PLGA-PEG Encapsulated sitamaquine nanoparticles drug delivery system against Leishmania donovani

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Abstract

Targeted drug delivery systems are a promising technology to increase the maximum mechanism of action with smaller dose of requirement. Therefore we have developed sitamaquine encapsulated PLGA-PEG nanoparticle (NP) attach with antibody to CD14 to target macrophage of infected tissues against leishmaniasis. The analyses of detailed structural characterization performed by transmission electron microscopy (TEM) and dynamic light scattering (DLS) confirmed the nano-size of the particle (size range of 20–30 nanometer scale) and fourier transform infrared spectroscopy (FTIR) for NP encapsulation confirmation. The ED$_{50}$ value of sitamaquine showing less than sitamaquine antileishmanial drug alone and three fold decrease of macrophage infection in which hamster treated with PLGA-PEG encapsulated sitamaquine (0.0039 ± 0.0012) as compare to hamster treated alone with sodium sitamaquine (0.0123 ± 0.0047). Further significantly inhibition of amastigotes in the splenic tissue with PLGA-PEG encapsulated sitamaquine was significantly more than with conventional (89.01±6 verse 71.39 ±12).

Keywords: Amphoterecin B, CD14, Electron microscope, Nanometer, Amastigotes.

Introduction

Leishmaniasis is a group of diseases with a wide range of clinical manifestations ranging from self-healing cutaneous ulcers to more severe forms visceral disease and sometimes even death.¹ Leishmaniasis is an infectious disease caused by the protozoan of the genus Leishmania which is an obligate intracellular parasite of mammalian macrophages.² Parasite the metacyclic promastigotes form is transmitted to the mammalian host during biting of sand flies. In the mammal, the parasites are in macrophage where they develop into non-motile amastigotes.³⁻⁴ Visceral leishmaniasis, also known as Indian Kala-azar and dum dum fever was first isolated by Scottish doctor Leishman and by Irish physician Donovan in 1903.⁵ Kala azar is most severe and lethal of leishmaniasis, specially to those patients in which, patient particularly confected with AIDS.⁶ Visceral leishmaniasis (VL), potentially caused by Leishmania donovani, has limited and unsatisfactory treatment option. Availability of drugs is mostly parenteral and has serious toxicity. This problem is further intense in Bihar (India) where widespread resistance to pentavalent antimonials persists.⁷ The first-line treatment has been used by Amphoterecin B in these regions because of its nearly 100% cure rates; however, adverse drug reactions (ADRs) are a major limiting factor. A total dose of 15–20 mg/kg has to be given as intravenous infusions either daily or on alternate days, necessitating prolonged hospitalization. In addition, ADRs are universal,
which can occasionally be serious. The development of lipid formulations of amphotericin B, especially liposomal formulations, has alleviated this problem. This strategy targets intra macrophage organism, increasing bioavailability and reducing toxicity. However, AmB has shown poor gastrointestinal absorption and low bioavailability due to the hydrophobicity of the polyene structure. It can also interact with the mammalian cell membrane causing cellular dysfunction. Formulation of deoxycholate complexes AmB micelles (Fungi zone) most commonly used drug is highly toxic to patients, often causing decreased renal function, anaphylaxis, chills, high fever, nausea, phlebitis, anorexia and other adverse effects. Usefulness as in anti-infective therapy in general limit by this adverse reaction coupled with long therapeutic regime. Lipid formulations of AmB reduce toxicity to non-target tissues but development of resistance cannot be degraded by body. The prohibitive cost of these formulations puts them beyond the reach of most of the patients in the endemic areas of VL, which represent the poorest areas of the world. A different approach to drug formulations could be nanonization of the drug. The nanoparticles are recognized as foreign bodies and phagocytosed by the macrophages leading to target specific delivery, as Leishmania harbours inside the macrophage phagocytic system. Furthermore; the solid nanoparticles are characterized by high weight per volume, which is an ideal situation for sustained drug release by gradual diffusion from the depot. Thus, the drug can be delivered in higher doses and over a shorter duration to achieve cure of the disease. We have developed PLGA-PEG encapsulated sitamaquine by co precipitated method. The present study is aimed at evaluating this formulation of PLGA-PEG nanoparticle was carried out and then cross linking has been carried out with antibody CD14 and further encapsulation with antileishmanial drug sitamaquine has been done. Nanoparticle syntheses were characterized and the size of the particles was confirmed by transmission electron microscope (TEM), dynamic light scattering (DLS) and further confirmation of chemical group specific to PLGA-PEG by FT-IR (FT-IR) analysis has been done to determine successful synthesis.

Materials and Methods

Chemical

Chemical like PLGA, COOH-PEG-NH2, methylene chloride, N-hydroxysuccinimide, 1 Ethyl-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Sigma-Aldrich. Deionizer water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Nanoencapsulation

Carboxylated-functionalized copolymer PLGA–b–PEG was synthesized by the conjugation of COOH–PEG–NH2 to PLGA–COOH. PLGA–COOH (1g) in methylene chloride (2 ml) was converted to PLGA-NHS with excess N-hydroxysuccinimide (27 mg) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 46 mg). PLGA–NHS was precipitated with ethyl ether (1 ml), and repeatedly washed in an ice-cold mixture of ethyl ether and methanol to remove residual NHS. After drying under vacuum, PLGA–NHS (1g) was dissolved in chloroform (4 ml) followed by addition of NH2–PEG–COOH (250 mg) and N, N-diisopropylethylamine (28 mg). The co-polymer was precipitated with cold methanol after 12 h and washed with the same solvent (3x 5 ml) to remove untreated PEG. The resulting PLGA–PEG block co-polymer was dried under vacuum.

Bioconjugation Antibody to CD14 to Polymeric Surface

PLGA–b–PEG NPs (10μg/ml) were suspended in water and were incubated with EDC (400 mM) and NHS (200 mM) for 20 min. NPs were then repeatedly washed in DNase-, RNase-free water (30 ml) followed by ultrafiltration. The NHS-activated NPs were reacted with amine terminal of CD14 (1 μg /ml). The resulting NP-CD14 bioconjugate were washed with ultrapure water (15 ml) by ultrafiltration, and the surface-bound CD14 were denatured at 90°C and allowed to assume binding conformation during snap-colling on ice. The NP suspensions were kept at 4°C until use.

Formulation of Amphoterecin B Drug-encapsulation PLGA-PEG

The nano precipitation method was employed for the formation of drug encapsulated carboxylated PLGA–b–PEG NPs. Briefly, miltefosine (Sigma-Aldrich) was dissolved in various organic solvents that are miscible with water. Polymer was likewise dissolved and mixed with the drug. NPs were formed by adding the drug–polymer solution to water, a non-solvent. The resulting NP suspension was allowed to stir uncovered for 6 h at room temperature. NPs were purified by centrifugation (10 min, 10,000 g). The PLGA–b–PEG NPs were resuspended, washed with water, and collected likewise.
Characterization of nanoparticles

Characterization of nanoencapsulated antileishmanial compound mainly was done by Transmission electron microscope, Dynamic light scattering and Fourier transform–Infra red.

Transmission electron microscope

TEM measurements were performed using Philips CM 200 TEM in SAIF department of IIT Bombay.

Dynamic Light Scattering

Size Distribution determined by DLS using Beckman Coulter in chemistry department of IIT Patna.

Fourier Transform Infrared (FTIR) Spectroscopy

In infrared spectroscopy, were characterized by FTIR using shimadzu in chemistry department of IIT Patna.

Promastigote assay

In vitro assay on *L. donovani* promastigotes (Promastigotes of Indian *Leishmania donovani* strain MHOM/IN/83/AG83) working solutions of drugs, nano-amphotericin B and amphotericin B, at concentrations of 0.8 mg/L were prepared. Aliquots of 200 mL of the drugs in duplicate were dispensed in the first row of a 96-well plate (Corning Inc., COSTAR). In all the remaining wells of selected columns, 100 mL of medium was dispensed. From the drug wells, 100 mL of drugs was aspirated and transferred to the successive well of the second row using a multi-channel pipette. The process was continued to obtain 2-fold dilutions, and the last row was left as the control row. The parasite culture in the stationary phase was washed with RPMI-1640 medium and resuspended to obtain 1 _ 106/mL in the same medium containing 15% HIFBS, Ph 7.2. Aliquots of 100 mL of the parasite suspension were dispensed in all the medicated rows and the non-medicated row so as to obtain drug concentrations from 0.4 to 0.025 mg/L and the control in duplicate. The plate was incubated at 258C for 24 h in a cold incubator.

Hamster studies

Female hamsters were infected, via the tail vein, with 2x10-7 amastigotes of Ag83 L. After 7 days, patency of infection has been checked and drug dose has given. Groups of five mice (50–60 g), received Amphotericin B (5 mg/kg) and PLGA-PEG encapsulated amphotericin B also received (5 mg/kg) days; Liver impression smears were made 14 days post infection and activity determined by counting, microscopically, the number of amastigotes per liver cell in treated and untreated mice.

Statistical analysis

$ED_{50}$ and inhibition of amastigotes *in vivo* (spleen) and suppression of parasite growth values were calculated Graph Pad Prism5 version was used for statistical analysis.

Results

Nanoparticle Synthesis

Syntheses of PLGA–b–PEG copolymer were carried out by direct conjugation of PLGA–COOH with NH2–PEG–COOH, both having fixed block length, to generate PLGA–b–PEG–COOH. The carboxyl group in the copolymer is located at the terminal end of the hydrophilic PEG block; therefore, upon NP formulation, the PEG should facilitate the presentation of the carboxyl groups on the NP surface making them available for surface chemistry. After preparing the nanoparticle, the size of this nanoparticle was characterized by TEM (Transmission Electron Microscope) and DLS (Dynamic Light Scattering). TEM micrograph (the inset shows the particle size distribution) exhibited nearer to spherical nature. Analysis of both TEM and DLS confirmed size of PLGA-PEG nanoparticles and PLGA-PEG encapsulated sitamaquine are between 25to 30nm and 30 to 35 nm respectively (figure 1-3). According to TEM and DLS analysis, with the attachment of sitamaquine, the diameter of the PLGA-PEG encapsulation amphoterecin B nanoparticles (than PLGA-PEG NPs alone) exhibited marked increase in diameter.

Figure 1: TEM image of PLGA-PEG Nanoparticle showing size of 30 nm scale
Bioconjugation to CD14 to the polymeric surface

FT-IR spectroscopic analysis of PLGA-PEG NPs CD14 conjugate demonstrates that CD14 was indeed covalently linked to the PEG via amide bond free carboxyl group on the surface of PLGA nanoparticles through carbodiimide chemistry (figure 4). FT-IR spectra of the pure PLGA-g-PEG NPs can be seen that the PLGA-g-PEG composite preserved the characteristic peaks of each component. The peak around 1748 cm$^{-1}$ was attributed to the stretching of carbonyl groups (C = O) from the polymer. The peak at 1084 cm$^{-1}$ belonged to the stretch band of the C–O bond in PLGA-PEG. Peaks appeared at 1650 cm$^{-1}$ and 1530 cm$^{-1}$ assigned to C=O stretching vibration and -NH- bending vibration of amide, respectively. These results suggest that a stable amide bond formed as a result of the reaction.

**In vitro**

In vitro EC$_{50}$ for *L. donavani* amastigotes was determined after 3 days exposure to different concentration of sitamaquine and PLGA-PEG encapsulated sitamaquine. In all vitro test PLGA-PEG encapsulated sitamaquine was significantly more effective than sitamaquine alone. The mean (0.1203 + 0.016) of extracellular promastigote of sitamaquine is significantly higher than PLGA-PEG encapsulated sitamaquine (0.0897 + 0.026) has shown in table 1.

<table>
<thead>
<tr>
<th>Parasites/ macrophages</th>
<th>nano sitamaquine (+SD)</th>
<th>amphotericin B (+SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular promastigotes</td>
<td>0.0897 + 0.026</td>
<td>0.1203 + 0.016</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**Effect of test drug on in vivo intracellular amastigotes**

Formulations of PLGA-PEG encapsulated amphotericin B against *L. donovani* infections were significantly more
active than the amphoterecin B alone as measured by parasite load in the liver and mean values were taken for further analysis (Table 2). The load of the parasites was found to be significantly lower for group A (89.01 ± 6) compared with group B (71.39 ± 13.29).

Table 2: In vivo activity of Sitamaquine and PLGA-PEG encapsulated sitamaquine

<table>
<thead>
<tr>
<th>Condition</th>
<th>Before treatment</th>
<th>Nano Sitamaquine</th>
<th>AMB Group</th>
<th>Control Group</th>
<th>P value (nano versus AMB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight (g)</td>
<td>1.012</td>
<td>0.486</td>
<td>0.6756</td>
<td>1.479</td>
<td></td>
</tr>
<tr>
<td>Amastigotes/500 nuclei +SD</td>
<td>369.82 ± 117.42</td>
<td>25.21 ± 23</td>
<td>92.09 ± 52.7</td>
<td>2506.69 ± 1459</td>
<td>0.05</td>
</tr>
<tr>
<td>Percentage inhibition +SD</td>
<td>89.01 ± 6</td>
<td>71.39 ± 13.29</td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
</tbody>
</table>

Discussion

Treatment of visceral leishmaniasis (VL) involves several limitations such as high cost, specific toxicities, parenteral administration (technical efficiency required) and spread of drug resistance, and relapses in HIV–Leishmania co-infected patients. Low cost treatments have requirement of smaller doses of drugs with optimum mechanism of action. Thus targeted drug delivery systems are a promising date, Therefore we have developed PLGA-PEG nanoparticle (NP) conjugated with antibody to CD14 (targeting macrophage) with antileishmanial compounds amphoterecin B to overcome these drawbacks. These nanoencapsulated systems have greater potential to convert poorly soluble, poorly absorbed substances to biologically active substances with promising deliverable drugs. Due to the particulate form of polymer, the drug is preferentially engulfed by the macrophages, leading to targeted drug delivery. The encapsulated sitamaquine (with PLGA-PEG) nanoparticles are considerably smaller (25–35 nm) and these particles didn’t lose their original characters (premature degradation), leading to engulfment of these particles preferentially by the cells of the macrophage phagocyte system this function may further be increased as nanosizing adhesion properties of cells to tissues. When evaluated by in vitro studies, PLGA-PEG nanoconjugates retained their antileishmanial activities against promastigotes. ID_{50} values of PLGE-PEG encapsulated sitamaquine nanoparticles are lower than that of sitamaquine; it gives higher efficacy at lower dose. The reason behind the encouraging results of these PLGE-PEG encapsulated sitamaquine nanoparticles, especially with regard to lower dose, is probably due to the targeted delivery to tissues due to nanosizing.

This study signifies that there is an increased contact surface area of the drug and significant reduction in size, improved its efficacy than that of normal miltefosine, sodium antimony gluconate liposomal amphotericin B. It is well targeted to macrophage. Thus, smaller doses of PLGE-PEG encapsulated amphoterecin B nanoparticle are required to achieve the better rate of success in treatment in future. Hence, PLGAPEG encapsulated amphoterecin B nanoparticles are likely to be taken up by the macrophage phagocytic system, thereby considerably reducing the systemic side effects of miltefosine and amphotericin B akin to that seen with liposomal amphotericin B. If the cost of the production of nanoparticles of PLGA-PEG encapsulated amphoterecin B nanoparticle works out to be considerably less than that for liposomal amphotericin B, it will be worthwhile to investigate a large-scale in vivo study to look at the efficacy and toxicity of PLGA-PEG encapsulation amphoterecin B nanoparticle.

Conclusion

Polymeric nanoparticles generally described as nanospheres and nanocapsules, have been proposed for use as passive drug delivery to macrophages because of their long circulation time in the body and rapid clearance from the plasma by the mononuclear phagocyte system (MPS). The polymer drug conjugates were synthesized from a polymeric precursor by aminolysis followed by substitution at the terminal amino group of the antileishmanial drug and during synthesis the target moiety was introduced. Improved antileishmanial efficacy and bioavailability of bioactive drug miltefosine in poly (lactide-co-glycolide) PLGA was observed by nanoencapsulation, nanoparticles were in a size range of 30 to 35 nm with an increased localization in macrophages predominantly infested with Leishmania parasite. The phagolysosomes acidic pH may be accelerating the degradation of PLGA, promoting specific release of the drug in the vicinity of the amastigotes. In vitro antileishmanial activities of PLGA-PEG encapsulated sitamaquine nanoparticles (NPs) were found to be more effective than that of free sitamaquine in terms of therapeutic efficacy as ED_{50} promastigote of leishmania parasite significantly decrease and moreover inhibition of amastigotes in the splenic tissue with PLGA-PEG encapsulated sitamaquine was significantly more than with
conventional sitamaquine and amphotericin B antileishmanial drug

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References


