (*i.e.* AUC and Cmax) return to the baseline, that is to say, are similar to free AM corresponding parameters (Fig. 6). Thus we conclude that the SNEDDS effect on absorption mechanisms is reversible and lasts no more than two hours.

Several studies have previously demonstrated superior bioavailability of compounds administered in SNEDDS. In this aspect it is important to distinguish between compounds which have poor oral bioavailability mainly due to solubilization limitation (*i.e.* griseofulvin) (14,15) and compound which solubility is fair, but they are extensively metabolized by enterocyte microsomes and subjected to P-gp efflux. In this case the contribution of SNEDDS to their improved bioavailability is probably mediated by additional mechanisms rather than solely higher solubilization. The net outcome of SNEDDS modulation of these absorption mechanisms resembles the effect of grapefruit juice (GFJ) co-administration. The main mechanism for (GFJ)–drug interaction is the inhibition of the CYP3A4 metabolic enzyme (67). Contrary to other orally administrated CYP3A4 inhibitors that affect predominantly metabolic processes that take place in the liver, GFJ key site of action is in the enteric CYP3A4, resulting in a significant reduction of the presystemic metabolism (68,69). Additional studies shown that the effect of GFJ also involves the modulation of the intestinal P-gp efflux activity (70,71). This modulation is still ambiguous, since some authors have reported activation(72,73) and others inhibition of P-gp (74). However, most recent studies that assessed this matter conclude that GFJ and its components inhibit P-gp activity and thus have the potential to modifying the disposition of drugs that are P-gp substrates (75). Hence, drugs that are mostly prone to be affected by GFJ consumption are those that are subjected to extensive intra-enterocyte metabolism and efflux, compounds that can be collectively defined as "GFJ effect substrates". Widely studied compounds belonging to this group that showed increased bioavailability following incorporation into SNEDDS are CsA (17), Tacrolimus(18), simvastatin(76) and Nimodipine (77). Hence, various "GFJ effect substrates" can benefit from incorporation into SNEDDS both in terms of improved solubilization, preferable PK profile and reduced inter and intra-subject variability that reduces the potential to obtain plasma concentrations above or below the therapeutic window.

## CONCLUSIONS

Our mechanistic studies have revealed the underlying processes that are associated with the nano particulate drug delivery system and together account for the elevated oral bioavailability of poorly water soluble compounds. Our key finding is the identification of each of these processes and the synergistic, synchronized and concerted action they produce at the absorption site. We conclude that our SNEDDS improve oral bioavailability of Class 2 compounds by multi-processes mechanism, both physical and biological: increased GI milieu solubilization, reduced intra-enterocyte metabolism and reduced P-gp efflux activity. Importantly, we determined that the latter two mechanisms occur at concentrations lower that those reported earlier for each of the SNEDDS ingredients assessed separately. The nano-scale particle size is also of a great importance, since the particles share the advantage of gaining access to increased surface area

available for absorption (*i.e.* inter-villous space). These mechanistic findings bridge the gap in knowledge regarding the mode of SNEDDS effects on oral bioavailability of poorly water soluble compounds. In addition to increased bioavailability, SNEDDS reduces the high variability that is typically associated with the absorption pattern of Class 2 compounds. This finding is of extreme importance for compounds with a narrow therapeutic index, especially in cases when deviation from the therapeutic plasma drug levels is of severe clinical consequences. Additionally, SNEDDS utilization can improve the bioavailability of poorly water soluble drug candidates, which would otherwise fail to precede towards advanced stages of development and clinical use.

Importantly, we have demonstrated that the improved oral bioavailability achieved by our SNEDDS doesn't result from intestinal tissue or cell membrane damage, and its effects are reversible and non-toxic in biorelevant concentrations.

The net outcome of SNEDDS modulation of these absorption mechanisms resembles the effect of GFJ co-administration, which also affects the enteric CYP3A4 and the intestinal P-gp efflux activity, resulting in significant reduction of the presystemic metabolism. These notable findings contribute to our understanding of the previously reported phenomenon of the impact of SNEDDS on oral bioavailability that was demonstrated in clinical studies and enable us to design a drug delivery platform that will provide improved oral bioavailability and reduced variability for Class 2 compounds and drug candidates, in particular those affected by pre systemic CYP3A4 and P-gp absorption barriers.

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