Original Article

PREPARATION AND CHARACTERIZATION OF ANDROGRAPHOLIDE NANOPARTICLES FOR VISCERAL LEISHMANIASIS CHEMOTHERAPY: *IN VITRO* AND *IN VIVO* EVALUATIONS

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ABSTRACT

Objective: To overcome low physiological solubility, poor bioavailability, the short plasma half-life of andrographolide (AG), a delivery system based on poly (D, L-lactide-co-glycolide) (PLGA) nanoparticles (NPs) were developed to increase the efficiency of AG against visceral leishmaniasis (VL).

Methods: Andrographolide-PLGA nanoparticles (AGnp) were prepared with Pgp efflux inhibitor vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate) by emulsion solvent evaporation method and characterized. Antileishmanial activity was evaluated using *in vitro* and *in vivo* VL infection model.

Results: The particle size of AGnp was found to be171.4±11.5 nm with an encapsulation efficiency of 81%. The AGnp reduced AG cellular toxicity, retained it's *in vitro* antileishmanial activity and lead to a reduction (99.9%) of parasite burden in the *Leishmania donovani* infected spleen and liver. AGnp was more active in infected mice liver at low dose than in spleen. Therapeutic indexes (TI) were 6.9-fold greater in AG and 68-fold in AGnp compared to amphotericin B (AmB) when evaluated in *L. donovani* infected spleen.

Conclusion: Incorporation of AG in PLGA nanoparticles, provided controlled and improved *in vivo* performance against VL

Keywords: Andrographolide, PLGA nanoparticles, *In vitro* activity, *In vivo* antileishmanial activity, Macrophage, Therapeutic index

INTRODUCTION

Leishmaniasis is a neglected tropical disease, indigenous to tropical and subtropical areas and affects about 12 million people around the world. However, it's also becoming more common in developed countries, because of the increasing immunosuppressed population [1]. The current therapy against this parasite infection is not adequate and has the following limitations: toxicity, high cost, non-effectiveness, resistance or hospitalization requirement. The first line chemotherapy treatments are based on injectable pentavalent antimonials (Pentostam® and Glucantime®), which are highly toxic and drug resistance has already proved to be an issue [2].

AmB deoxycholate (Fungizone®) normally considered as second-line drug, which has become the first line therapy in Bihar, India. Following the loss of effectiveness of antimonial drugs, it requires careful and slow intravenous administration due to its high toxicity [3]. Liposomal AmB formulations (Ambisome®) have been developed in order to improve toxicological and pharmacokinetic properties. Virtually, these lipidic formulations reduced toxicity exhibited a better half-life and a higher efficacy in the leishmaniasis treatment. Unfortunately, their high cost and relapse in immunocompetent patients are the major limitations for their large-scale use especially in under developing countries [4].

Oral antineoplastic agent, miltefosine (MIL) was introduced as the first effective oral treatment for VL, and as an alternative treatment for HIV patients [5]. Teratogenic potential and resistance development are the major limitations of MIL in women of child-bearing age [5, 6]. A parenteral formulation of aminoglycoside antibiotic paromomycin (PMM), showed 94% efficacy in clinical trials and introduced for VL in India [7]. A strategy of co-administration of available antileishmanial drugs was carried out by the drugs for Neglected Diseases initiative to provide safety and efficacy for the treatment of VL [8].

It is roughly estimated that of the discovered 17,000 species of plants, nearly 3,000 species are used in the medicinal field. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments [9]. Numerous plant derived products from different structural classes have been investigated as antileishmanial candidates including various alkaloids, terpenoids, flavonoids and quinonoid. AG, a labdane diterpene was isolated from the leaves of Indian medicinal plant *Andrographis paniculata*, exert significant antiproliferative effects on the life cycle of *L. donovani*, the causative agent of VL [10, 11]. However, this compound has drawbacks, such as low aqueous solubility, poor bioavailability, and short plasma half-life, making their potential development as chemotherapeutic agent prohibitive. By using Nano Drug Delivery System (NDDS), one can reduce their cytotoxicity side effects, site-directed drug delivery, and aqueous solubility properties, hence contributing to their bioavailability.

Leishmania parasites are dimorphic, alternating between promastigote and amastigote form during their life-cycle. In macrophage cells of mammals, *Leishmania* cells reside and multiply [12]. Fibroblasts play an important role during the chronic phase of parasitic infection due to their inability to sustain parasite killing and may provide a safe target for the parasites in clinically latent diseases [13]. Indeed, phagolysosomal localization of the parasites, prevent drugs from readily diffusing into the target and hence represents a major problem in the development of effective antileishmanial agent. Therefore, the use of NDDS, capable of delivering antileishmanial compounds to infected cells should improve the therapeutic efficacy (therapeutic index,) of these drugs and at the same time, reduce the toxicity by altering the pharmacokinetic and bio distribution profile of the drug.

Many NDDS have already been used for antileishmanial compounds [14]. In general, these systems tend to increase drug efficacy while decreasing toxicity, resulting in an improved therapeutic index (TI) [15]. The biggest improvement, the cost-efficacy benefit, is believed to come from nano drug carriers. Recently, polylactide NPs containing basic acid were more effective than microemulsions in an *in vivo* hamster model of VL [16]. Other example includes AmB-loaded in poly (ε-caprolactone) NPs [17] or in mannose-anchored, PLGA NPs for the efficient delivery of AmB to macrophages [18].

The aim of this study was to develop and characterize a controlled drug delivery system for the treatment of VL, using an *in vitro* active AG [10, 11]. Therefore, hydrophobic AG was encapsulated in the biodegradable polymer PLGA, in order to increase its solubility, reduce cytotoxicity and enhance TI. The physicochemical characterization, the biological evaluation and its delivery system of this new antileishmanial drug were investigated. The AG load PLGA NPs were able to reduce cellular drug toxicity, retaining it's *in vitro* antileishmanial activity and improving efficacy by 8 to 10 fold when compared with free AG on *L. donovani* infected BALB/c mice, in the spleen.

MATERIALS AND METHODS

Animal and ethics statement

Six-week-old BALB/c mice of either sex, weighing 20–25 g and of approximately the same age were used for the study and they were obtained from a biological supply vendor (Rita Ghosh and Co., Kolkata, India). The experimental protocols were approved by the Jadavpur University Animal Ethics Committee (Id # JU/Pharm./384/11), and procedures followed were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Government of India and followed the standards as described in the *Guide for the Care and Use of Laboratory Animals*. Animals were housed five per cage for acclimatization one week before experiments at the animal house facility of Jadavpur University. Mice were fed with a standard diet and water ad libitum. Mice were exposed to a normal day and night cycle.

Experimental materials: medicines and chemical reagents

PLGA (lactide: glycolide = 50:50, molecular weight: 40,000-75000 Da), TPGS, $C_{33}O_5H_{54}(CH_2CH_2O)_{23}$, AG, PMM, AmB, MIL, fluorescein 5(6)-isothiocyanate (FITC), medium 199, RPMI 1640, dialysis tubing D9652 (MW cut of 12400) and fetal calf serum (FCS) were purchased from Sigma-Aldrich (USA). Sodium stibogluconate (SSG) was a generous gift from Albert David Ltd. (Kolkata, India). All other chemicals and reagents were reagent grade or higher.

Equipment and instruments

Standard glassware of Borosil® were used for experimental purpose. A 700 MW sonicator, model vibra cell vex 750 (Sonics, USA); homogenizer, model TH 02 (Omni International, USA); a precision balance 0.00001 g, Mettler Toledo AL54 (Mettler, USA); an ultracentrifuge, Himac CS 120 GHXL (hitachi Koki, Japan) were used in the preparative processes. Chemical analysis were carried out by dual pump HPLC model (UHPLC+, Dionex UltiMate 3000, Thermo Scientific); Zetasizer nano ZS (Malverm, UK); atomic force microscope nanoscope 3A (Veeco, England); FEI Tecnai TM Transmission Electron Microscopy (Netherland) and fluorescence microscope (B X 51, Olympus, USA) were used for particle characterization and imaging experiments. GraphPad Prism 5.01 and Sigmaplot 6.0, (Zendal Scientific, USA) were used for the data analysis purposes.

Preparation of PLGA NPs containing AG

The encapsulation of AG in PLGA NPs was obtained by emulsion solvent evaporation method as described previously by Roy *et al.* [10]. Briefly, 100 mg PLGA and 5 mg of AG were dissolved in 6 ml chloroform. This phase was emulsified by sonication for 5 min in 0.04% (w/v) of aqueous TPGS solution at 20 KHz. Resultant o/w emulsion was homogenized for 30 min at 20000 r. p. m under external cooling in ice water. The solvent evaporation was continued for 12 h over a magnetic stirrer at room temperature. An initial centrifugation at 5000xg for 15 min at 4 °C was performed to remove larger particle aggregates. The resulting supernatant was recovered and centrifuged at 30000 r. p. m. for 25 min at 4 °C. AGnps were washed with water, recentrifuged and preserved after decontamination by 30 min UV exposure, at-20 °C up to 6 mo. Stability of NPs in terms of morphology and drug content was not affected either by storage at-20 °C or the decontamination process (data not shown).

Preparation of fluorescein-loaded PLGA NPs (FITCnp)

FITC was used as a model drug and was encapsulated into PLGA NPs by emulsion solvent evaporation method described above. FITC was dissolved in dimethylformamide (DMF) at 100 mg/ml and 0.1 ml of

solution was added into 10 mg of polymer in 2 ml of chloroform, and rest of the process was performed as described above.

Nanoparticle characterization: size, polydispersity and ζ-potential

Frozen NPs were diluted in distilled water at 1 mg/ml. NPs were characterized in terms of size, polydispersity, and surface potential by dynamic light scattering with a 4 mW He-Ne laser beam of 633 nm wavelength (Zetasizer, Melvern Instruments). A minimum of three different sets was examined at 25 °C, with a detection angle of 173 ° using NIBS technology.

Morphology

The morphology of NPs was observed by transmission electron microscopy (TEM: JEM-1400, Jeol Tokyo, Japan) and atomic force microscopy (AFM: Veeco Nanoscope IIIa, England). AGnp suspension (100 μl) in water were deposited into fused mica substrates by drop cast method. The particles were visualized in AFM in tapping mode using RTESP tip with 267 328 KHz resonance frequency at a scan speed of 1.2 Hz. The morphology of NPs examined by the conventional negative staining method, operated with an accelerated voltage of 80 kV. One drop of NPs was placed over the carbon-coated copper grid, and then that grid was washed with distilled water. Then staining was performed with uranil acetate solution. The sample was air-dried before observation.

AG encapsulation efficiency and drug loading

AG nanoformulations were evaluated in terms of encapsulation efficacy (EE), according to equation (1).

$$\text{EE (\%)} = \frac{\text{Mass of AG originally taken} - \text{Mass of AG in supernatant}}{\text{Mass of AG originally taken}} \times 100 \quad (1)$$

Estimation of AG in all cases was carried out by a reverse phase HPLC system. The mobile phase was acetonitrile (0.1% v/v) and phosphoric acid in water (40:60 v/v) at a flow rate of 1 ml/minutes. The analysis was carried out by using 250 X 4.6 mm C_{18} column. A peak area (y) vs. concentration (x) graph for AG was first prepared, y= 30145, x= 68911, R^2 = 0.9930, retention time was 4.5 min. This was used to detect AG concentration throughout. Mass of AG in solution before and after nanoparticulation in the supernatant was determined by HPLC experiments for calculation of entrapment efficiencies [11].

In vitro drug release assay

The drug-loaded NPs were dispersed in phosphate buffered saline (PBS) (0.1 M, pH 7.4 or pH 5.5) and were then placed in an orbital shaker, shaking at 300 r. p. m. at 37 °C. At designated time intervals, the suspension was centrifuged at 30000 r. p. m. for 20 min. The pellet was resuspended in the corresponding fresh buffer to continue the drug release, and the supernatant was used for drug quantification. The quantity of AG was determined by the same HPLC procedure as mentioned above. The error bars were obtained from the triplicate samples.

Cell culture

The mice peritoneal monocyte cells were grown at 37 °C with 5% CO_2 in RPMI 1640 medium, complemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For macrophage differentiation, mice monocyte cells were incubated in the presence of 20 mg/ml phorbal 12-myristate 13-acetate (PMA, Sigms-Aldrich) for 18 h at 37 °C and left another 24 h with fresh medium containing no PMA to induce maturation.

Cellular uptake of FITC-loaded NPs

In order to study nanoparticle uptake in macrophage cells, the desired cell concentration (3 X 10^5 cells/well) was seeded with FITCnp (0.5 mg/ml). After incubation of specific times in CO_2 incubator at 37 °C, cells were washed twice with PBS to eliminate unbound NPs, centrifuged (1000 r. p. m., 5 min) after trypsinization, then suspended in 0.5 ml of PBS and observed in FITC channel under fluorescence microscope.

Parasite and culture conditions

L. donovani AG83 (MHOM/IN/83/AG83) was VL isolate obtained as a gift from Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India. *L. donovani* AG83 promastigotes were grown at 25 °C in medium 199 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, 20 mmol Hepes, 100 U/ml penicillin and 100 µg/ml streptomycin. *L. donovani* axenic amastigotes were derived from promastigotes by culturing them in MMA/20, pH 5.5 (medium for axenic amastigotes) culture medium as described previously [10]. Amastigotes were maintained in culture, at 37 °C in an atmosphere of 5% CO_2 by sub-passage (10^5 parasites/ml), every 5 d.

Evaluation of drug susceptibility on intracellular *L. donovani* amastigotes

The growth of the AG83 wild-type amastigotes on the mouse macrophages was evaluated as described [11]. Briefly, adherent cells grown in 24-well plates at 37 °C with 5% CO_2, were incubated with stationary-phase promastigotes for 4 h at 37 °C at a 1: 5 cell: parasite ratio. After the parasite internalization period, cells were washed at least twice with fresh media to remove non-phagocytosed parasites and kept with variable concentrations of free AmB, SSG, PMM, MIL, AG or AGnp for 72 h. The amastigote number in macrophages was assessed after staining with Giemsa, 100 cells on the glass disks were counted by inverted microscope. Three independent experiments in triplicate for each concentration were performed for the efficacy of drugs and NPs. Results were presented as the ratio between the infection proportions of treated and untreated macrophage cells. Drug concentration necessary to decrease the number of intracellular amastigotes to 50%, inhibitory concentration 50% (IC_{50}), was determined by linear regression analysis.

Cytotoxicity assay and selectivity index

Macrophages cells were cultured in RPMI-1640 supplemented with 10% FCS, 20 mmol L-glutamate, 16 mmol NaHCO3, penicillin (50 U/ml) and streptomycin (50 µg/ml). The assay was performed in 24-well tissue culture plates in the presence of standard counts of macrophages. The wells were seeded with test solutions, and the viable macrophages were counted microscopically.

A key part of drug discovery and development is the characterization and optimization of the safety and efficacy of drug candidates and to identify those that have an appropriately balanced safety-efficacy profile for a given indication. The selectivity index (SI) which is typically considered as the highest exposure to the drug that results in no toxicity to the exposure that produces the desired efficacy, is an important parameter in efforts to achieve this balance. In the present study, the degree of selectivity of the drug or its formulation is expressed as SI = cytotoxicity (CC_{50}) of a drug or its formulation in a macrophage cell line/IC_{50} of the same drug or its formulation, where CC_{50} is the concentration required to kill 50% of the host cell population and IC_{50} is the concentration required to kill 50% of the parasites inside the host cell. When the SI value is≥10, that drug or formulation will present promising activity, that is higher than its CC_{50} [19].

In vivo studies

For infection of mice, stationary phase promastigotes of AG83 wild-type were collected, washed and suspended with sterile PBS. A volume of 200 µl of sterile PBS containing 10^7 parasites was injected through tail-vain, which will lead to a systemic distribution of parasite throughout the body. The mice from all infected groups were treated after 45 d of infection by intraperitoneal injection of 200 µl membrane sterilized drug solutions or nanoformulation. Dosing schedule was either one dose/day for 5 consecutive days or one dose/week. Each dose was given to 5 mice. Treatment groups were as follows:

Group I: Infected and drugs or NPs untreated negative control;

Group II: Infected and treated with drugs or NPs solution as positive control;

Group III: Uninfected and drugs or NPs untreated control.

Seven days after the last treatment administration, the animals were euthanized and their spleens and livers were aseptically removed for parasite quantification by limiting dilution, as described [20].

Statistical analysis

Experimental results were expressed as mean±standard deviation. Student's t-test was used to calculate the statistical difference of mean values. Differences were considered significant at a level when p<0.01. For toxicity studies, an unpaired t-test was used to compare groups. A p value<0.05 was considered to be statistically significant.

RESULTS

The physicochemical characteristics of the formulated nanoparticles such as particle size, polydispersity, ζ-potential and drug encapsulation efficiency are summarized in table 1. Among the various physicochemical properties of NPs, the drug loading, particle size and surface property may be the most important parameters that determine theirs in vitro and in vivo performance [15]. The NPs were prepared from the PLGA by emulsification solvent diffusion method. The optimized amount of 0.04% (w/v) of TPGS used as an emulsifier exhibited negative surface charge (-35.8 mV) indicating near stable dispersion of NPs with a narrow size distribution (171.4±11.5 nm) identical to unloaded PLGA NPs (168.5±10.3 nm) (table 1).

The polydispersity index characterizes the nanoparticulate system in terms of size homogeneity by a value of the relative variance of sizes in a NP population [21]. The characterization of the unloaded and AG loaded NPs by TEM and AFM (fig. 1 and 2, respectively) showed a monodispersed size distribution and good re-dispersibility suggesting that their surface is stabilized by the deposition of TPGS, which prevent aggregation. Before release study, AGnp showed no change or perforation at the surface (fig. 2A). Whereas AGnp showed cleavage perforation at the surface (fig. 2B) after 600 h release study.
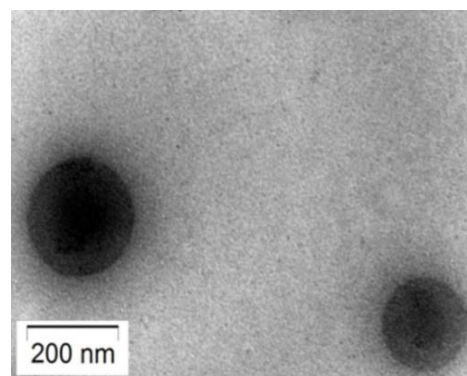

Fig. 1: TEM micrograph of AGnps with TPGS obtained from the emulsion solvent evaporation method. One drop of NPs was placed over the carbon-coated copper grid. The sample loaded copper grid was washed with distilled water. Then staining was performed with uranil acetate solution and the sample was air dried before observation. Magnifications: 30000X and acceleration voltage 80 kV

Table 1: Size, polydispersity index and ζ-potential characterization of nano-formulations[a]

Nanoformulations	Size (nm)	Polydispersity index	ζ-potential (mV)	Encapsulation efficiency (%)
Empty PLGA NPs	168.5±10.3	0.24±0.03	-38.3±1.7	NA
AGnps	171.4±11.5*	0.23±0.02	-35.8±1.5	81.1±3.4

[a]Results are expressed as mean±SEM, (n=4). *p<0.001, statistically significant difference when compared with empty NPs. NA: Not Applicable.
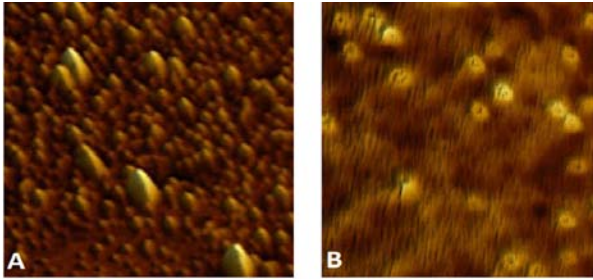
Fig. 2: Atomic force microscopy of AGnp before and after dissolution. A) AGnp before release, B) AGnp 600 h after AG release. AGnp showed no change or perforation at the surface (fig. 2A). Whereas after 600 h AGnp showed cleavage perforation at the surface (fig. 2B) indicating release of AG from AGnp

Furthermore, storage stability studies revealed that the physical structure of the NPs is stable up to 3 mo at 4 °C [11]. The release profile of AGnps under physiological (7.4) pH and acidic (5.5) pH (similar to the phagolysosome) are shown in fig. 3.

It was found that a significant initial burst release profile was obtained for both conditions during the first half day. Especially, in the case of pH 5.5, AGnps released approximately 50% of its content over the first day of the study, while under the physiological pH the same quantity was released after 1 w. After the initial burse release,

there was a phase of constant AG release. The AG release from PLGA NPs reached a plateau after 150 h. We have investigated the *in vitro* antileishmanial activity of standard antileishmanial drug; AmB, SSG, PMM and MIL along with free AG and AGnp against intracellular wild-type *L. donovani* amastigote cells and compared their CC_{50} in macrophage cells (table 2).
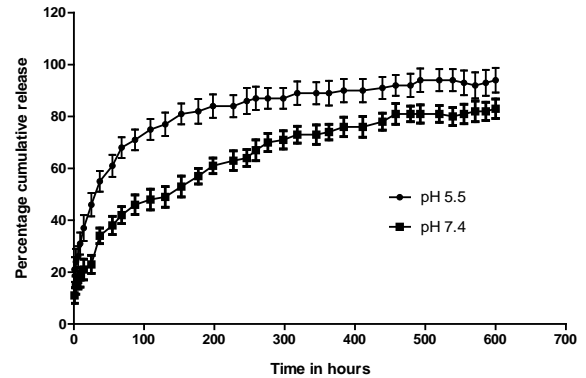
Fig. 3: Release profile of AGnp under physiological pH (7.4) and phagolysosomal pH (5.5). *In vitro* release profile of AGnp at pH 7.4 (—■—) and pH 5.5 (—●—). Results expressed as mean±SD (n=3)

Table 2: Antileishmanial activity profile of standard antileishmanial drugs, AG and AGnp against intracellular *L. donovani* wild-type amastigote cell line

IC_{50} μM^a			
Drug	Cellular AG83 evaluation model		CC_{50} μM(Macrophage cells)
	Wild-type	SI^c	
AmB	0.14±0.05*	93	13±2.1**
SSG (SbV)^b	1.5±0.20	17	25±3.9
PMM	7±2*	34	235±32*
MIL	0.34±0.04*	97	33±6*
AG	134±20*	7.1	950±160*
AGnp	26±2*	73	1900±300*

Results are expressed as mean±SD, (n=4)^{a}Assays are described in Materials and Methods, ^{b}Values for antimonial agents are in µg SbV/ml, ^{c}SI, Selectivity Index was CC_{50}/IC_{50}., *p<0.001, significant difference compared with SSG, **p<0.5, no significant difference compared with SSG.

Using a homogenization solvent evaporation technique, FITC-loaded PLGA NPs were obtained with 173±10.7 nm and-36±1.8 mV, characteristics identical to AGnp, and those were used to evaluate

cellular uptake of FITCnp by macrophage cells. Fluorescence microscopy was applied to confirm the internalization of the FITC-loaded PLGA NPs by macrophages (fig. 4).
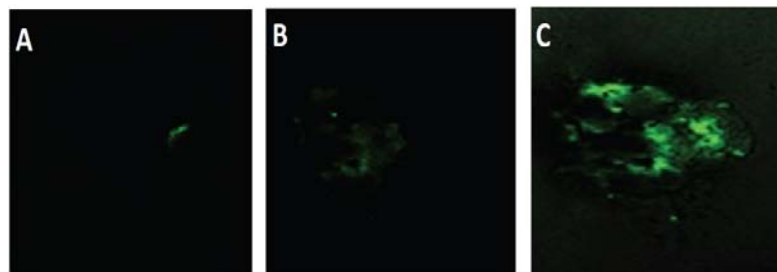
Fig. 4: AGnp uptake in macrophage cells. Fluorescence image. FITCnp were used to study the cellular uptake of FITCnps in macrophage cells as the amastigote cells reside within the phagolysosomes of macrophages. 4 x 10^5 macrophage cells/well in three batches were studied with FITCnps (0.5 mg/ml) and incubated in CO$_2$ incubator at 37 °C. For (A), 15 min; (B), 30 min; and (C), 60 min. Samples were withdrawn after specific time intervals, washed and observed in FITC channel under a fluorescence microscope. FITCnps showed considerable cellular internalization within 60 min of incubation and insignificant internalization within 15 min. Cellular internalization of FITCnps only took place in viable cells. Fig. represented time-dependent uptake of FITCnps

Macrophage cells under investigation exhibited increasing FITC fluorescence intensity from 15 min to 60 min. In the present study, the effect of AG and AGnp were evaluated using the acute

murine model of VL by treating the mice at 6 w post infection with AG, AGnp and AmB or SSG as the positive control (table 3 and 4).

Table 3: Comparison of antileishmanial effects of free AG and AGnp on the parasite burden of wild-type *L. donovani* infected mice

% Suppression of amastigote burden				
Drug	Dose, ip mg/kg/week	Dosing (number of week)	Spleen	Liver
			Wild-type	Wild-type
SSG (SbV)	40	4	82±13	93±2
AG	2	2	23±3*	24±4*
AG	2	4	36±5*	38±7*
AGnp	2	2	84±12***	91±4***
AGnp	2	4	99.9±0.1**	99.9±0.1***

Results are expressed as mean±SEM, (n=5). ip: intraperitoneal, *p<0.001, significant difference compared with SSG, **p<0.5, no s significant difference compared with SSG, ***p>0.5, no significant difference compared with SSG.

Table 4: Antileishmanial activity profile of standard antileishmanial drugs, AG and AGnp on the parasite burden of *L. donovani* wild-type amastigote cell line in mice

ED_{50} mg/kg[a]				
Antileishmanial drug and nanoformulation	Route of administration (consequtive dose)	AG83 evaluation model		LD_{50} (mg/kg)[b]
		Wild-type	TI[c]	
AmB	5 x i. p	0.3±0.06*	90	27±7*
SSG (SbV)[d]	5 x i. p	9.5±2.21	8.9	85±23
AG	5 x i. p	17±4.32**	618	10500±2420*
AGnp	5 x i. p	3.6±0.77*	6111	22000±5860*

Results are expressed as mean±SEM, (n=5), [a]Determination of 50% suppression of parasite burden in spleen of mice by drugs (ED_{50}) are described in Materials and Methods and defined as the dose that produces a quantal effect in 50% of the population that takes it., [b]Determination of 50% death of mice by drugs (LD_{50}) are described in Materials and Methods., [c]Therapeutic Index (TI) is a quantitative measurement of the relative safety of drugs, which is the ratio of LD_{50}/ED_{50}., [d]Values for sodium stibogluconate are in mg SbV/kg as given in Materials and Methods., *p<0.001, significant difference compared to stibogluconate., **p<0.5, no significant difference compared to stibogluconate.

Weekly intraperitoneal administration of all the treatments showed a significant (p<0.001) lower parasite burden in the visceral organs, spleen and liver when compared with the mice in control groups that received either PBS/DMF or empty PLGA NPs (table 4).

DISCUSSION

The multidisciplinary field of nanotechnology is bringing the science of the almost incomprehensibly small device closer and closer to reality. Nanotechnology offers the ability to build large numbers of products that are incredibly powerful by today's standards [22].

VL can be caused either by *L. donovani* (in Asia and Africa) or *L. infectum* (in southern Europe). In this study, *L. donovani* was selected for the development of the new antileishmanial nanoformulation, since it has been the objective of our research. For the therapeutic application of an antileishmanial agent, high selectivity compared to its host cell is the primary requirement. Selectivity is expressed in terms of selectivity index (SI). To be a safe drug, SI should be greater than 10 [19]. It appears from table 2 that AmB and MIL followed by AGnp were most selective for wild AG83 strains when evaluated *in vitro* in intracellular amastigotes. The AGnp have demonstrated significant advantages compared to free AG in reducing cellular toxicity (CC_{50}) and increasing SI. These results are in accordance with other *in vitro* and *in vivo* studies, in which the encapsulation of a compound into a nanocarrier was proven effective in reducing the toxicity of the drug [13, 15]. The polydispersity index values being less than 0.3 (table 1), indicating that the NPs were of relatively uniform size. The encapsulation of AG into the NPs, resulted in a statistically significant (p<0.01) decrease in the negative surface charge (table 1) indicating *in vitro* stable dispersion of NPs. In general, it is thought that NP suspension with large positive or negative ζ-potential values $≮ ±30$ mV) are less likely to suffer from aggregation phenomena due to electrical repulsion. Upon the *in vivo* administration, the physiological condition will mask the almost neutral surface charges of the nanoformulations, contributing to the equilibrium of the system. Drug loading of the nanoformulations was optimized to control the size of NPs. Due to the fact that the amount of encapsulated drug is crucial in a drug delivery system, high drug loading rate is desirable to reach therapeutic necessities. Optimized AGnp was prepared with a drug loading of 8% (w/w) and an encapsulation efficiency of 81.1±3.4. The high encapsulation efficiency obtained is a consequence of hydrophobic nature of the AG. The treatment with the AGnp was significantly more effective than free AG in reducing parasite burden in spleen and liver, at an equivalent drug dose of 2 mg/kg/week either for 2 w or 4 w for wild-type *L. donovani*. Most strikingly, AGnp at 2 mg/kg/week dose for 4 w showed 99.9% lowering of parasite burden in both spleen and liver infected with wild-type *L. donovani*. Therapeutic applicability of any chemotherapeutic agent depends on its safety which is quantitatively expressed as a therapeutic index (TI). TI is the ratio of LD_{50}/ED_{50}. LD_{50} is defined as 50% death of mice by drug and ED_{50} as the dose that produces 50% suppression of parasite burden. TI of free AG and AGnp was determined considering AmB and SSG as a control group in 5 consecutive i. p. dosing with variable drug or nanoformulation concentrations (table 4). For this purpose ED_{50} of AG and AGnp was determined to compare with an infected untreated animal in spleen burden of wild-type *L. donovani* infected mice and AmB and stibogluconate as a positive control. Comparing AmB, stibogluconate, and AG, AGnp was clearly more safe and active for wild-type *L. donovani* infected animals. It appears from table 4 that TI of AGnp was 68-fold in wild *L. donovani* infected mice when compared with AmB, a first line drug of choice for Kala-azar (VL). TI of stibogluconate was less than 10 for wild strains of *L. donovani*, and thus not safe for VL treatment.

CONCLUSION

In conclusion, the *in vivo* activity data exhibited by AGnp provides evidence that intraperitoneal administrations to mice very efficiently reduced parasite burden in the visceral organs of acute infected BALB/c mice. A facile approach to preparing biodegradable, biocompatible, and FDA approved PLGA NPs with high drug load and encapsulation efficiency of AG using the homogenization solvent evaporation method is described. Fluorescence microscopic observation provides compelling evidence for complete internalization of FITC-PLGA NPs in macrophages, which are the host of *Leishmania* amastigotes. Interestingly, AG-loaded NPs turned out to be the most effective and selective in killing intracellular *L. donovani* amastigotes in macrophage cells model than first and second line antileishmanial drugs. *In vitro* results translate well into the *in vivo* model of visceral leishmaniasis, whereas phagocytic macrophage cells are the major constituent of visceral organs. Indeed, AG-loaded PLGA NPs were very efficient in reducing parasite burden in the visceral organs of acute infected BALB/c mice. Acute

and subacute toxicity profile of AGnp were much less than the free AG, and first and second antileishmanial drugs. In summary, we showed that AGnp has great advantages as a delivery system for AG, providing controlled, effective and safe delivery for treatment of visceral leishmaniasis.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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