Solid lipid nanoparticles for delivery of andrographolide across the blood-brain barrier: in vitro and in vivo evaluation.

Solid lipid nanoparticles for delivery of andrographolide across the blood-brain barrier: *in vitro* and *in vivo* evaluation

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Abstract

Andrographolide is a major diterpenoid of *Andrographis paniculata* and possesses several biological activities, including protection against oxidative stress mediated neurotoxicity, inflammation-mediated neurodegeneration, and cerebral ischemia. However, this molecule shows low bioavailability, poor water solubility, and high chemical and metabolic instability. The present study reports preparation of solid lipid nanoparticles (SLN) to deliver andrographolide (AG) into the brain.

SLN were prepared using Compritol 888 ATO as solid lipid and Brij 78 as surfactant and applying emulsion/evaporation/solidifying method as preparative procedure. Nanoparticles have a spherical shape, small dimensions, and narrow size distribution. Encapsulation efficiency of AG-loaded SLN was found to be 92%. Nanoparticles showed excellent physical and chemical stability during storage at 4 °C for one month. The lyophilized product was also stable at 25 °C during the same period. SLN remained unchanged also in the presence of human serum albumin and plasma. *In vitro* release at pH 7.4 was also studied. The release of AG was prolonged and sustained when the compound was entrapped in SLN. ~~and Higuchi model was shown to be the best fit model (R^2>0.96) to describe the kinetics of release~~. The ability of SLN to cross the blood-brain barrier (BBB) was evaluated first *in vitro* by applying a permeation test with artificial membrane (parallel artificial membrane permeability assay, PAMPA) to predict passive and transcellular permeability through the BBB, and then by using hCMEC/D3 cells, a well-established *in vitro* BBB model. *In vitro* results proved that nanoparticles improved permeability of AG compared to free AG. Fluorescent nanoparticles were then prepared for *in vivo* tests in healthy rats. After intravenous administration, fluorescent SLN were detected in brain parenchyma outside the vascular bed, confirming their ability to overcome the BBB.

Keywords: Andrographolide; stealth SLN; stability; PAMPA test; hCMEC/D3 cells*; in vivo* evaluation.

1. Introduction

Andrographolide (AG) is the major diterpenoid in the traditional Asian medicinal plant *Andrographis paniculata* (AP, Burm. f.) Wall. ex Nees (Acanthaceae) and it has received increasing attention due to its various pharmacologic activities as it is hepatoprotective, antiviral, antibacterial, as well as protective against oxidative stress mediated neurotoxicity, inflammation-mediated neurodegeneration, and cerebral ischemia [1-3]. Recently, Serrano et al. [4] showed that AG protects against damage induced by amyloid-β oligomers *in vitro*, lowers amyloid-β levels and tau phosphorylation in mice, modulates the formation of amyloid plaques, and recovers spatial memory functions in Alzheimer disease transgenic mouse model.

In vitro and *in vivo* studies proved that AG modulates complex oxidative stress-related pathways involved in stroke pathogenesis in primary cerebral endothelial cells and it provides positive protection against ischemic stroke [5]. *In vitro* studies also demonstrated that AG inhibits the proliferation of human glioblastoma cells [6]. Furthermore, recent studies suggested that it possesses anti-tumor activity, with various mechanisms being involved. The high lipid solubility of AG permits penetration of the blood–brain barrier (BBB) on the one hand, but on the other reduces its bioavailability due to poor water solubility and high chemical and metabolic instability. Indeed, these factors are the greatest limitations with regard to development of new formulations for clinical use [7,8].

Several approaches have been applied to overcome these obstacles. Xie et al. [9] developed AG nanocrystals to improve solubility and evaluated the impact of different stabilizers and matrix formers on their redispersibility. Chellampillai et al. [10] demonstrated that pH-sensitive nanoparticles significantly improve the dissolution rate and bioavailability of AG after oral administration in male Wistar albino rats. Several nanosystems have been proposed to enhance the anti-inflammatory effect and hepatoprotective properties of AG in recent years. In particular, toward this aim, Roy et al. developed heparin functionalized poly(D-L-lactide-co-glycolic acid) nanoparticles able to alleviate paracetamol hepatotoxicity in mice [11] and cationic modified poly(D-L-lactide-co-glycolic acid) nanoparticles to increase the solubility, the hepatic residence and the cytokine regulation of AG in hepatotoxicity status [12]. Das et al. demonstrated that poly (D-L-lactide-co-glycolic acid) nanocapsules reduced arsenic-induced liver damage in mice compared to free AG [13]. Furthermore, just this year, Qiao et al. proved that amorphous AG nanosuspensions are effective at enhancing oral bioavailability and biological efficacy [14], and Mishra et al. developed a multi-layered nanoemulsion and demonstrated improved hepatoprotection and absorption of AG when orally delivered [15]. Other recently published studies have

shown that the anti-leishmanial activity and anticancer efficacy of AG were considerably ameliorated when it is encapsulated in poly(D-L-lactide-co-glycolic acid) nanoparticles [16]. In addition, two attempts to incorporate AG into solid lipid nanoparticles (SLN) have been proposed: Yang et al. carried out *in vitro* and *in vivo* studies to prove that nanoparticles enhance the antihyperlipidemic property of AG [17], while Parveen et al. evaluated the antitumor activity of AG and obtained better results with SLN compared to the free-molecule [18]. PLGA-nanoparticulation of AG enhanced its anti-cancer properties three fold and the chitosan coating of nanoparticles further accentuated cellular localization and increased cellular toxicity and apoptosis in MCF-7 cells [19].

The challenges regarding poor pharmacokinetic properties are even more evident for drugs directed toward the CNS due to the presence of the BBB [20,21]. For this reason, various strategies have been developed to increase the bioavailability of neurotherapeutics: recent developments of nano-sized delivery systems seem to be the solution to this great problem [22]. These systems provide sufficient drug permeability into the brain and can be functionalized and interact with specific receptors of the BBB, favouring the crossing over of active molecules [23,24].

In this context, we evaluated the use of solid lipid nanoparticles (SLN) to deliver AG through the CNS and ameliorate its biopharmaceutical characteristics.

Essential ingredients for SLN preparation include solid lipids at room temperature and at body temperature, surfactant/s, and water; the lipid matrix is made of biocompatible and biodegradable materials, which decrease the risk of acute or chronic toxicity. SLN present many advantages such as long-term stability, increased bioavailability of encapsulated active ingredient, the possibility to obtain a controlled or targeted release, versatility in encapsulating both lipophilic or hydrophilic drugs, and high efficiency of encapsulation [25,26]. SLN are also potential drug delivery systems for brain targeting [27] as their small size prolongs circulation time in the blood and decreases the burst effect [23,28,29].

Continuing our studies on the development of drug delivery systems able to cross the BBB [30,31], the present study includes the incorporation of AG into SLN. The nanoparticles were prepared by emulsion/evaporation/solidifying method [32] and made using Brij 78, a non-ionic surfactant employed to obtain "stealth" and "long circulating" nanoparticles. This surfactant protects nanoparticles from interaction with plasma components, prolongs their half-life in blood circulation, and increases drug permeability by fluidization of cell membranes [29]. SLN were characterized in terms of average diameter, polydispersity, ζ-potential, morphology, and physical and chemical stability; *in vitro* tests were also performed.

The ability of nanoparticles to increase the permeability of AG was evaluated by Parallel Artificial

Membrane Permeability Assay (PAMPA) [33] and hCMEC/D3 cells, as a well-established *in vitro* BBB model [34,35]. Finally, fluorescent SLN were developed to study *in vivo* distribution and fate after intravenous administration in healthy rats and to evaluate the ability of the delivery system to cross the BBB and reach brain tissues.

2. Experimental

2.1 Materials

Compritol 888 ATO, a mixture of mono-, di- and triglycerides of behenic acid, was a gift of Gattefossé (Milan, Italy). Andrographolide, Brij 78, Fluorescein isothiocyanate (FITC), Human Serum Albumin (HSA), Caffeine, Piroxicam, Progesterone and Phosphate Buffered Saline (PBS 0.01 M) powder (29 mM NaCl, 2.5 mM KCl, 7.4 mM $Na_2HPO_4 \cdot 7H_2O$, 1.3mM KH_2PO_4) pH 7.4 were from Sigma Aldrich (Milan, Italy). 96-well Multi-Screen PAMPA filter plates (pore size 0,45 μm) were purchased from Millipore Corporation (Tullagreen, Carrigtwohill, County Cork, Ireland). Porcine polar brain lipid was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All the solvents from Sigma Aldrich (Milan, Italy) were HPLC grade. Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA) and phosphotungstic acid (PTA) was purchased from Electron Microscopy Sciences (Hatfield, USA).

2.2 Preparation of SLN

Empty SLN were prepared by the emulsion/evaporation/solidifying method [32]. Compritol 888 ATO was dissolved in 5 mL of acetone under magnetic stirring and heated at 50 °C ± 2 °C. Brij 78 was dissolved in 30 mL of water at 75 °C ± 2 °C. The organic phase was added dropwise to the aqueous phase under fast magnetic stirring to obtain an emulsion that was concentrated to 7 mL to induce the evaporation of the organic solvent. The emulsion was added quickly to 7 mL of cold distilled water in an ice bath under fast magnetic stirring to obtain solidified nanoparticles. The colloidal dispersion was frozen and freeze-dried for one night.

AG-loaded SLN (AG-SLN) or FITC-loaded SLN (FITC-SLN) were prepared using the same method, adding AG or FITC (λ_{ex}=492 nm, λ_{em}=518 nm, green) to the lipid phase.

Different percentages of drug were loaded to obtain AG-SLN: 2.5%, 5%, and 10% (w/w respect to the amount of lipid and surfactant). Fluorescent SLN contained 5% of FITC (3.67 mM).

2.3 Characterization of SLN in terms of size, polydispersity index and ζ-potential

Particle size was measured by Light Scattering, Zsizer Nano series ZS90 (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fibre-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water-bath) set at 25 °C. Time correlation functions were analysed to obtain the hydrodynamic diameter of the particles ($Z_{average}$) and the particle size distribution (polydispersity index, PdI) using ALV-60X0 software V.3.X provided by Malvern. ζ-potential was measured using the same instrument and was calculated from the electrophoretic mobility. For all samples, opportunely diluted with distilled water, an average of three measurements at stationary level was taken. The temperature was kept constant at 25 °C by a Haake temperature controller.

2.4 Morphological characterization of nanoparticles

SLN were analysed by transmission electron microscopy (TEM). The opportunely diluted aqueous dispersion was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film, which was stained with phosphotungstic acid solution (1% w/v) in sterile water. Samples were dried for 3 min and were then examined under a JEOL 1010 electron microscope and photographed at an accelerating voltage of 64 kV.

2.5 Differential scanning calorimetry (DSC)

DSC was carried out using a Mettler TA4000 apparatus equipped with a DSC 25 cell. Samples (about 5-10 mg) were accurately weighed (Mettler M3 Microbalance) directly in pierced aluminium pans and scanned between 30 and 250°C at a heating rate of 10 K/min under static air. DSC thermograms of pure AG, Compritol, Brij 78 and AG-SLN were compared.

2.6 Determination of encapsulation efficiency and loading capacity

The amount of AG or FITC entrapped in SLN was calculated using the indirect method [29]. SLN were purified from free drug by ultracentrifugation for 60 min at 11330 x g and 4 °C. Encapsulation efficiency (EE%) and loading capacity (LC%) were calculated considering the difference between the total amount of drug loaded and the amount of AG or FITC determined in the supernatants obtained after purification of the SLN, using the following equations:

$$EE\% = \frac{(Total\ Drug - Free\ Drug)}{Total\ Drug} \cdot 100$$

$$LC\% = \frac{(Total\ Drug - Free\ Drug)}{Weight\ of\ NPs} \cdot 100$$

The content of AG or FITC was quantified by HPLC-DAD or HPLC-FLD analysis, respectively.

2.7 HPLC-DAD and HPLC-FLD methods

Quali-quantitative determinations were carried out using a HP 1200 liquid chromatograph equipped with a DAD and FLD detector. A 150 mm x 4.6 mm i.d., 5 μm Zorbax Eclipse XDB, RP18 column (Agilent Technologies) was used. The mobile phase was (A) CH_3CN and (B) formic acid/water pH 3.2, and flow rate 0.8 ml/min; temperature was set at 26 °C. The following gradient profile was used: 0-2 min, 5-15% A, 95-85% B; 2–5 min, 15% A, 85% B; 5-7 min 15-50% A, 85-50% B; 7-12 min, 50% A, 50% B; 12-15 min, 50-30% A, 50-70% B; 15-20 min, 30% A, 70% B; 20–25 min, 30-5% A, 70-95% B with equilibration time of 5 min. Injection volume was 10 μL. The chromatograms were acquired at 223 nm (for AG), 250 nm (for progesterone), 280 nm (for caffeine) and 350 nm (for piroxicam).

A HPLC-FLD method was used to quantify FITC (λ_{ex}=492 nm, λ_{em}=518 nm, green) of the fluorescent SLN applied in the *in vivo* test. The column was a Lichrosorb C18 (4.6 mm x 100 mm i.d., 5 μm) maintained at 27 °C. The mobile phase was (A) CH_3CN and (B) formic acid/water pH 3.2; flow rate 0.8 ml/min and injection volume 10 μl. The following gradient profile was used: 0-5 min, 10-40% A, 90-60% B; 5-10 min, 40-50% A, 60-50% B; 10-12 min 50-55% A, 50-45% B; 12-15 min, 55% A, 45% B; 15-18 min, 55-90% A, 45-10% B; 18-20 min, 10% A, 90% B with an equilibration time of 5 min.

To quantify NaF (λ_{ex}=460 nm, λ_{em}=515 nm, green), an HPLC-FLD method was used. The column was a Kinetex C18 (4.6 mm x 150 mm i.d., 5 μm) maintained at 27 °C. The mobile phase was (A) CH_3CN and (B) formic acid/water pH 3.2; flow rate 0.8 ml/min and injection volume 10 μl. The following gradient profile was used: 0-3 min, 20% A, 80% B; 3-23 min, 20-80% A, 80-20% B; 23–25 min 80–100% A, 20-0% B; 25–27 min, 100-20% A, 0-80% B with an equilibration time of 5 min.

Standard solutions were freshly prepared by dilutions of stock solutions (0.5 mg/mL) in HPLC grade CH_3OH. An external standard method was applied to quantify each compound. Quantification of individual constituents was performed using a regression curve and the analyses were performed in triplicate.

All compounds showed a linear response: AG from 0.5 to 250 ng/mL, FITC from 6.40 to 520 ng/mL, and NaF from 0.5 to 460 ng/mL. All the curves had coefficients of linear correlation $R^2 \geq 0.999$.

A dilution of standard solutions was used to determine limits of detection (LOD) (S/N ≥ 3) and limits of quantification (LOQ) (S/N ≥ 10). For AG, LOD was 2.6 ng and LOQ 5.3 ng.

2.8 Determination of yield%

The yield of the preparation process of SLN was calculated as weight of the product obtained after the freeze-drying compared to the weight of the components used in the reaction:

$$Yield\ \% = \frac{real\ weight\ (mg)}{teoric\ weight\ (mg)} \times\ 100$$

2.9 Stability studies

Stability of empty and AG-SLN was studied over a one-month period in both nanoparticles as colloidal dispersion and lyophilized product. Aqueous dispersion was stored at 4 °C, while lyophilized SLN were kept at room temperature (25 °C). At determined intervals, the samples were assayed for physical and chemical stability. Physical stability was checked by monitoring size and polydispersity by DLS; chemical stability was assessed by quantification of drug content by HPLC-DAD analysis.

SLN were also incubated in a solution of human serum albumin (HSA) at body temperature under magnetic stirring to mimic *in vivo* conditions. Briefly, 200 µL of dispersion were exposed to 2 mL of albumin solution (40 mg/mL) for 2 h [36,37].

A stability study of AG-SLN was also performed in rat plasma. We collected whole blood into heparinized tubes. Cells are removed from plasma by centrifugation for 10 minutes at 1.000-2.000 x g using a refrigerated centrifuge. Following centrifugation, we immediately transferred the liquid component (plasma) into a clean polypropylene tube using a Pasteur pipette.

Stability of AG-SLN and FITC-SLN was evaluated by adding 250 µL of rat plasma to 50 µL of nanoparticles suspension. After 2 h of incubation, the resulted mixture was opportunely diluted for DLS analysis and particle size and PdI were measured.

2.10 In vitro *release*

In vitro release of AG and FITC from optimized formulations was carried out using dialysis membrane (Cellulose regenerated, cut-off 3-5KD) in PBS/Ethanol 70:30 mixture, in absence and in presence of

HSA at a concentration of 40 mg/mL to mimic *in vivo* conditions [38]. Two millilitres of AG saturated solution (65 μg/mL in distilled water), AG solution (2.80 mg/mL in methanol), AG suspension (2.80 mg/mL in distilled water), AG-SLN (30 mg/mL in distilled water, corresponding to 2.80 mg/mL of AG) or FITC-SLN (15 mg/mL in distilled water) were inserted into pre-soaked dialysis tubes and placed in 200 mL of release medium. The medium was stirred at 37 °C. At pre-determined intervals, 1 mL of release medium was withdrawn and replaced with an equal volume of fresh release medium. The concentration of AG or FITC was defined by chromatographic analysis. ~~The *in vitro* release data were applied to assess release kinetics of the mechanism of drug release.~~

2.11 PAMPA studies

PAMPA studies were performed using the method reported in the literature [33], optimized for our experiments. Caffeine, piroxicam and progesterone were used as standards and solubilised in ethanol at a concentration of 0.5 mg/mL and diluted five times in a mixture of PBS/Ethanol (70/30 v/v). A 96-well filter plate was used as the permeation donor compartment and a 96-well receiver plate was used as acceptor. A solution (2% w/v) of Porcine Polar Brain Lipid (PBL) in n-dodecane was prepared and the mixture was sonicated to ensure complete dissolution. PBL solution (5 μL) was added to each donor plate well [33]. Immediately after the application of the artificial membrane, 250 μL of the free drug or formulation were added to each donor compartment; the acceptor compartment was filled with PBS/Ethanol solution (250 μL). The drug-filled donor compartment was then placed in the acceptor plate. After 18 h of incubation, samples were withdrawn from the donor and acceptor plates and standards or AG concentrations were assessed by HPLC-DAD analyses. In the case of the formulation, 150 μL were withdrawn from both compartments, diluted with methanol and placed in the ultrasonic bath for 30 min, and finally ultracentrifuged for 1 h at 11330 x g (4 °C). Permeability of compounds was calculated using the following formula [39]:

$$P_e = -\ln[1 - C_A(t)/C_{equilibrium}]/A \times (1/V_D + 1/V_A) \times t$$

where P_e is permeability (cm/s), A= effective filter area ($f \times 0.3$ cm^2), where f = apparent porosity of the filter, V_D = donor well volume (mL), V_A = receptor well volume (mL), t = incubation time (s), $C_A(t)$ = compound concentration in receptor well at time t, C_D (t) = compound concentration in donor well at time t, and

$$C_{equilibrium} = [C_D(t) \times V_D + C_A(t) \times V_A]/(V_D + V_A)$$

Experiments were performed in triplicate and the mean of three samples was used for data analysis.

2.12 Blood-Brain Barrier hCMEC/D3 cell culture

Immortalized hCMEC/D3 cells were obtained from Millipore (Cat. # SCC066). This cell line is derived from human temporal lobe microvessels isolated from tissue excised during surgery for control of epilepsy. Cells were seeded in a concentration of 2.5×10^4 cells/cm^2 and grown at 37 °C in an atmosphere of 5% CO2 in 25 cm^2 rat tail collagen type I coated culture flasks. EndoGRO ™- MV Complete Media Kit (Cat. # SCME004) supplemented with 1 ng/mL FGF-2 (Cat. #GF003) was changed every three days and cells were grown until they were 90 % confluent. Cells were passaged at least twice before use. Confluent hCMEC/D3 cells were split by Accumax ™ Cell Counting Solution in DPBS.

2.13 MTT assay

To assess cell viability after AG and AG-SLN exposure, an MTT assay was performed. Cells were seeded in a 24-well plate (6 x 10^4 cells /cm^2) pre-coated with Collagen Type I, Rat Tail (Cat. #08-115) and grown at 37 °C in an atmosphere of 5% CO2 in EndoGRO ™ Basal Medium (EBM-2). When the cells were approximately 70-80% confluent they were incubated with different concentrations of AG (5, 25 and 125 µM) and SLN (0.3 and 0.06 mg/mL, obtained by redispersion in the EBM-2 of the lyophilized formulation) for 2 h. The SLN formulation was previously filtered through 0.4 µm sterile filter units in order to maintain sterile conditions and minimize contaminations. The medium of each well was separated from the cells and stored for LDH assay, and cells were treated with 1 mg/mL of MTT for 1 h at 37 °C and 5% CO2. Finally, DMSO was added to dissolve MTT formation and absorbance was measured at 550 and 690 nm. Cell viability was expressed as a percentage compared to the cells incubated only with EBM-2 (positive control). Triton X-100 was used in the MTT assay as the negative control since detergent action disrupts the cells.

2.14 LDH assay

The LDH assay was performed to determine cytotoxicity after AG and SLN exposure. The medium resulting from incubation of AG and SLN with cells was centrifuged (250 g, 10 min at RT) and the supernatant separated from the deposited cells in each well. This centrifugation process allowed us to remove any wastes and cellular debris as well as AG and SLN. The release of LDH into culture supernatants was detected by adding catalyst and dye solutions of a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Indianapolis, USA). The absorbance values were read at 490 nm and 690 nm. Cytotoxicity was expressed as a percentage compared to the maximum LDH release in the presence of

triton X-100 (positive control). EBM-2 was used as negative control since no cytotoxicity was detected in such conditions.

2.15 hCMEC/D3 cell culture for transwell permeability studies

For all transcytosis assays, high density pore (2 x10^6 pores/cm^2) transparent PET membrane filter inserts (0.4 μm, 23,1 mm diameter, Falcon, Corning BV, Netherlands) were used in 6-well cell culture plates (Falcon, Corning, Amsterdam, Netherlands). Prior to cell barrier coating, the transparent PET membrane filter inserts were coated with rat tail collagen type I at a concentration of 0.1 mg/mL and incubated at 37 °C for 1 h. Inserts were subsequently washed with PBS and incubated for 1 hour after which time PBS was removed and replaced with the assay medium. The inserts were calibrated with assay medium at 37 °C for at least 1 hour. Optimum media volumes were calculated to be 1 mL and 1.2 mL for apical and basolateral chambers respectively. After the transwell inserts were calibrated with assay medium for 1 h, medium was removed and hCMEC/D3 cells seeded onto the apical side of the inserts at a density of 6 x 10^4 cells /cm^2 in 1 mL assay media. 1.2 mL of fresh medium was added to the basolateral chamber. The assay medium was changed every three days following transwell apical insert seeding with hCMEC/D3. Cells were grown to confluence for seven days. hCMEC/D3 monolayers were used as a permeability assay for AG and AG-loaded SLN. Fluorescein sodium salt (NaF) at a concentration of 10 μg/mL was used as integrity control marker with a known permeability coefficient (P_{app}) for this cell line [35]. The integrity of monolayer cells was also confirmed by observation of cultures under phase-contrast microscopy or under bright-field optics using of transparent membranes. The image was viewed using an inverted microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) with a low-power objective (20X). Images were digitized using a video image obtained with a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA).

For permeability studies, AG (5, 25 and 125 μM) and AG-SLN (0.3 mg/mL, corresponding to AG 80 μM) obtained by redispersion in the EBM-2 of the lyophilized formulation were tested and incubated in the apical donor compartment for 1 h. At the end of incubation, the amount of NaF and AG were quantified both in apical and basolateral compartments by HPLC-FLD or HPLC-DAD method. In the case of the formulation, EBM-2 was diluted with methanol and placed in the ultrasonic bath for 30 min, then ultracentrifuged for 1 h at 11330 x g (4 °C). The apparent permeability coefficients (P_{app}) of free AG and AG encapsulated in SLN was calculated according to the equation [40]:

$$P_{app}\ (cm/s) = V_D / (A\ M_D) \times (\Delta M_R / \Delta t)$$

where V_D = apical (donor) volume (cm^3), M_D = apical (donor) amount (mol), $\Delta M_R/\Delta t$ = change in amount (mol) of compound in receiver compartment over time.

Recovery for AG and NaF was calculated according to the equation [35]:

$$Recovery\ (\%) = C_{Df}V_D + C_{Rf}V_R / (C_{D0}\ V_D) \times 100$$

where C_{Df} and C_{Rf} are the final concentrations of the compound in the donor and receiver compartments, C_{D0} is the initial concentration in the donor compartment and V_D and V_R are the volumes in the donor and receiver compartments, respectively. All experiments were performed at least in triplicate.

2.16 In vivo *experiments*

One-month-old, 100-150 g Wistar rats (Harlan, Milan, Italy) (n=3-4/group) were used, divided into the following groups:

group 1): sacrificed after 3 h from injection of 200 μL of nanoparticles

group 2): sacrificed after 24 h from injection of 200 μL of nanoparticles

group 3): sacrificed after 72 h from injection of 200 μL of nanoparticles

group 4): controls (200 μL of saline)

group 5): sacrificed after 3 h from injection of FITC solution (200 μL of 0.1 mg/mL of FITC dissolved in saline)

Animals were housed in macrolon cages with ad libitum food and water and maintained on a 12-h light/dark cycle at 23 °C. All experiments were carried out according to the EC Directive 86/609/EEC for animal experiments and national guidelines for animal care (Permit Number: 152/2014-B). All efforts were made to minimize the number of animals used and their suffering.

2.17 Intravenous administration of fluorescent SLN

To evaluate the ability of SLN to cross the BBB, rats were acutely dosed with SLN, FITC solution or vehicle (0.9% NaCl) by intravenous injection via the tail vein. At the time of sacrifice, rats were perfused transcardially with 500 mL of saline solution (0.9 %) to remove blood components and then with 4 % ice-cold paraformaldehyde in 0.1 M PBS, pH 7.2, under deep anaesthesia (chloral hydrate, 400 mg/kg, i.p.). After sacrifice, brains were quickly extracted and fixed in 4% paraformaldehyde for 24 h. Subsequently, brains were rinsed with PBS for 24 h, dehydrated using an automated machine, and embedded in paraffin. Coronal sections (5.0 μm) were cut using a microtome and mounted on slides.

2.18 Immunohistochemistry

Immunohistochemistry was performed on 5.0 μm coronal paraffin-embedded sections, as previously described [31]. The sections were incubated overnight at 4 °C with the primary antibody (Ab) anti-CD34 (1:50) and anti-IBA1 (1: 200) diluted in 0.1 M PBS, pH 7.4, containing 0.3% Triton X-100 and 5.0 mg/mL BSA. On day 2, sections were incubated for 2 h in the dark with the appropriate fluorescent secondary Ab (Alexa Fluor 594 conjugated monoclonal anti-mouse and polyclonal anti-rabbit Ab; Invitrogen, New York, USA) diluted 1:400. At the end of incubation, sections were washed in PBS-Triton and subsequently covered with a cover slip using a specific aqueous upright for viewing preparation by the microscope in fluorescence (Vectashield mounting medium plus nuclear marker DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), VectorLaboratories, Burlingame, CA). Sections were then analysed using an epi-fluorescence microscope connected to a digital camera (Olympus BX63, MI, Italy) and the most significant images were acquired using the CellSensDimension Software (Olympus, MI, Italy).

2.19 Statistical analysis

Experiments were repeated three times and results expressed as a mean ± standard deviation. Statistical significance was calculated by Student's t-test with statistical significance level set at $P < 0.05$.

The results of LDH and MTT tests were expressed as a mean ± standard error of the mean.

3. Results and discussion

3.1 Preparation and characterization of SLN

Natural products have played a key role in treating and preventing human diseases since ancient times and they still represent a significant source of modern drugs. Drug discovery has undergone transformation over time with it becoming clear that targeting single sites has low therapeutic value against multifactorial and complex diseases, especially cancer and diabetes [41,42]. By contrast, various natural products can modulate multiple targets, activating multiple signalling or functional pathways [43,44]. However, their efficacy can be limited due to their low hydrophilicity and instability. In addition, they can present scarce absorption [45], poor pharmacokinetics and bioavailability, and low targeting efficacy. Novel nanoformulations based on drug delivery systems offer significant promise in overcoming these limitations. Nanoparticles have emerged as versatile, biodegradable, biocompatible, non-antigenic nano-carriers for the specific and targeted delivery of drugs to organs and tissues, which

is particularly relevant in cancer therapy where most of the biological processes occur at the nanometer level [45,17]. Many of these studies are very promising and they have resulted in nanoformulations with sustained release and improved bioavailability at much lower doses than conventional preparations, and in many cases presenting a better safety profile.

In this context, we designed SLN using Compritol and Brij 78 to deliver AG to the brain and to overcome its biopharmaceutical limitations. The components under study were selected for their biocompatibility, biodegradability, and versatility [32,46]. Compritol is a mixture of glycerol with behenic acid esters, while Brij 78 is a non-ionic and non-toxic surfactant, conferring "stealth" characteristic to the nanoparticles, with the possibility to realize a constant and prolonged release [32]. Different lipid/surfactant ratios were tested (Table 1).

In the presence of a greater quantity of lipid, nanoparticles were larger (300 nm), highly polydispersed and not suitable for parenteral administration. Nanoparticles became more homogeneous by increasing surfactant amount. SLN obtained with the gravimetric ratio 1:3 showed the best technological parameters and they resulted suitable for administration by the intravenous route (Table 2). Their high ζ-potential confirmed stability of the system.

Different percentages (2.5%, 5% and 10%) of drug were loaded to obtain AG-SLN. Nanoparticles with AG 10% have similar characteristics to the empty carrier (Table 2). For this level, EE% and LC% were of 92.4% ± 1.77% and 8.4% ± 0.16% respectively. TEM images confirmed DLS results and they reveal that all nanoparticles were nearly spherical and uniform in shape (Supplementary material, Fig. S1).

In view of an *in vivo* administration, fluorescent nanoparticles (5% of FITC, [47]) were prepared, using the same method described for AG-SLN. Fluorescent SLN maintained dimensions below 300 nm with a good size distribution suitable for parenteral administration (Table 2), with EE% and LC% resulting as 78.7% ± 0.670% and 3.7% ± 0.04%, respectively.

3.2 Stability studies

The conservation of SLN as lyophilized product offers advantages in terms of stability and formulation versatility. Toward this aim, AG-SLN were lyophilized overnight, producing a voluminous and spongy solid which was easily dispersible in water. The yield percentage of the whole preparative process, including the lyophilisation, was 99% ± 0.053, EE% and LC% resulted 92% ± 0.56% and 8.3% ± 0.012% respectively.

As lyophilized powder or an aqueous dispersion, AG-SLN were maintained at a temperature of 4 °C and 25 °C, respectively, for one month and analysed at regular intervals. No significant changes were observed in terms of size, PdI and ζ-potential values for both the products (Fig. 1). In addition, the EE% remained close to 92-95% (Supplementary material, Fig. S2). The presence of the surfactant gives a high ζ-potential value that prevents precipitation and aggregation of nanoparticles.

The stability of SLN in presence of HSA at physiological concentration was also evaluated. DLS analysis confirmed that sizes were not affected, thus revealing a coexistence of free serum proteins and optimized nanocarrier without any protein corona effect [48, Supplementary material Table S1]. This can be attributed to the protective action of the hydrophilic PEG chain present in the chemical structure of Brij 78.

In the case of stability in rat plasma, no aggregation phenomena occurred after 2 h of incubation. DLS analysis revealed that particle size remained around 260 nm (262±5.34) for AG-SLN and 270 nm (273±4.57) for FITC-SLN and no significant changes in PdI values were observed (0.20±0.010 and 0.19±0.020, respectively), confirming the good results obtained in presence of HSA solution and stealth properties of developed SLN.

3.3 DSC studies

DSC analyses were performed to assess drug-lipid interactions [49]. The following samples were analyzed: pure AG, Compritol, Brij 78, physical mixture of empty SLN and AG, and AG-SLN. Figure 2 depicts the DSC thermograms obtained. The observed melting peak at 72 ºC might be due to a mixture of metastable polymorphic β and β' forms of Compritol. DSC analysis of AG indicates the melting point at 230 ºC and at 46 °C for Brij 78. In the thermogram of AG-SLN, an AG melting peak was not recorded due to its amorphisation or nearly complete molecular dispersion into the lipid matrix. As previously reported, the absence of a melting peak for a crystalline drug is due to its solubilisation and/or amorphisation in the nanoparticle formulation [38]. On the contrary, the melting peak of Compritol was detectable both in empty and in AG-SLN, even though it appeared clearly reduced in intensity and slightly shifted, indicating a loss of crystallinity with respect to the pure lipid. This is due both to the presence of other components in the formulation and to the preparation method of nanoparticles [38,50]. To exclude that AG is present as a crystalline compound in the SLN, we also analysed the mixture of empty SLN and AG (Fig. 2, PM free AG + SLN). In this case, the AG melting peak is visible, demonstrating that the drug is present in an amorphous state when loaded into SLN.

3.4 In vitro *release*

The dialysis bag method was used to evaluate the AG and FITC *in vitro* release from optimized formulations [38,51]. The release profiles (Fig. 3) of AG saturated solution in water, AG solution in methanol, AG suspension in water, AG-SLN in water and in the presence of HSA are reported.

The release rate of AG from SLN was slower than that from the solutions and faster than that from the suspension, as also reported in the literature [52]. In the case of aqueous saturated solution, about 50% of AG was released after 4 h; the percentage increased to 75.3% at 24 h and reached 85.6% at 72h. 86.7% of AG was released from methanol solution at 4 h, whereas the percentage decreased to 35.3% at 6 h for AG-SLN, and the release was prolonged for approximately 72 h until a percentage of 67.0%. The release of AG from suspension was slower compared with AG-SLN, only 15.6% of AG was released within 72 h.

AG-SLN showed a rapid release (burst release) in the first six hours. This is due to the fast release of the adsorbed and/or precipitated molecules from the drug-enriched shell around the particles. In the remaining time, a slower release over 72 h (referred to as sustained release) was observed. During this time AG dispersed within the core was released slowly from the lipid matrix. A slow release was expected due to the solid nature of the nanoparticles [53]. In this context, the use of SLN increases the solubility of AG and guarantees a prolonged release. Finally, given the low aqueous solubility of AG (0.07 mg/mL), its encapsulation into SLN allows the administration of the molecule at a higher concentration (30 mg/mL contained 2.80 mg/mL of AG), an important aspect for parenteral administration and for a possible reduction of the administered dosage with the aim to decrease the toxicity of AG at high dosages, as reported in the literature [54].

~~Drug release mechanism was defined by fitting AG release data to various kinetic models. By comparing the correlation coefficient values R^2 (Zero order: 0.7953, First order: 0.8852, Korsmeyer-Peppas: 0.5789, Hixson: 0.8571, Higuchi: 0.9604) from the applied models, the Higuchi model ($R^2 = 0.9604$) was shown to be the most adequate to describe the kinetics of SLN formulation. Thus, the diffusion process controlled the release mechanism. If analyzed according to Korsmeyer–Peppas model, the release exponent n was 0.7862, corresponding to anomalous release.~~

In presence of HSA, the AG release from SLN remains functional for a sustained release of molecule and is identical to release without HSA.

The release of FITC from fluorescent SLN was evaluated to determine the stability of the probe within nanoparticles during the *in vivo* experiments. The test confirmed that FITC is not released until 24 hours (Supplementary material, Fig. S3).

3.5 PAMPA studies

PAMPA assay is a particularly helpful complement to the cellular permeability model for its speed, low cost and versatility, and readily provides information about passive-transport permeability. Different solvents (DMSO, MeOH, EtOH and PBS) were considered to optimize the test for standard compounds. Ethanol resulted the best one, as also reported by Salado et al. [55]. After the incubation time (18 h), solutions were taken from acceptor and donor compartments and analysed to estimate the permeability values (Pe). The values obtained for standards reflected the classification reported in the literature for PAMPA-BBB test [36,47]: caffeine and piroxicam have low permeability and progesterone resulted as a compound with high BBB permeability.

In PAMPA-BBB assay, AG resulted as a molecule with low permeability, with a P_e value of $0.487 \pm 0.155 \times 10^{-6}$ cm/s and therefore a perfect candidate to be encapsulated into SLN. P_e of AG loaded into SLN was found to be increased ($3.25 \pm 0.086 \times 10^{-6}$ cm/s). The value confirmed that nanoparticles also increased the permeability of AG compared to AG alone in aqueous solution. PAMPA is a useful test to assess the passive permeability of both single molecules and their formulations, as previously reported [56].

3.6 MTT and LDH assays

MTT and LDH assays were performed in hCMEC/D3 cell line to evaluate the effect of AG and AG-SLN in cell viability and cytotoxicity, and permeability studies were also conducted in transwell devices using the same cell line. The *in vitro* cytotoxicity of the developed SLN and AG was assessed by cell viability determination and membrane integrity evaluation using the hCMEC/D3 cell line in MTT and LDH assays, respectively (Fig. 4). When cells were exposed to different concentrations of AG (25 and 125 µM) and AG-SLN (0.06 and 0.3 mg/ml) for 2 and 4 h, no significant changes were observed in MTT metabolisation or LDH release when compared to cells exposed to the EBM-2 medium alone, indicating that AG and AG-SLN affected neither the metabolic activity of the cells nor the membrane integrity at these time points. On other hand, when the cells were incubated for 24 h with AG at a dose of 125 µM and AG-SLN (0.06 and 0.3 mg/ml) we observed cellular death. These data are in accordance with Yen and colleagues who observed AG induced cellular toxicity in a dose-dependent manner in CEC cell lines [54]. The same authors evidenced *in vivo* that AG at a rather low dose (0.1 mg/kg) protects against brain injury, but worsens it at a higher dose (5 mg/kg).

3.7 BBB permeability studies

hCMEC/D3 brain microvascular endothelial cell line is an easily grown model of human BBB that is amenable to cellular and molecular studies on pathological and drug transport mechanisms with relevance to the CNS [34,57]. hCMEC/D3 cells retain the expression of most transporters and receptors expressed *in vivo* at the human BBB.

hCMEC/D3 permeability coefficients (P_{app}) correlate well with *in vivo* permeability data therefore permeability studies were performed to predict the permeability of free AG and AG-SLN across the BBB. NaF was used as negative control and its P_{app} was determined during all transport experiments in absence and in presence of AG or AG-SLN to monitor the integrity of the cell layer. The integrity was also assessed by observation of cultures under phase-contrast microscopy or under bright-field optics using transparent membranes.

The P_{app} of NaF after 1 h was 11.1 ± 1.81 x 10^{-6} cm/s, in agreement with the literature value (12.5 ± 0.326 x 10^{-6} cm/s, [35,58]). This data confirms the confluence of the monolayer and assesses the tight junction integrity of the cell layer, because this fluorescent molecule has low BBB penetration and it was used as barrier integrity marker for *in vivo* models. Permeability results (P_{app} values) after 1 h of experimentation are reported in Table 3.

The first experiments were done with free AG at 5, 25 and 125 μM, with co-incubation with NaF at 10 μg/mL as negative control. The P_{app} of AG 5 μM for the apical to basolateral transport could not be calculated because AG was below the limit of detection. At higher concentrations, P_{app} of AG was 10.2 ± 1.70 x 10^{-6} cm/s and 14.5 ± 1.70 x 10^{-6} cm/s for 25 and 125 μM concentrations, respectively, proving that the molecule did not significantly permeate the hCMEC/D3 cell monolayer because its P_{app} value was similar to that of NaF.

After one hour of transport across the hCMEC/D3 cell monolayer [58], the P_{app} value recorded with SLN loaded with 80 μM of AG significantly increased the permeability of AG, reaching a value of 26.8±4.17 x 10^{-6} cm/s, about three-fold higher than for free AG. This data correlated well with the PAMPA results. The P_{app} of negative control remained unchanged and barrier integrity was preserved during the test. Furthermore, during the experiment, the hCMEC/D3 monolayer remained intact with no relevant morphological changes, as demonstrated also by observation of cultures under phase-contrast microscopy or under bright-field optics before and after each permeability test (Supplementary material, Fig. S4). The recovery of AG and NaF was above 80% in all experiments, suggesting an acceptable *in vitro* prediction of P_{app} values. As reported in the literature [57, 59], the main mechanism involved in the

SLN uptake by hCMEC/D3 cells was found to be clathrin-mediated endocytosis. Nanoparticles may partially follow lysosomal and endosomal trafficking inside cells. In fact, the lysosomal pathway is responsible for particle degradation and release of drug content inside cells, while the endosomal trafficking may be involved in the transport of intact drug loaded NPs from one side to the other of the cell barrier.

The stability of AG-SLN during the experiment was assessed by TEM analyses of the medium of apical and basolateral compartments at the end of the assay. The images (Fig. 5) confirmed the integrity of nanoparticles after permeation through the cell layer and the endocytosis as main transport mechanism.

3.8 In vivo *experiments*

Observation of fluorescent SLN localization after administration into the caudal vein of rats was carried out by analysing preparations under fluorescence microscope. Initially the presence of SLN was evaluated in groups of rats sacrificed at 3, 24 and 72 h after injection (Fig. 6 A). Based on histochemical analysis, with DAPI (blue), a high presence of SLN (green) was observed in the brain of animals sacrificed 24 h after injection (Supplementary Material, Fig. S5). In animals sacrificed 3 and 72 h after injection, a smaller number of SLN was identified probably due to the fact that in the few hours from administration, the "long circulating" nanocarriers are located predominantly into the blood stream and after three days they are mostly degraded. In the control group (saline) no green fluorescence was observed, thus confirming the presence of SLN in the other groups and excluding a possible autofluorescence of the tissue. Subsequently, in the group of rats sacrificed at 24 h and characterized by an abundant presence of nanoparticles, the localization of SLN was further evaluated. By immunohistochemical analysis with CD34 antibody, an endothelial cell marker, nanoparticles were found both at vessel level (white arrows) and in brain parenchyma (yellow arrows) (Fig. 6 B). In the saline injected rats (Fig. 6 B) no fluorescence was detected in either the vessels or brain tissue, further excluding the presence of nonspecific tissue autofluorescence in the SLN-administered rats. In the FITC solution injected rats some fluorescence was detected within few brain vessels (arrow) but not in the brain parenchyma (Supplementary Material, Fig. S6). Then, by means of IBA1 antibody that recognizes microglial cells, we evaluated whether the administration of SLN leads to any microglia activation. As shown in Fig. 6 C, the morphology of microglial cells (red) in different brain areas of rats, sacrificed 24 h after intravenous injection of SLN (green), is typical of animals at rest and not different from that of saline-injected rats. This lack of microglia activation likely indicates that the brain macrophages do not recognize SLN as foreign bodies.

These findings in agreement with data reported in the literature about the transport mechanism of SLN across the BBB [57]. The relative strategy employed by SLN involves endocytosis influenced by developing concentration gradient followed by membrane fluidity and transcytosis of particles through the endothelium.

Kaur et al. [60] reported that SLN may likely permeate the BBB. Once the nanoparticle enters the endothelium it has to counteract the microvascular environment, which consists of astrocytes, pericytes, neurons, and extracellular matrix in order to cross the BBB. Our formulation possesses a highly lipophilic characteristic and charge, which helps the nanoparticles overcome astrocyte and pericyte attack, thereby facilitating the permeation of drug across the neurovascular junction.

Altogether, these preliminary data indicate that the formulation containing "stealth" SLN is able to pass the BBB and reach the brain tissues that do not recognize them as a foreign agent. In fact, the surface coating with Brij 78 reduces opsonization, phagocytosis, and clearance by the liver and reticuloendothelial system; consequently, the retention time of SLN in plasma was increased. Furthermore, the improved blood residence time in brain capillaries, with absorption to the capillary walls, creates a higher concentration gradient that enhances penetration across the endothelial cell layer. In addition, SLN could cross the BBB intact by transcytosis [60-63].

4. Conclusions

Novel nanoformulations based on drug delivery systems offer significant promise in overcoming the limitations of AG. In the present study, we have established that stealth-SLN were successful in enhancing AG permeation in *in vitro* BBB models, PAMPA, and hCMEC/D3 cells. Furthermore, the ability of nanoparticles to cross the BBB and reach brain tissues was confirmed by *in vivo* test in healthy rats.

SLN have physical characteristics for systemic administration in terms of particle size, polydispersity, encapsulation efficacy, and ζ-potential. *In vitro* drug release studies have revealed that SLN release AG in a sustained and controlled manner. SLN show excellent chemical and physical stability as both suspension and lyophilized product. *In vitro* transport studies performed with PAMPA and hCMEC/D3 cells revealed that SLN were successful in enhancing the permeation of AG while *in vivo* studies confirmed that nanoparticles could cross the BBB and reach the brain tissues. This suggests that the developed nanocarriers represent new potential brain delivery systems to increase the efficacy of AG in the field of neurodegenerative diseases.

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Captions to Figures

Fig. 1. Particle size, polydispersity index (PdI) and zeta-potential of AG-SLN as dispersion or lyophilized product after one-month storage at 4 °C (dispersion) and 25 °C (lyophilized product). (Data displayed as mean ± SD; n=3).

Fig. 2. DSC curves of pure components lyophilized AG-SLN and mixture of empty SLN and AG.

Fig. 3. *In vitro* release profiles of AG solution, AG saturated solution, AG suspension, AG-SLN and AG-SLN in the presence of human serum albumin (HSA). (Average values ± S.D. of n=3 experiments)

Fig. 4. hCMEC/D cell viability evaluated by MTT assay (A) and cytotoxicity by LDH assay (B) when exposed for different time periods (2, 6 and 24 h) to AG (25 and 125 µM) or AG-SLN (0.06 and 0.3 mg/mL). Data are expressed as percentage of control (EBM-2 medium) and Triton-X (TTX) which represent, respectively, the maximum cell viability and cell cytotoxicity. Values represent the mean ± SEM of at least three experiments performed in triplicate. *$P < 0.05$ and **$P < 0.01$ vs. EBM-2 alone.

Fig. 5. TEM images of AG-SLN into apical (left) and basolateral (right) compartment after 1 h of the *in vitro* permeation test (scale 300 nm).

Fig. 6. A) Intravenous administration of fluorescent SLN: representative photomicrographs showing the presence of SLN (green) in brain parenchyma of rats 3, 24 and 72 h after injection. Note the high presence of fluorescent SLN at 24 h after injection. Scale bar = 20 µm applies to all images. B) Immunohistochemical detection of SLN (green) with the endothelial marker CD34 (red) in the brain of rats 24 h after injection. SLN are detected both within blood vessels (white arrows) and brain parenchyma (yellow arrows). A-B) No SLN are detected in the brain of rats injected with saline. Scale bar = 20 µm applies to both images. C) Immunohistochemical detection of SLN (green) with the microglia antibody IBA1 (red) in the brain of rats 24 h after injection. SLN are not detected within microglial cells and no microglia activation is detected compared to saline injected rats. Scale bar = 20 µm applies to both images. A-C) DAPI is in blue.

Compritol:Brij 78 (w/w)	Size (nm)	Pd	ζ-potential (mV)
2.5:1	321 ± 7.07	0.277 ± 0.00710	-31.2 ± 0.416
2:1	408 ± 5.08	0.443 ± 0.0862	-30.2 ± 0.794
2:1.5	384 ± 27.7	0.492 ± 0.0601	-32.2 ± 0.974
1:1.5	293 ± 6.61	0.229 ± 0.0210	-32.2 ± 0.737
1:2	283 ± 3.54	0.212 ± 0.0120	-36.6 ± 0.643
1:3	266 ± 3.97	0.184 ± 0.00321	-36.4 ± 0.532

Table 1. Physical characterization of empty SLN obtained with different Compritol:Brij 78 w/w ratios. (Data displayed as mean ± SD; n=3).

Sample	Size (nm)	PdI	ζ-potential mV	EE%	LC%
SLN	266±3.97	0.18±0.031	-36.4±6.32	-	-
FITC-SLN	278±9.55	0.18±0.0020	-36.1±0.921	78.7±0.670	3.7±0.04
AG-SLN 2.5%	278±5.17	0.19±0.032	-29.6±0.254	81.2±0.984	1.9±0.21
AG-SLN 5%	264±1.65	0.13±0.040	-30.4±0.481	86.0±1.77	4.2±0.12
AG-SLN 10%	262±7.23	0.18±0.029	-36.0±4.12	92.4±1.77	8.4±0.16

Table 2. Physical characterization of empty, AG and FITC-loaded SLN. (Data displayed as mean ± SD; n=3).

Sample	P_{app} (x 10^{-6} cm/s)		
NaF	11.1 ± 1.81		
AG 5 μM	N.D.	NaF	11.3 ± 0.674
AG 25 μM	10.2 ± 1.70	NaF	13.2 ± 1.91
AG 125 μM	14.5 ± 2.90	NaF	12.4 ± 1.84
AG-SLN	26.8 ± 2.17	NaF	12.7 ± 0.702

Table 3. Apparent permeability coefficients (Papp) for AG at different concentrations, for AG-loaded SLN and for NaF as barrier integrity control. (Data displayed as mean ± SD; n=3).