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Full Length Article

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. 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.

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 research suggested that it possesses antitumor activity, with various mechanisms being involved. The high lipid solubility of AG permits penetration of the blood–brain barrier

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(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 $Na_2HPO_4{\cdot}7H_2O$, 1.3 mM 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-Qplus 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-60 × 0 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,