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RESEARCH PAPER

In Vitro Lipolysis and Intestinal Transport of β-Arteether-Loaded Lipid-Based Drug Delivery Systems

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ABSTRACT

Purpose We aimed to assess the fate of β-arteether lipid-based drug delivery systems (AE-LBDDS) in terms of resistance to lipolysis and permeation across intestinal cells.

Methods AE-LBDDS contained Tween 80 or Cremophor EL as surfactants, ethanol, Maisine 35-1 and vegetable oil. The solubilization behavior of AE was investigated during dynamic *in vitro* lipolysis. The permeation of AE-LBDDS was evaluated using Caco-2, HT29-MTX and M cell monolayers.

Results A higher level of AE precipitation was observed for formulations containing Cremophor EL (~30%) compared to formulations containing Tween 80 (~10%) after lipolysis. However, rapid re-dissolution of the precipitated AE from LBDDS containing Cremophor EL in the intestinal biorelevant media was observed. The transport of AE loaded in LBDDS was enhanced in comparison to that of free drug due to the increased AE solubility. The apparent permeability of all AE-LBDDS across Caco-2 cell monolayers was approximately 3.10^{-6} cm/s. A decrease in the permeability was observed at 4°C. M cells did not influence the transport of AE-LBDDS, and mucus decreased AE permeability when formulated with Tween 80. Furthermore, AE is not a P-glycoprotein substrate.

Conclusion LBDDS that are partly resistant to *in vitro* lipolysis significantly increased the transport of AE across intestinal cell monolayers.

KEY WORDS Arteether lipid-based drug delivery systems · lipolysis · Caco-2 cells · M cells

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INTRODUCTION

β-arteether, the ethylether derivative of artemisinin, is a potent and highly specific erythrocytic schizontocidal agent used for the treatment of patients with uncomplicated and severe malaria. Due to its high lipophilicity, its poor water solubility and its low stability in gastric media, β-arteether (AE) is marketed only as oil-based intramuscular injectable formulations (1). These intramuscular injections are characterized by slow and prolonged absorption rates at the injection sites (2). Intramuscular injections are also painful and are associated with a risk of infection for the patient due to possible contamination. The development of more convenient, painless and patient-friendly oral formulations would be beneficial for malaria patients. Hence, we developed oral lipid-based drug delivery systems (LBDDS), which were used to solubilize the daily dose of AE (3). In gastrointestinal medium, these formulations enhance the drug's solubilization and stability. When tested in *Plasmodium berghei*-infected mice, AE-LBDDS demonstrated an antimalarial efficacy comparable to that of an intramuscular oily solution of AE and a significantly higher efficacy than that of an oily solution of AE administered orally at the same dose (3).

Due to the complexities associated with oral lipid-based formulations, a "holistic" approach using several complementary techniques is crucial to maximize their development potential and commercial future (4). Lipid-based systems circumvent the dissolution step and maintain the drug in solution as it travels through the gastrointestinal tract. However, complex and ill-defined processes occur *in vivo*. These processes include possible loss of solubilization capacity of the formulation, which is associated with a risk of drug precipitation due to the partitioning of excipients in the aqueous phase (5), the digestion of excipients and the formation of a different colloidal structure (6). In particular, during the digestion process, mixed micelles and several types of colloidal vesicles maintain and enhance the solubilization of co-administered hydrophobic

drugs (7). The nature of the colloidal structure formed and the drug affinity for each colloidal species are critical determinants for enhancing the drug's solubilization and preventing the precipitation of the drug (8). However, the precipitation of the drug during digestion could occur once the drug solubilization capacity of the colloidal structure is exceeded (9).

In addition to resistance to lipolysis, the *in vivo* efficacy of lipid-based systems also depends on the absorption of free and/or encapsulated drug that occurs in the gastrointestinal tract. A clear understanding of transport mechanisms is also crucial for the optimization of lipid-based systems.

The aims of this study were to assess the fate of AE-LBDDS in term of resistance to lipolysis and absorption across intestinal cells. Therefore, we evaluated the solubility of AE after lipolysis of LBDDS and the permeability of AE-loaded LBDDS across Caco-2, HT29-MTX (mucus secreting cells) and M cell monolayers.

MATERIALS AND METHODS

Materials

AE (≥ 99%) was purchased from Calyx Chemicals and Pharmaceuticals, Ltd. (Mumbai, India). Sodium taurodeoxycholate, L-α-Phosphatidylcholine from egg yolk (~60%), pancreatin from porcine pancreas (activity 8×USP specifications), pepsin, Tris-maleate, 4-bromophenylboronic acid and tributyrin were obtained from Sigma-Aldrich (Bornem, Belgium). Tween 80 (Polysorbate 80) and sesame oil were purchased from Certa (Braine-l'Alleud, Belgium), and groundnut oil was obtained from Fagron (Waregem, Belgium). Absolute ethanol (99.2%, w/w) was supplied by Merck (Darmstadt, Germany). Cremophor EL (polyoxyl 35 castor oil) was kindly provided by BASF (Burgbernheim, Germany). Maisine 35-1 (glyceryl monolinoleate) was a kind gift from Gattefossé (Saint-Priest, France). Methanol and 0.1% ammonium acetate were HPLC grade, and all other chemicals were reagent grade.

Preparation and Characterization of AE Lipid-Based Formulations

All lipid-based formulations were prepared as described previously (3). Briefly, 3 g of Maisine 35-1 was melted at 60°C and mixed with 3 g of sesame oil (or groundnut oil) by stirring for 5 min at room temperature. Three grams of Cremophor EL (or Tween 80) and then ethanol (1 g) were added and mixed until homogenized. Two grams of AE was introduced to the solution (drug loading = 200 mg/g) and stirred for 120 min at room temperature. In humans, the dose regimen of artemisinin derivatives orally administered is 100 mg to 200 mg/day (1). The compositions of AE lipid-based formulations are indicated in Table I. Before the start of the experiments, AE-LBDDS were kept in sealed glass vials at room temperature with protection from light.

The size of the AE lipid-based formulations in transport (Hank's Balanced Salt Solution, HBSS) (Invitrogen Gibco®, Belgium) and in lipolysis media was determined by photon correlation spectroscopy using the Zetasizer Nano ZS model ZEN 3600 (Malvern Instrument, Ltd., UK), as described previously (3).

Solubility of AE

In 250 ml of gastric (0.1 N HCl and Fasted State Simulated Gastric Fluid, FaSSGF), intestinal (Fasted State Simulated Intestinal Fluid, FaSSIF), lipolysis and transport (HBSS) media at 37°C, 1.2 g of the formulation was dispersed under gentle agitation for 60 min. FaSSGF contained 80 μM sodium taurodeoxycholate, 20 μM lecithin, 0.1 mg/mL pepsin and 34.2 mM sodium chloride (pH 1.6) (11). FaSSIF contained 50 mM Tris-maleate, 150 mM sodium chloride, 5 mM calcium chloride dihydrate, 5 mM sodium taurodeoxycholate and 1.25 mM lecithin at pH 6.8 (10). The lipolysis medium contained 50 mM Tris-maleate, 150 mM sodium chloride, 5 mM calcium chloride dihydrate, 5 mM sodium taurodeoxycholate and 1.25 mM lecithin at pH 7.5 (5,10). Aliquots of the dispersion were sampled and centrifuged for 15 min at 5,000×g and 37°C. The supernatants were then diluted with methanol and assayed for AE content by HPLC analysis. All experiments were performed in triplicate.

The equilibrium solubility of AE was determined in water, in intestinal and in lipolysis media by dispersing a large amount of AE in these solutions. After 24 h with stirring at 37°C, the samples were centrifuged (5,000×g, 15 min, 37°C), and the supernatants were filtered through a 0.45 μm filter. The filtrate was diluted twice in methanol and then analyzed by HPLC. To determine aqueous solubility, the filtrate was

Table I Compositions of AE Lipid-Based Formulations

Formulation A	Formulation B	Formulation C	Formulation D
Groundnut oil (3 g)	Sesame oil (3 g)	Groundnut oil (3 g)	Sesame oil (3 g)
Maisine 35-1 (3 g)	Maisine 35-1 (3 g)	Maisine 35-1 (3 g)	Maisine 35-1 (3 g)
Cremophor EL (3 g)	Cremophor EL (3 g)	Tween 80 (3 g)	Tween 80 (3 g)
Ethanol (1 g)	Ethanol (1 g)	Ethanol (1 g)	Ethanol (1 g)
AE (2 g)	AE (2 g)	AE (2 g)	AE (2 g)

evaporated to remove all liquid using a SpeedVac, reconstituted in a methanol-water (90:10, v/v) mixture and then analyzed by HPLC. All solubility experiments were performed in triplicate.

HPLC Analysis of AE

An Agilent 1100 Series HPLC system with a diode array and a multiple wavelength detector was used for these experiments. The column used was a CC 125/4 Nucleodur 100-5 C18 ec (Macherey-Nagel, Germany) (3). The mobile phase (methanol: ammonium acetate buffer (10 mM, pH 4), 90:10, v/v) was eluted at a flow rate of 0.5 ml/min (12). The detection wavelength was 216 nm. The injection volume was 50 µl. The retention time of AE was approximately 5.9 min under these conditions. The limit of detection (LOD) and the limit of quantification (LOQ) of AE were 1.3 µg/ml and 4 µg/ml, respectively. The coefficients of variation (CV) for intra- and inter-assay were all within 5%.

Lipolysis of AE Lipid-Based Formulations

Using a pH-stat titration system (Metrohm Titrando 842; Software Tiamo 1.3, Herisau, Switzerland), dynamic *in vitro* lipolysis was performed as previously described (5). Briefly, 1.2 g of AE-LBDDS was dispersed in 36 ml of lipolysis medium and stirred for 20 min at 37°C. The enzymatic digestion was initiated by the addition of 4 ml of freshly prepared pancreatin extract (lipase activity of 1,000 tributyrin units per ml digest). The fatty acids released during *in vitro* lipolysis were automatically titrated with 0.2 M NaOH to maintain the pH at 7.5. At different time (15, 30, 45 and 60 min), aliquots were sampled and a lipolysis inhibitor (0.5 M 4-bromophenylboronic, 9 µl/ml digestion medium) immediately added to each sample. Subsequently, aliquots were centrifuged for 60 min at 37°C and 48,000×g to separate the sample into an aqueous phase, a pellet phase and/or a lipid phase (6,7). After an appropriate dilution with methanol, samples obtained from each separated phase were assayed for AE content by HPLC analysis. Control experiments with the lipolysis medium alone were also performed as described above.

Analysis of the Pellet Obtained After Lipolysis

The dissolution of the pellet obtained from centrifugation of all the media (~50 ml) sampled at the end of lipolysis was performed at 37°C using the USP II Apparatus with the paddle rotating at 100 rpm. Two pellets were dispersed in 10 ml of water. The dispersion was then added to 500 ml of intestinal medium (pH 6.8, 37°C). During the dissolution study, 3 ml samples were withdrawn at each sampling time point (5, 10, 20, 30 and 60 min), and the volume was substituted with fresh intestinal medium. Subsequently, the samples were filtered through 0.45 µm filters (Acrodisc®, Pall, France) and then analyzed by HPLC for drug content following an appropriate dilution with methanol. These experiments were conducted in triplicate.

To elucidate the solid state of AE present in the isolated pellet, X-ray powder diffraction (XRPD) was performed on a Siemens D5000 diffractometer using the Kα radiation of Cu ($\lambda = 1.5418$ Å). The 2θ range between 2° and 60° was scanned at a rate of 0.02°/s with an applied voltage and current of 40 kV and 40 mA, respectively. The isolated pellets (AE-pellets) were spread on a zero-loss sample holder (Si single crystal) and then allowed to air dry. Control experiments were conducted using blank pellets obtained from the lipolysis of unloaded-LBDDS and blank pellets spiked with the corresponding amount of crystalline drug present in the AE pellets (8).

In Vitro Intestinal Cell Models Used to Study the Transport of AE

Cell Culture

The Caco-2 cell line was obtained from the University of Milan-Bicocca, Italy (13). Caco-2 cells were maintained in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) (Invitrogen Gibco®) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Hyclone®, UK), 1% (v/v) L-glutamine (Invitrogen Gibco®) and 1% (v/v) non-essential amino-acids (Invitrogen Gibco®) at 37°C in an atmosphere of 10% CO_2. Caco-2 cells were used between passages $x+17$ and $x+30$ (3,13). HT29-MTX cells were kindly provided by Dr. T. Lessufleur, Paris, France and were used between passages $x+16$ and $x+20$ (14). HT29-MTX cells were maintained at 37°C and 5% CO_2 in the medium listed above further supplemented with 1% (v/v) 10,000 U/ml Penicillin-10,000 µg/ml Streptomycin (PEST) (Invitrogen Gibco®) (15). The human Burkitt's lymphoma Raji-B cell line (American Type Culture Collection, VA, USA) was maintained in RPMI 1640 (Invitrogen Gibco®) supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acids at 37°C under a 5% CO_2 humidified atmosphere. Raji-B cells were used at passage numbers 106–110 (13,16).

Caco-2 and HT29-MTX Models

Approximately 500,000 Caco-2 or HT29-MTX cells at various ratios (1:0, 7:3, 5:5, 3:7 and 0:1) were seeded onto Matrigel® (BD Biosciences, Belgium) (10 µl/ml in DMEM)-coated 12-well cell culture inserts (1 µm pore diameter, 0.9 cm^2 area) (Corning Costar®, New York, USA)

and were grown in supplemented DMEM+1% PEST for 21 days. The culture medium was changed every 2 days (3,13,18). The integrity of the cell monolayer was controlled before starting the experiment and at the end of the permeability assay by Transepithelial Electrical Resistance (TEER) measurements, which were performed with an electrode connected to an EVOM® volt ohm meter (World Precision Instruments, USA). For transport studies, only Caco-2 and HT29-MTX cell monolayers with initial TEER values higher than 400 Ω cm^2 and 200 Ω cm^2, respectively, were used.

Follicle-Associated Epithelium Model

An *in vitro* model of human follicle-associated epithelium (including M-like cells) was obtained according to the protocol published by des Rieux *et al.* (13,16). Briefly, 500,000 Caco-2 cells were seeded onto Matrigel®-coated 12-well cell culture inserts (3 μm pore diameter, 0.9 cm^2 area) (Corning Costar®) and cultivated for 5 days in supplemented DMEM+1% PEST. The inserts were inverted, and a piece of silicon tube was plated on the basolateral side of each insert. The inverted inserts were then transferred into prefilled Petri dishes with supplemented DMEM+1% PEST and maintained for 11 days. The basolateral medium was refreshed every 2 days. Approximately 250,000 Raji-B cells, resuspended in supplemented DMEM+1% PEST, were added to the basolateral compartment of the inserts. The co-cultures were maintained inverted for 5 days. Monocultures of Caco-2 cells, cultivated as described above but without the addition of Raji-B cells, were used as controls. The silicon tube was then removed, and the inserts were placed in BD Falcon® 12-well multiwell plates (Becton Dickinson, France) in their original orientation for the transport experiment. The integrity of the cell monolayers was checked prior to and after the experiment by determination of the TEER. Only cell monolayers with initial TEER values greater than 200 Ω cm^2 for the monocultures and greater than 100 Ω cm^2 for the co-cultures were used for the permeation studies.

Permeation Studies of AE Lipid-Based Formulations

For permeation studies using Caco-2 cells, the cell monolayers were washed twice with preheated HBSS (37°C) and equilibrated for 30 min at 37°C with 0.5 ml and 1.5 ml of HBSS in the apical and basolateral compartments, respectively. For the apical (A) to basolateral (B) transport experiments (A to B), 0.5 ml of the AE solution (20 μg/ml in HBSS containing 2% of absolute ethanol) or 0.5 ml of dispersed formulations in HBSS (2.4 mg/ml of AE-LBDDS *i.e.* 0.4 mg/ml of AE, unless otherwise stated) were added to the apical compartments of the inserts, while the basolateral compartments were filled with 1.5 ml of HBSS. For the basolateral to apical transport experiments (B to A), 1.5 ml of AE solution (20 μg/ml) was added at the basolateral side, while the apical side was filled with 0.5 ml of HBSS. HBSS buffer was used in the donor compartment because the intestinal medium (FaSSIF, 5 mM bile salts) and FeSSIF are not suitable for use in the Caco-2 system due to their cytotoxicity, as previously reported by Ingels *et al.* (44). At a predetermined time, sampling of the acceptor compartment (basolateral for A to B transport or apical for B to A transport) was obtained to determine the permeability of AE in solution or loaded in LBDDS. After solid phase extraction in the Oasis HLB cartridge (Waters, Belgium), evaporation using a SpeedVac and reconstitution in methanol-water (90:10, v/v), the amount of AE that had crossed the Caco-2 monolayer was determined using pooled samples by HPLC. The cumulative amount of AE that permeated the cells was plotted as a function of time. To determine the energy-dependent mechanisms, A to B transport of free AE and AE-LBDDS were also performed at 4°C.

The AE apparent permeability coefficient was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_o A}$$

where dQ/dt (transport rate) is the amount of AE (μg) appearing per time unit (s) in the receiver compartment, C_o is the initial concentration in the donor compartment (μg/ml) and A is the surface area of the monolayer (A=0.9 cm^2).

During the transport studies in the HT29-MTX and follicle-associated epithelium models, A to B transport was investigated as described above.

Statistical Analyses

Significant differences between the droplet sizes, permeability coefficients and percent distribution of AE into the various digestion phases were compared by one-way ANOVA with Tukey's post-hoc test (with a level of significance of $p<0.05$).

RESULTS

Characterization and Dispersion of AE-LBDDS

To check whether the lipid-based formulations of AE could self-emulsify in transport and in lipolysis media, 1.2 g of each formulation was diluted in 50–250 ml of HBSS and lipolysis medium. Consistent with previous results, the lipid formulations with Tween 80 had size and particle distributions higher than those of the formulations with Cremophor EL

(data not shown) (3). The lipid formulations A and B (0.48% to 2.4%, w/v) had a size of approximately 75 nm with a polydispersity index of 0.12 both in HBSS and lipolysis medium. At different dilutions in HBSS, formulations C and D had a size of approximately 350 nm with a polydispersity index larger than 0.5 whereas in lipolysis medium, multidispersed aggregates were formed (data not shown).

The solubility of AE in water was 16±3 μg/ml, whereas it increased to 75±4 μg/ml in FaSSIF (n=3). Hence, the presence of bile salts and phospholipids in FaSSIF enhanced the solubility of AE by a factor of 4.7 compared to water. The solubility of AE in FASSIF and in lipolysis medium was similar; the variation of pH did not influence the solubility of AE which is a unionized compound. In LBDDS, up to 700 μg/ml of AE was dissolved in water, a 10-fold increase compared to its solubility in FaSSIF and in lipolysis medium (3).

Dispersion experiments were carried out in 250 ml of gastric (HCl, FaSSGF), transport (HBSS), lipolysis and intestinal media. After centrifugation, no sign of phase separation was observed for the formulations containing Cremophor EL. In all the media, more than 96% of AE was recovered in the aqueous phase, which is in agreement with our previous results (3). After centrifugation, the formulations containing Tween 80 were separated into different phases. In any dispersion medium, 0.5-2.0% of AE was recovered in the aqueous phase, whereas 84–88% was recovered in the creamed phase (data not shown).

In Vitro Dynamic Lipolysis

In Vitro Lipolysis

To determine whether AE precipitates due to intestinal digestion of the oils and surfactants present in our LBDDS, a dynamic lipolysis procedure was performed as previously described (5). The course of NaOH addition as a function of time during lipolysis of AE-LBDDS is shown in Fig. 1a. The quantity of NaOH used to titrate the fatty acids released by the lipid formulations ranged from 1.04–1.32 mmol (formulations with Cremophor EL) and 0.68–0.90 mmol (formulations with Tween 80).

After centrifugation of the formulations containing Tween 80, a lipid phase appeared, suggesting that the lipids were digested incompletely. For formulations containing Cremophor EL, no oil phase was visible after centrifugation (19). The concentrations of AE in lipid phase of formulations

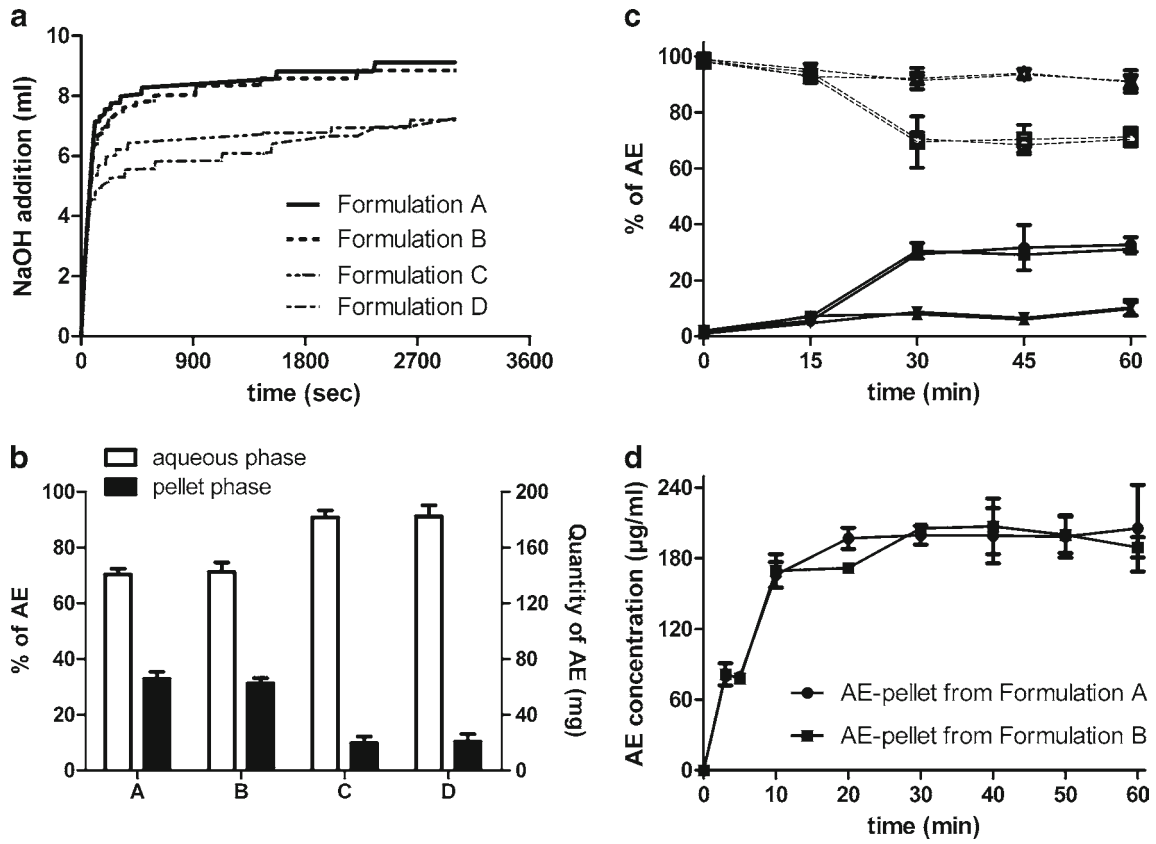

Fig. 1 *In vitro* dynamic lipolysis. (**a**) Quantity of 0.2 M NaOH added to titrate the fatty acids released during lipid digestion. (**b**) Distribution of AE among the digestion phases at the end of lipolysis. (**c**) Distribution profile of AE in the aqueous phase (*open shapes* and *dotted lines*) and in the pellet phase (*filled shapes* and *full lines*) as a function of lipolysis time. The self-emulsifying systems were represented by *circles* (Formulation A), *squares* (Formulation B), *triangles* (Formulation C) and *inverted triangles* (Formulation D). (**d**) Dissolution rate of AE in the pellet at endpoint of lipolysis (60 min) ($N=2–3$, $n=4$).

containing Tween 80 were too low to be determined with accuracy.

The amount of non-precipitated AE in the aqueous phase and the amount of precipitated AE in pellet after lipolysis and centrifugation are shown in Fig. 1b. In formulations containing Tween 80, approximately 90% of AE was present in the aqueous phase as compared to approximately 70% for the formulations containing Cremophor EL, indicating a strong contribution of the surfactant, but not the oil, to resistance to lipolysis. These percentage correspond to a concentration of approximately 2.8 mg/ml (140 mg) and 3.6 mg/ml (180 mg) of AE in the aqueous phase for formulations containing Cremophor EL and for formulations containing Tween 80, respectively (Fig. 1b). Throughout *in vitro* lipolysis, the distribution of AE in the aqueous phase decreased from approximately 98% to 70% for Formulations containing Cremophor EL and from approximately 98% to 90% for Formulations containing Tween 80, which are consistent with the increase of AE in the pellets (Fig. 1c). The formulations with Cremophor EL or Tween 80 displayed nearly identical distribution of AE between the aqueous phase and the pellet phase as a function of time (Fig. 1c). The dispersion of 1.2 g of AE-LBDDS in lipolysis medium (36 ml, no lipase) maintained AE in a solubilized state; less than 6 mg (*i.e.* 3%) of AE precipitated for all the formulations.

To assess whether the degree of AE saturation in the formulation might influence the loss of solubilization capacity of the lipid formulations with Cremophor EL, the initial content of AE (200 mg/g excipient, 95% of saturation solubility) was reduced to 150 mg/g of excipients (70% of saturation solubility), and *in vitro* lipolysis was repeated as described above. The proportion of AE in the aqueous phase increased to 92% ($n=4$). Hence, the precipitation was dependent on drug loading and occurred when the solubilization capacity of the vesicular and micellar species was exceeded (9).

Dissolution and X-ray Powder Diffraction of AE in the Pellet After Lipolysis

Potential re-dissolution of the precipitated AE in the intestinal medium prior to absorption was assessed by performing a dissolution test of the pellet obtained from lipolysis of LBDDS containing Cremophor EL (95% of saturation solubility). Interestingly, 80–90% of the AE present in these pellets was re-dissolved in intestinal medium after less than 30 min, corresponding to 204 ± 19 µg AE/ml of intestinal medium ($n=4$) (Fig. 1d). The presence of various colloidal structures containing bile salts, phospholipids and lipid digestion products improved the solubilization of AE compared to its solubility in water (16 µg/ml) and in intestinal medium containing bile salts and phosphatidylcholine but no lipid digestion products (75 µg/ml) (see "Characterization and Dispersion of AE-LBDDS").

To determine the solid state of precipitated AE, X-ray powder diffraction was performed on the AE pellet. A series of peak positions detected at lower 2θ values revealed the organization of the fatty acid phases present in all the precipitates (Fig. 2). The corresponding d-spacing values (23.6, 15.7, 11.8 and 9.4 Å) showed the regular periodicity of a multi-order diffraction with the characteristic ratio (2:3:4:5) of a lamellar phase with a lamellar distance of about 47 Å which is in accordance with the results previously reported by Sassene *et al.* (8). The first order diffraction peak was not detected because its position was out of the scanned range ($2\theta < 2°$). In the blank and the AE pellets, the presence of an additional peak with a d-spacing of 28.5 Å may provide evidence of the coexistence of a non-lamellar configuration (6,20). This suggests that in the blank pellet spiked with AE, a possible effect of the drug on the organization of the phase containing the products of digestion may occur. Additionally, the typical X-ray powder diffraction pattern of crystalline AE showed similar characteristic reflections to those present in the pellets from lipolysis of AE lipid-based formulations (95% and 70% drug saturation solubility), suggesting that AE precipitated in a crystalline form during *in vitro* lipolysis (Fig. 2).

Permeation of AE Across Intestinal Cell Monolayers

Transport of Free AE Across Caco-2 Cell Monolayers

To evaluate the permeability of free AE (20 µg/ml in 2% ethanol), absorptive (apical (A) to basolateral (B)) and secretive (B to A) transport across Caco-2 cell monolayers were performed for 120 min. The P_{app} (A to B) of free AE was $26.93 \pm 3.24 \times 10^{-6}$ cm/s at 37°C (Fig. 3). At 4°C, a similar P_{app} was measured ($p<0.05$), suggesting the absence of active transport (Fig. 3). The existence of potential active efflux was also investigated during the secretive transport. The P_{app} (B to A) of AE was $28.1 \pm 3.32 \times 10^{-6}$ cm/s (Fig. 3). The ratio P_{app} (B to A)/P_{app} (A to B) corresponded to 1.04, indicating that AE was not a substrate of the PgP efflux transporter (ratio < 2).

Transport of AE-LBDDS Across Caco-2 Cell Monolayers

To determine the intestinal permeability of AE formulated in 4 LBDDS, transport studies were carried out in Caco-2 cells. Cell viability was previously investigated in order to choose the range of concentration used for the transport experiments (3). Based on these cytotoxicity studies (3), Caco-2 monolayers were incubated for 120 min at 37°C with 2.4 mg/ml of AE-LBDDS (unless otherwise stated), equivalent to 0.4 mg/ml of AE. As shown in Fig. 4a, the P_{app} of AE was between 2.10^{-6} and 3.10^{-6} cm/s. The quantity of AE transported across the Caco-2 monolayers

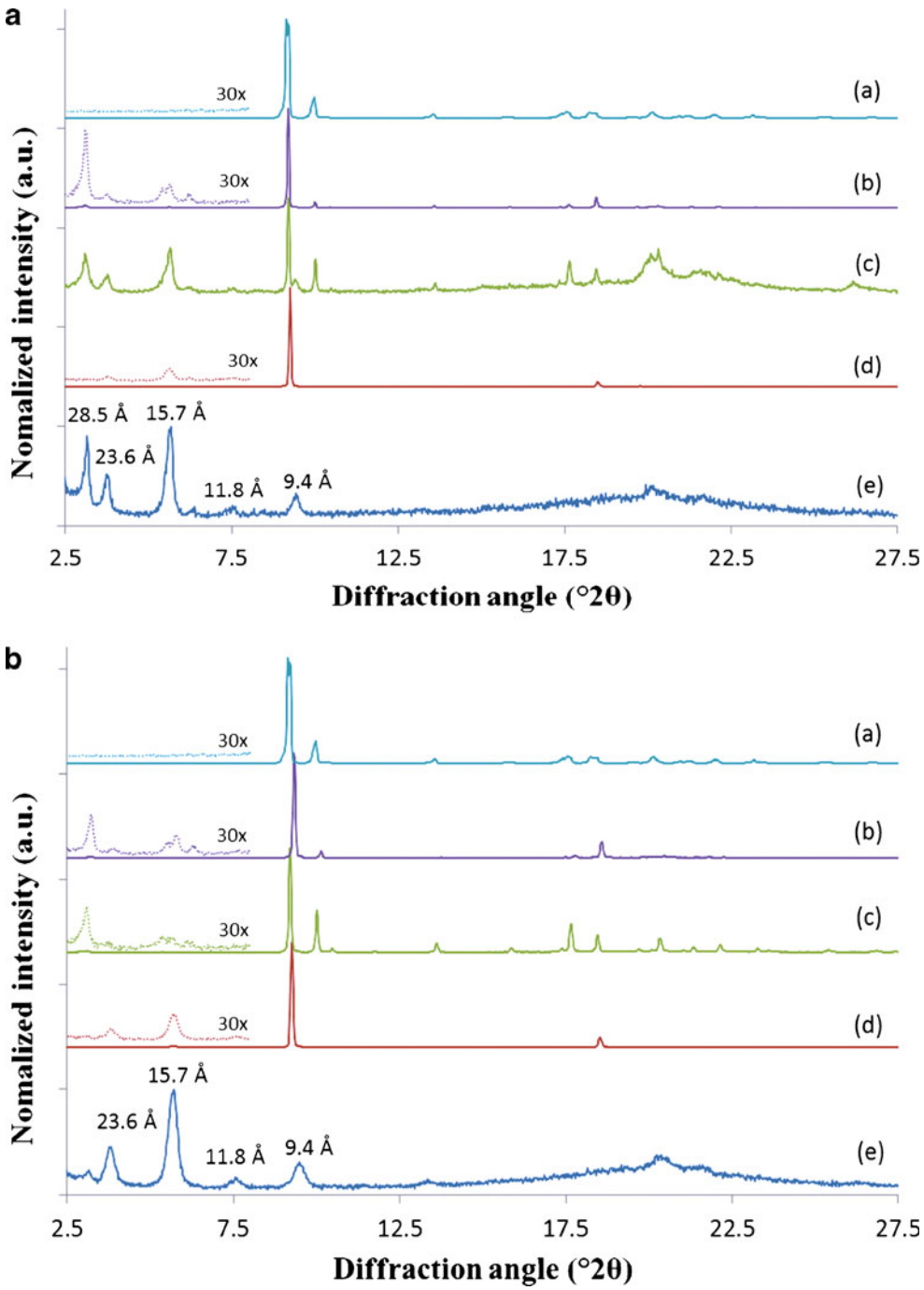

Fig. 2 X-Ray powder diffraction pattern of (**a**) crystalline AE, (**b**) AE-pellet (200 mg/g drug loading), (**c**) AE-pellet (150 mg/g drug loading), (**d**) blank pellet spiked with AE and (**e**) blank pellet from the lipolysis of formulation A (**a**) and formulation D (**b**). The numbers over the peaks indicate d-spacings.

ranged between 2.5 and 3.5% of the donor AE-LBDDS. In contrast, the transport of AE in an oily solution (groundnut or sesame oil) was below the limit of detection (data not shown). The oils and surfactants employed in the AE-LBDDS did not affect the AE transport across Caco-2 monolayers ($p > 0.05$). The size of the oil droplets also did not affect AE permeation ($p > 0.05$). Hence, the following transport studies were only performed with formulations A and D.

To assess the transport of AE-LBDDS as a function of time, the concentration of AE in the basolateral side was determined at 30, 60, 90 and 120 min. Figure 4b shows that AE permeating into the receiver compartment increased linearly ($R^2 > 0.98$), indicating that non-saturable transport occurred as a function of time without any influence of the surfactant.

The effect of the concentration of AE-LBDDS on AE permeation across the Caco-2 cells was investigated. At 0.2, 0.4 and 0.6 mg/ml of AE-loaded LBDDS, the transport rates were 3.60×10^{-4} µg/s, 8.39×10^{-4} µg/s and 7.83×10^{-4} µg/s for formulation A and 3.56×10^{-4} µg/s, 8.03×10^{-4} µg/s and

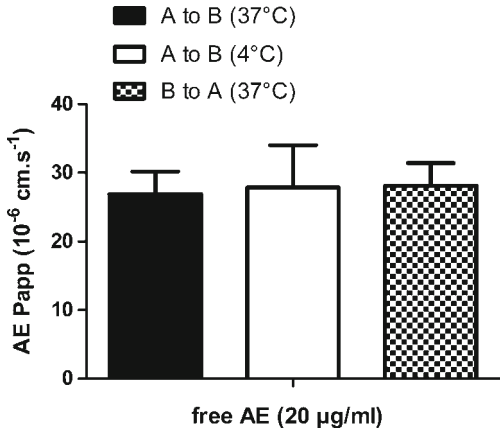

Fig. 3 Apparent permeability coefficient of free AE across Caco-2 monolayers in the apical to basolateral direction (at 37°C and 4°C) and in the basolateral to apical direction (at 37°C). Each value represents the mean ± SD ($n = 3$–4).

9.39×10^{-4} µg/s for formulation D. These results indicate that the transport rates were similar at the same concentration but were not linearly dependent on drug concentration (Fig. 4c).

To examine whether AE-LBDDS were actively transported across the intestinal epithelium, permeation studies were also performed at 4°C. As shown in Fig. 4a, the apparent permeability coefficient of AE-LBDDS across Caco-2 cells at 4°C decreased by 55% on average compared to those obtained at 37°C, suggesting the simultaneous presence of active and passive transport of AE-loaded LBDDS during intestinal absorption, as described previously (10,21–23).

Transport of AE-LBDDS by M Cells

To determine whether M cells, which specialize in the uptake of particles, influence the transport of AE-LBDDS across intestinal mucosa, the inverted co-culture technique using Caco-2 cells and Raji cells developed by des Rieux *et al.* (16) was used as a model. The transport rates of AE-LBDDS obtained in the co-cultures were compared to monocultures of Caco-2 cells, which served as a control. Before and after the experiment, the average TEER values were 250 ± 30 Ωcm^2 and 150 ± 30 Ωcm^2 for the monocultures and co-cultures, respectively. No significant difference in the P_{app} of AE was observed between the co-culture and monoculture ($p > 0.05$, Fig. 5), suggesting that M cells do not enhance the transport of AE-loaded LBDDS.

The AE P_{app} values of the inverted monoculture ($3.05 \times 10^{-6} \pm 0.02 \times 10^{-6}$ cm/s and $4.05 \times 10^{-6} \pm 0.29 \times 10^{-6}$ cm/s for formulation A and formulation D, respectively; Fig. 5) were higher than the mean P_{app} values obtained with the normal monoculture ($2.33 \times 10^{-6} \pm 0.63 \times 10^{-6}$ cm/s and $2.23 \times 10^{-6} \pm 0.35 \times 10^{-6}$ cm/s for formulation A and formulation D, respectively; Fig. 4a) ($p < 0.05$).

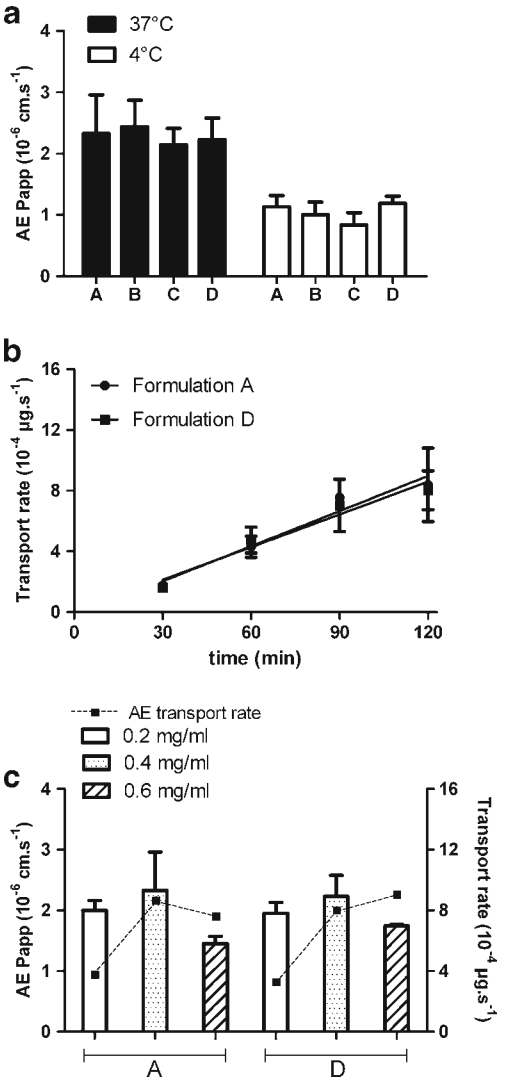

Fig. 4 Transport of AE-loaded LBDDS across Caco-2 cells. (**a**) Apparent permeability coefficients of AE-loaded LBDDS (0.4 mg/ml AE) at 37°C and 4°C. ($N = 2$–3, $n = 3$). (**b**) Kinetics of AE transport at 37°C (0.4 mg/ml AE, formulation A and D) ($n = 4$). (**c**) Concentration dependency of AE-LBDDS across the Caco-2 cell monolayer in the apical to basolateral direction at 37°C. ($N = 2$, $n = 3$). Data are presented as the mean ± SD.

Influence of Mucus on AE-LBDDS Transport

To examine the significance of the mucus layer as a barrier to AE-LBDDS absorption, transport experiments with a mucus-secreting cell culture model using HT29-MTX and Caco-2 cells at different ratios were performed. In presence of mucus, the P_{app} values of AE lipid formulations containing Cremophor EL were not significantly different in comparison to those of the Caco-2 monoculture ($p > 0.05$, Fig. 6a). For formulations containing Tween 80, a significant decrease in the P_{app} values of AE was observed in comparison to the monocultures of Caco-2 cells ($p < 0.05$, Fig. 6b).

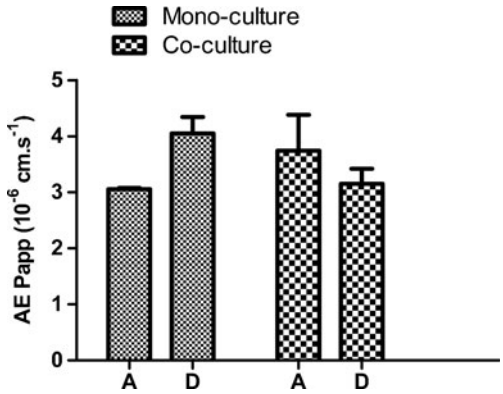

Fig. 5 Influence of M cells on the transport of formulation A and formulation D in Caco-2 monolayers (monocultures) and the follicle-associated epithelium model (co-cultures). Data are presented as the mean ± SD ($n = 3$).

DISCUSSION

The aim of this study was to assess the resistance to lipolysis and the permeation across intestinal cells of β-arteether lipid-based drug delivery systems (AE-LBDDS) developed for the oral delivery of AE (3).

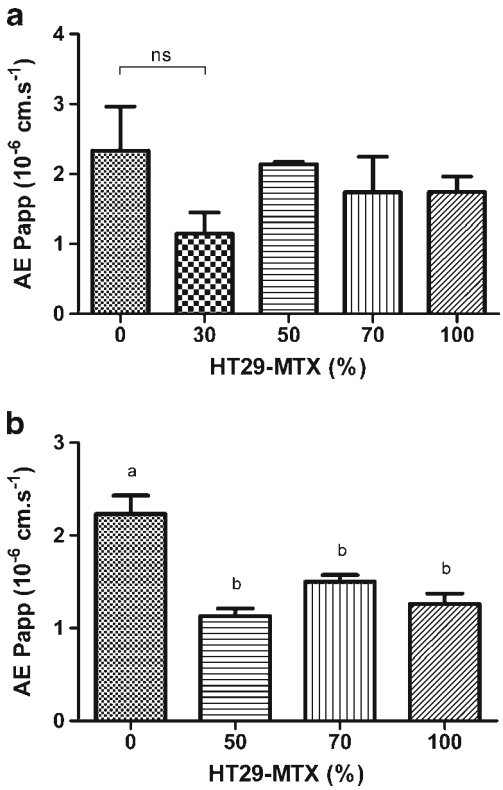

Fig. 6 Influence of mucus on formulation A (**a**) and formulation D (**b**) transport. Each value represents the mean ± SD ($n = 3$). *ns* not significant ($p > 0.05$). Data with different superscript letters are significantly different ($p < 0.05$).

Characterization of AE Lipid-Based Formulations

All the tested AE-LBDDS were capable of self-dispersion in aqueous media, as reported previously (3). These formulations were kinetically stable; no significant modification in oil droplet size was observed after dilution (24). The formulations containing Cremophor EL, which disperse in smaller droplets (< 100 nm), could be considered self-nanoemulsifying drug delivery systems (SNEDDS) (24) . However, formulations containing Tween 80 would be considered self-emulsifying drug delivery systems (SEDDS) because they spontaneously formed oil-in-water (macro)emulsions and were separated into different phases after centrifugation (24,25).

In Vitro Lipolysis of AE Lipid-Based Formulations

During *in vitro* lipolysis, a higher level of AE precipitation was observed for formulations A and B containing Cremophor EL (~30%) compared to formulations C and D containing Tween 80 (~10%) (Fig. 1b and c). The difference observed in drug precipitation may be attributed to the different colloidal structures with different drug solubilization capacities that were generated during the digestion of the various surfactants used in the LBDDS (22,26–28). The formulations containing Cremophor EL presented a larger surface area created by the dispersed oil droplets, which facilitated their enzymatic digestion by pancreatic lipase. Additionally, in equal mass, Tween 80 is less susceptible to enzymatic digestion by pancreatic enzymes and can more efficiently inhibit lipase activity and the lipolysis of triglycerides as compared to Cremophor EL (29,30).

The dissolution rate and solid state of the pellet obtained after lipolysis of AE-LBDDS containing Cremophor EL were analyzed. The data indicated that AE was rapidly solubilized in intestinal medium at a higher concentration than its solubility in FaSSIF, even if AE precipitated in the crystalline form. The re-dissolution could occur as lipid digestion progresses (9). Faster dissolution rates of LBDDS pellets were also observed with cinnarizine and halofantrine, which precipitated as an amorphous solids or molecular dispersions (8,9). The faster dissolution of the crystalline-precipitated AE may be explained by the coexistence of lamellar and non-lamellar phases in the pellet (Fig. 2). Solubilization capacity and re-dissolution of the drug influence the performance of the lipid formulations and are dependent on the bile salt/phosphatidylcholine ratio, the composition of the formulation, the colloidal structure, the nature of the drug and the drug's affinity for the colloidal structure (20,22,26–28,31)

Permeation of Free AE and AE Lipid-Based Formulations Across Intestinal Cells

Artemisinin derivatives (dihydroartemisinin and artesunate) are known to avoid P-gp efflux and to be highly permeable

across intestinal cells (32). However, to the best of our knowledge, this is the first time that the permeability of AE across Caco-2 cells and the influence of P-gp efflux on its permeation have been studied. Because AE has a high permeability coefficient ($P_{app} > 10^{-6}$ cm/s) (33) and poor water solubility, it should be classified under the Biopharmaceutics Classification System as class 2, similar to dihydroartemisinin (32).

The permeability of AE loaded in LBDDS was investigated using Caco-2 cell monolayers. The tested concentration of AE (0.4 mg/ml *i.e.* 100 mg/250 ml) is compatible with the range of human dose (100 mg of AE twice a day). A high level of permeability (P_{app} between 2 and 3.10^{-6} cm/s) was measured and suggested to result from concomitant passive and active diffusion. Due to the presence of a microclimate acidic pH adjacent to the unstirred water layer, the protonation of long chain fatty acids followed by the dissociation of droplet oils may occur to release the lipids and the lipophilic drug that will be transported passively (10,22,34). The hydrolysis of lipid excipients could take place in the apical medium by brush border enzymes (45). These processes would not depend on the size of the droplets, but could be influenced by the depletion of temperature at 4°C. Furthermore, the solubilization of AE in the dispersed oil phase could facilitate its partitioning and diffusion into the aqueous boundary layer and provide a concentration gradient for its absorption (10,21). However, in the absence of dissociation, the colloidal species containing AE could be absorbed actively by endocytosis (10,21,23). The extent of the passive diffusion of lipids compared to their active transport across intestinal epithelium may depend on the prandial state (35).

The influence of M cells on the transport of drug-loaded LBDDS has not been well studied. In the inverted M cell model, there was no significant difference in the P_{app} of AE between the co-culture and monoculture ($p > 0.05$), which suggests that even though self-emulsifying LBDDS are in particulate form and of the appropriate size, their uptake was not enhanced by M cells, in contrast to what has been extensively reported for polymeric nanoparticles (17). This emphasizes the role of the surface of the particles in their interaction with M cells, and our results are consistent with the similar transport of saquinavir when it was loaded onto nanostructured lipid carriers (43). The decrease in TEER observed in this model by the conversion of Caco-2 cells into M cells had no influence on the P_{app} of AE, likely because the intercellular junctions remain tight in mono- and co-cultures (16). However, after eventual re-precipitation of the lipophilic drug in the gastrointestinal tract, the aggregate could be endocytosed by M cells (36,37). In the Caco-2 cell monolayers, a difference was observed between the AE P_{app} of the inverted and normally oriented inserts ($p > 0.05$), which may be explained by the TEER decrease observed in the inverted inserts (200–300 Ωcm^2) compared to the normally oriented inserts (> 700 Ωcm^2) and the larger pore diameter of the insert membranes (13,16).

The transport of AE in the formulations containing Cremophor EL was not significantly affected by the mucus barrier. Several hypotheses can address this observation. First, due to its acidic pH, the mucin unstirred water layer could protonate the long chain fatty acids and then dissociate the (mixed) micellar and vesicular species to release the drug, which will be absorbed passively (10,22,34). Second, the mucoadhesive properties of Maisine 35-1 (38) could help to enhance this phenomenon. Finally, the size and good dispersibility of this formulation could maximize the rate of the drug partitioning into the aqueous boundary layer, thereby providing consistent absorption (21), which may explain why the P_{app} values of the AE lipid formulations containing Tween 80 were lower in the presence of mucus (Fig. 6b). Indeed, the larger droplets did not easily penetrate the mucin mesh, resulting in reduced AE permeation and prolonged absorption (39,40).

The predictive permeability of AE-LBDDS observed in the *in vitro* Caco-2 cells system could be largely influenced by the absence of sink conditions and of a series of complex processes or factors occurring *in vivo* (*e.g.*, bile salts, phospholipid micelles, lipid digestion). Therefore, we attempted assessing the permeability of AE across intestinal cells after lipid digestion of AE-LBDDS. However, this experiment could not be performed due to the cytotoxic effects of bile salts and lipase with and without lipase inhibitors (data not shown, (41,42)). Neither the inactivation of enzymes by heat nor the dilution of digested AE lipid formulations to non-cytotoxic concentrations of enzymes and bile salts (to maintain cell integrity) could be performed in the appropriate experimental settings (data not shown).

CONCLUSION

The present study demonstrated that lipid-based formulations could increase the oral bioavailability of AE, a BCS class 2 drug, across intestinal cells. LBDDS that are partly resistant to *in vitro* lipolysis significantly increased the transport of AE across intestinal cell monolayers. The precipitation of AE from LBDDS containing Cremophor EL, which occurred during *in vitro* lipolysis, should not affect *in vivo* performance because the rapid dissolution profile of precipitated AE was observed in the intestinal medium. The transport of AE loaded in LBDDS was enhanced in comparison to that of free drug due to the increased AE solubility. The apparent permeability (P_{app}) of all AE-LBDDS across Caco-2 cell monolayers was high (approximately 3.10^{-6} cm/s). The intestinal absorption was not influenced by the presence of M-cells or the P-glycoprotein transporter. Further

pharmacokinetic studies are required to confirm that the AE-LBDDS enhance the oral bioavailability of AE, to determine which surfactant would be the most suitable and to investigate whether the precipitation of AE and its redissolution will influence its bioavailability.

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