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PLGA nanoparticles loaded with the antileishmanial saponin β-aescin: Factor influence study and *in vitro* efficacy evaluation

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ABSTRACT

Colloidal carriers are known to improve the therapeutic index of the conventional drugs in the treatment of visceral leishmaniasis (VL) by decreasing their toxicity whilst maintaining or increasing therapeutic efficacy. This paper describes the development of poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles (NPs) for the antileishmanial saponin β-aescin. NPs were prepared by the W/O/W emulsification solvent evaporation technique and the influence of five preparation parameters on the NPs' size (Z_{ave}), zeta potential and entrapment efficiency (EE%) was investigated using a 2^{5-2} fractional factorial design. Cytotoxicity of aescin, aescin-loaded and blank PLGA NPs was evaluated in J774 macrophages and non-phagocytic MRC-5 cells, whereas antileishmanial activity was determined in the *Leishmania infantum ex vivo* model. The developed PLGA NPs were monodispersed with Z_{ave} < 500 nm and exhibited negative zeta potentials. The process variables 'surfactant primary emulsion', 'concentration aescin' and 'solvent evaporation rate' had a positive effect on EE%. Addition of Tween® 80 to the inner aqueous phase rendered the primary emulsion more stable, which in its turn led to better saponin entrapment. The selectivity index (SI) towards the supporting host macrophages increased from 4 to 18 by treating the cells with aescin-loaded NPs instead of free β-aescin. In conclusion, the *in vitro* results confirmed our hypothesis.

1. Introduction

Leishmaniasis is a protozoan infection that is transmitted by means of sandfly bite to vertebrate hosts including man. The potentially fatal visceral form, *i.e.* visceral leishmaniasis (VL), is caused by *Leishmania donovani* in the Indian subcontinent, Asia and Africa, *Leishmania infantum* in the Mediterranean basin and *Leishmania chagasi* in South America. According to the World Health Organization (WHO) about 500,000 new cases of VL are considered to occur every year globally. Further, the incidence of VL is thought to increase and its endemic areas are thought to expand due to environmental changes, migration and HIV-VL co-infection. Control of VL relies almost exclusively on chemotherapy, which consists of a handful of drugs with serious limitations such as drug unresponsiveness, difficult route of administration, high cost and toxicity (Chappuis et al., 2007; Kedzierski et al., 2009; Maltezou, 2010). Although miltefosine and paromomycin were registered as clinical agents against VL in the last decade, the antileishmanial drug arsenal still requires improvement (Richard and Werbovetz, 2010). For instance, miltefosine monotherapy has failed to cure relapsing VL in HIV-infected patients and thus its role against the increasing problem of HIV-associated VL remains unclear (Ezra et al., 2010). In light of these limitations, WHO strongly recommends and supports research into new drugs including natural plant products (DNDi annual report, 2008; Frézard and Demicheli, 2010).

Saponins are widely distributed in higher plants and have a wide range of biological and pharmacological properties such as antimicrobial, anti-inflammatory and antitumor activities. These surface-active glycosides can be classified into three groups based on the nature of their aglycone skeleton: (i) triterpenoid saponins, (ii) steroidal saponins and (iii) steroidal alkaloids (Sparg et al., 2004). The former group is most common and triterpenoid saponin extracts from *Maesa balansae*, *Careya arborea* and *Astragalus oleifolius* were reported to possess antileishmanial activity (Dinda et al., 2010). Recently our research group added β-aescin, the main saponin from the seeds of *Aesculus hippocastanum* (horse chestnut), to this list (Van de Ven et al., in press). β-aescin

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appeared to be moderately active on the intracellular amastigote stage of *L. infantum* with an average IC_{50} of 1.55 ± 0.32 μg/ml and inactive on the extracellular promastigote stage ($IC_{50} > 16$ μg/ml), suggesting a macrophage-mediated mechanism as is the case for the pentavalent antimonials (Kedzierski et al., 2009). The exact mechanism of action of β-aescin remains unknown and deserves further research, but saponins were previously shown to exert their antileishmanial activity through the induction of apoptosis or programmed cell death in the parasite (Delmas et al., 2000; Dutta et al., 2007). Saponins, however, also have undesirable properties such as high cytotoxicity, rendering them less suitable candidates for drug development (Kedzierski et al., 2009). In this study, we emphasise the strategy of using polymeric nanoparticles (NPs) composed of poly(D,L-lactide-co-glycolide) (PLGA) as delivery vehicle for β-aescin to circumvent the saponin's toxicity. Colloidal carriers are known to improve the therapeutic index of the conventional drugs in the treatment of VL (Gupta et al., 2010). The mechanism is presumed to be a facilitated delivery of the drugs to macrophages of liver, spleen and bone marrow either through natural affinity of these carriers (passive targeting) or through macrophage-associated receptors (active targeting). Passive targeting is enabled by the inherent capacity of phagocytic cells to ingest carrier systems, which are recognised as substances foreign to the organism (Briones et al., 2008).

The aescin-loaded PLGA NPs were prepared using the W/O/W emulsification solvent evaporation technique and the influence of five preparation parameters on the NPs' physicochemical characteristics was investigated using a 2^{5-2} fractional factorial design. The five factors were: (i) presence of surfactant to stabilise the primary emulsion, (ii) presence of viscosifying agent in the inner aqueous phase, (iii) aescin concentration, (iv) PLGA concentration and (v) solvent evaporation rate. The fractional design allowed us to identify the major factors influencing the entrapment of the amphiphilic saponin in the PLGA NPs and to select the optimal aescin-loaded PLGA NP formulation with the highest entrapment efficiency (EE%). The latter was withheld for the *in vitro* cytotoxicity and drug sensitivity assays. We determined the selectivity index (SI) for both β-aescin and the selected NP formulation and used it as a tool to test our hypothesis.

2. Materials and methods

2.1. Materials

The PLGA polymer was Resomer® RG 503 (Boehringer Ingelheim, Germany) with a molecular weight of 40 kDa, D,L-lactide:glycolide 52:48 molar ratio and inherent viscosity of 0.32–0.44 dl/g. Aescin was obtained from Fluka (Sigma, Belgium). Poly(vinylalcohol) (PVA), with an average molecular weight between 30 and 70 kDa, Tween® and Span® sorbitan surfactants, *p*-anisaldehyde, $NaHCO_3$, potato starch, Giemsa stain and resazurin were purchased from Sigma (Belgium), whereas Dulbecco's Phosphate-Buffered Saline (D-PBS), Minimal Essential Medium (MEM), RPMI-1640 medium, Glutamax®, L-glutamine and foetal calf serum (FCS) were supplied by Invitrogen (Merelbeke, Belgium). Poloxamer 188 or Lutrol® F68 with a molecular weight of 7680–9510 Da was purchased from BASF (Germany). The solid phase extraction (SPE) C_{18ec} cartridges were obtained from Macherey-Nagel Filter Service (Eupen, Belgium), the silica gel 60 F_{254} HPTLC-plates were from Merck (Belgium). Milli-Q water was prepared with a Millipore water purification system (Millipore Co., Bedford, USA). The organic solvents dichloromethane (DCM) and methanol (MeOH) were of analytical grade and purchased from Sigma (Belgium).

2.2. Parasites, animals and cell cultures

Leishmania infantum MHOM/MA(BE)/67 was kindly provided by the Institute of Tropical Medicine in Antwerp (Belgium) and was maintained in the laboratory by serial passage in golden hamsters (*Mesocricetus auratus*). Fresh *ex vivo* amastigotes were obtained from the spleen of an infected donor hamster. *L. infantum ex vivo* amastigotes were allowed to transform to extracellular promastigotes as described previously (Vermeersch et al., 2009). Primary mouse macrophages (PMM) were collected from Swiss CD-1 mice (Elevage Janvier, France) 2 days after intraperitoneal stimulation with one millilitre of a 2% (w/v) aqueous potato starch suspension. Cells were collected and grown in RPMI-1640 medium supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$ and 5% (v/v) inactivated FCS at 37 °C under 5% CO_2. The MRC-5_{SV2} cell line, an SV40 immortalised human fibroblast cell line, was obtained from the European Cell Culture Collection and cultured in MEM with Earle's salts supplemented with 0.029% (w/v) L-glutamine, 0.16% (w/v) $NaHCO_3$ and 5% (v/v) inactivated FCS at 37 °C under 5% CO_2. The murine macrophage-like cell line J774A.1 was kindly provided by the Laboratory of Physiopharmacology of the University of Antwerp (Belgium) and was grown in RPMI-1640 enriched with Glutamax® and 10% (v/v) FCS at 37 °C under 5% CO_2. All animal experiments were approved by the Ethical Committee of the University of Antwerp (Belgium).

2.3. Preparation of water-in-DCM (W/O) emulsions

Two millilitres of Milli-Q water, containing the hydrophilic surfactants under investigation in the concentration range 1–10% (w/v), were emulsified in DCM by means of ice-cooled sonication for 1 min at ±20 W (amplitude set at 60%) (Branson Sonifier® Model S-450D, Branson, UK) at a 1:5 (W:O) ratio. Appropriate volumes of stock solutions of the hydrophobic Span® surfactants in DCM were added to the organic phase. The stability of the obtained W/O emulsion was investigated at room temperature. The time required for the initial formation of water globules (*i.e.* creaming) was determined (Leo et al., 1998; Mohamed and van der Walle, 2006).

2.4. Preparation of PLGA NPs

The NPs were prepared by means of a W/O/W emulsification solvent evaporation method. Two millilitres of an aqueous aescin solution (5%, w/v or 2.5%, w/v), in some cases also containing polysorbate 80 as surfactant and/or poloxamer 188 as viscosifying agent, were emulsified by means of ice-cooled sonication for 1 min at 23W (amplitude set at 60%) in an organic phase that consisted of 0.50 or 1.00 g of PLGA dissolved in 10 ml of DCM. The resulting W/O emulsion was dispersed in 25 ml 1% (w/v) PVA stabiliser solution and sonicated for 1 min at 20 W (amplitude set at 50%) on ice to obtain a multiple W/O/W emulsion. The multiple emulsion was then diluted in 120 ml 0.3% (w/v) PVA stabiliser solution. The organic solvent was allowed to evaporate at room temperature during 4 h under agitation (700 rpm) with a magnetic stirrer or during 2 h at reduced pressure (Rotavapor R-200 and Vacuum Controller V-800, Büchi, Switzerland). In case of the latter a gradient method starting from 600 mbar to 50 mbar was used for the first hour, pressure was subsequently maintained at 50 mbar. Each sample was prepared in duplicate. The obtained PLGA NPs were purified by cross-flow filtration to remove non-incorporated aescin and excess stabiliser, however, a fraction of the non-purified NPs was withheld for the indirect determination of aescin entrapment efficiency. The NPs were filtered two times over regenerated cellulose membranes with a MWCO of 100 kDa (Vivaflow® 50, Sartorius) using a Masterflex® L/S pump (model 7518-00) and tubing (Sartorius, Germany). After the addition of mannitol (5%, w/v) as