Parasitol Res (2010) 106:703–708
DOI 10.1007/s00436-010-1725-1

ORIGINAL PAPER

(−)−Hinokinin-loaded poly(D,L-lactide-co-glycolide) microparticles for Chagas disease

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Received: 10 October 2009 / Accepted: 4 January 2010 / Published online: 28 January 2010

Abstract The (−)−hinokinin display high activity against *Trypanosoma cruzi* in vitro and in vivo. (−)−Hinokinin-loaded poly(D,L-lactide-co-glycolide) microparticles were prepared and characterized in order to protect (−)−hinokinin of biological interactions and promote its sustained release for treatment of Chagas disease. The microparticles contain (−)−hinokinin were prepared by the classical method of the emulsion/solvent evaporation. The scanning electron microscopy, light-scattering analyzer were used to study the morphology and particle size, respectively. The encapsulation efficiency was determined, drug release studies were kinetically evaluated, and the trypanocidal effect was evaluated in vivo. (−)−Hinokinin-loaded microparticles obtained showed a mean diameter of 0.862 μm with smooth surface and spherical shape. The encapsulation efficiency was 72.46±2.92% and developed system maintained drug release with Higuchi kinetics. The preparation method showed to be suitable, since the morphological characteristics, encapsulation efficiency, and in vitro release profile were satisfactory. In vivo assays showed significant reduction of mice parasitaemia after administration of (−)−hinokinin-loaded microparticles. Thus, the developed microparticles seem to be a promising system for sustained release of (−)−hinokinin for treatment of Chagas disease.

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Introduction

Chagas disease, caused by *Trypanosoma cruzi*, affects approximately 15×10^6 people in South and Central America and thus continues to represent a health threat for an estimated in 28 million people, living mostly in Latin America (Who 2007). The parasite is transmitted to humans either by blood-sucking triatomine vectors and by blood transfusion or by congenital transmission.

The current treatment is relied on two nitroheterocyclic drugs, the nitrofuran nifurtimox, Lampit®, which production has now been discontinued, and the 2-nitroimidazole benznidazole, Rochagan® (Croft 1997; Paulino et al. 2005). Both drugs, if administered during the acute phase of the disease, could cure 50-70% of the patients. However, both drugs display limited efficacy in the treatment of the chronic phase of the disease and are quite toxic for the patients (Docampo 2001). Therefore, there is still a need for finding more efficient and less toxic drugs to act specifically against the pathogen (de Castro 1993; Rodriques Coura and de Castro 2002; Urbina and Docampo 2003).

Nowadays, the vegetal origin products represent approximately half of the drugs in used and interest in this

research area has been increased in view of the large number of compound biologically active that can be isolated from plants (Clark 1996; Montanari and Bolzani 2001). The (−)−hinokinin (HNK, Fig. 1) belongs to the dibenzylbutyrolactone lignan class of compounds, which is among the natural products of interest, since many of its compounds display a broad range of biological activities with therapeutic potential (Piccinelli et al. 2005; Charlton 1998; Souza et al. 2004; de Souza et al. 2004; da Silva et al. 2005; Saraiva et al. 2007).

Initially, (−)−cubebin was isolated from the hexane extract of the leaves of *Zanthoxyllum naranjillo* (Rutaceae) and its tripanocidal, anti-inflammatory, and analgesic activity were demonstrated by pharmacological assays (Bastos et al. 1996, 1999, 2001). In face of these results, (−)−cubebin was isolated of the seeds of *Piper cubeba* L and several derivatives also biologically active as HNK were obtained by synthesis in our laboratories (de Souza et al. 2005; Souza et al. 2004; da Silva et al. 2005).

HNK is the most active derivative of (−)−cubebin for Chagas disease with active higher than benzonidazol in in vivo and in vitro studies. Considering these promising results displayed by HNK, this lignan could be considered as a lead compound for the development of new drugs for the treatment of Chagas disease (Saraiva et al. 2007).

Many systems such as the microparticles system have been used as promising strategies to achieve modified release of many drugs (Ravi Kumar 2000). Several different methods for the preparation of the microparticles are described in the literature, but the suitability of a particular technique is mainly determined by the solubility of the polymer and the drug which is usually fixed (Herrmann and Bodmeier 1998).

The drug delivery systems were developed for the purposes of bringing, uptaking, retaining, releasing, activating, localizing, and targeting the drugs at the right timing, period, dose, and place (Ueda and Tabata 2003). The use of biodegradable polymers, as poly(D,L-lactic-co-glycolic acid; PLGA), for the controlled release of therapeutic agents is now well established. These systems have been extensively utilized for oral and parenteral administration. The physical properties and Food and Drug Administration approval of poly(lactide-co-glycolides) make them the most extensively studied commercially available biodegradable polymers (Birnbaum et al. 2000).

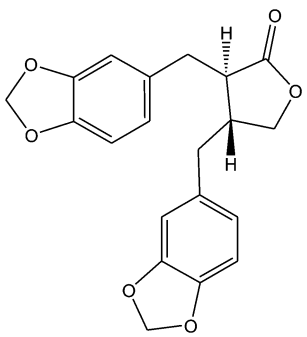

Fig. 1 Chemical structure of (−)−hinokinin

The microparticles can be able to sustain the release of the drug for a considerable period of time reducing the required frequency of administration increasing patient compliance, avoid plasmatic fluctuations, decrease side effects, and facilitate dosage administration (Hans and Lowman 2002).

In the present work, HNK-loaded PLGA microparticles was prepared and characterized in order to protect HNK of biological interactions and promote its sustained release for treatment of Chagas disease. The trypanocidal effect of microparticles contain HNK was evaluated in vivo.

Materials and methods

Chemicals

PLGA (copolymer ratio of D,L-lactide to glycolide 50:50; Resomer RG 502 (Mw=8,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA; Mw=26,000) was supplied by Mallinckrodt Chemicals (USA). Solvents high-performance liquid chromatography (HPLC) grade were obtained from Merck (USA). The solubility to water of HNK at 25°C is 453.3 mg/L. The water used was obtained from Milli-Q system (Millipore). All others chemicals were of analytical grade and obtained from Merck (USA).

Isolation of HNK

(−)−Cubebin was an isolate of *P. cubeba* L. according to Bastos et al. (1996). The HNK was obtained from (−)−cubebin by partial synthesis and purified by HPLC as previously described by our group (de Souza et al. 2005; Souza et al. 2004; da Silva et al. 2005). Its chemical structure was confirmed by its ^{1}H nuclear magnetic resonance and infrared data as published in the literature (Heleno et al. 2006). Its purity was estimated by both HPLC and spectral data analysis.

Preparation of microparticles

The HNK-loaded PLGA microparticles were prepared by the classical method of the emulsion/solvent evaporation (Birnbaum et al. 2000; Niwa et al. 1994). In general, 100 mg of PLGA and 10 mg of HNK (drug to polymer weight ratio of 1:10) were dissolved in 10 ml of dichloromethane and poured into 150 ml of 2.25% (*w*/*v*) PVA solution and then the solution was emulsified using an Ultraturrax® (13,000 rpm). The *O*/*W* emulsion was stirred at a controlled temperature of 25°C for 12 h to allow

solvent evaporation. The separation of the microparticles was performed by centrifugation (1,680×*g*; 15 min), thus, washing with distilled water for three times and lyophilized for 24 h in a Liobras® freeze-dryer (model L101, Brazil).

Characterization of microparticles

Particle size analysis and morphology

The mean diameters and polydispersity indices (PDI) of microparticles were evaluated by a light scattering using a Malvern Zetasizer Nano system ZS (Malvern Instruments, UK). The microparticles morphology was examined using a scanning electron microscope (SEM, Stereoscan 440, Leica). Microparticles were prepared on glass blades and coated with gold prior to examination by SEM. The voltage ranged from 10-25 kV during scanning.

Determination of encapsulation efficiency

To determine loading percentage of HNK in the microparticles, 2 mg of the freeze-dried microparticles was dissolved in 4 ml of dichloromethane, shacked vigorously during 1 min and sonicated for 1 min. Then, the polymer was precipitated with the addition of 19 ml of ethanol. The suspension was mixed, centrifuged, and filtered through a membrane filter to remove the polymer and analyzed by HPLC using Shimadzu liquid chromatograph model SPD 10AVP using a variable wavelength UV detector set at 285 nm, an LC-10 ADVP pump, a Reodyne injector and a model CR6-A integrator. Separation was performed on C_{18} reversed-phase column shim-pack (250×4 mm, particles of 5 µm) and a C_{18} pre-column shim-pack (4×4 mm, particles of 5 µm), at controlled room temperature (25°C). The mobile phase consisted of acetonitrile and water at a ratio of 60:40 (*v*/*v*), at flow rate of 1.0 ml/min. Under these conditions, the retention time of HNK was 10.0 min. When HNK solutions were injected, linearity was achieved over the concentration range between 1 and 20 µg/ml, presenting a correlation coefficient (R^2) of 0.9998. The error and the intra-day and inter-day variations were <5%. These values are considered adequate for an analytical method (Causon 1997).

The drug loading efficiency (EE) was calculated as follows: EE=(total amount of drug determinate in microparticles/total amount of drug theoretically associated with microparticles)×100%. All the measurements were conducted in triplicate and the mean values and standard deviations are reported.

In vitro drug release studies

The release rate of the microparticles was evaluated using a Hanson dissolutor model SR-8 Plus, with paddle assembly (USP apparatus 2 or BP apparatus II). Five milligram of microparticles were suspended in 400 µl of 30% ethanol in pH 7.4 phosphate buffer solution (PBS), and then placed within a dialysis membrane bags with closed extremities as described by Lira et al. (2008) with minor modifications. The bags were immersed into 100 ml of 30% ethanol in PBS solution as a dissolution medium and shaken at a rate of 100 rpm at 37°C. Dissolution medium (1 ml) was periodically drawn for analyzing HNK by HPLC as described. The same volume of fresh medium was simultaneously replaced to the vials. The samples were analyzed by HPLC (n=5). The data obtained from the drug release studies were kinetically evaluated using Statistica 6.0 program.

Trypanocidal activity in vivo

The undertaken experiments comply with the current laws of the country in which they were performed. All procedures were approved by the Ethical Committee for Animal Research from Ribeirão Preto, USP. Female Swiss mice (weight, 20-22 g) were infected intraperitoneally with 2×10^4 trypomastigotes forms of *T. cruzi* (clone CLB5). The treatment was initiated 48 h after infection and maintained for 20 days, as follows: group I was untreated control (n=5). Group II received HNK (n=5, concentration of 20 mg/kg/day). Group III received HNK-loaded microparticles (n=5, concentration of 40 mg/kg each 2 days). Group IV received empty microparticles (n=5). A mass of each formulation equivalent to dose was suspended in 0.3 ml of 0.5% DMSO/0.25% Tween 20/5% ethanol in PBS solution. The animals were treated once a day with 20 mg/kg HNK or each 2 days with 40 mg/kg of HNK-loaded microparticles by subcutaneous route. Parasitemia was evaluated by counting the trypomastigote forms of the parasite per 5 µl of fresh blood, following the method described by Brener (1962), with blood collected from the animal's tail, starting at the second day of infection. The groups were carried out in parallel. Statistical analyses were carried out with the Prisma 4.0 Software (GraphPad, San Diego, California), t test was conducted for the data analyses. Differences were considered statistically significant when $P<0.05$.

Results and discussion

Microparticles characterization

HNK-loaded microparticles were prepared with success by the emulsion evaporation method. In according, others authors showed that the conventional oil-in-water (*o*/*w*)

emulsion-solvent evaporation method is adequate to encapsulated lipid-soluble drugs, like HNK (Jain 2000).

Microencapsulation by solvent evaporation method involves two major steps: the formation of stable droplets of drug-containing polymer solution and the subsequent gradual removal of solvent from droplets leads to polymer precipitation, and hence drug entrapment. In practice, however, the reproducible manufacturing of microparticles with adequate properties (good encapsulation efficiency, suitable release profile, and particle distribution) can be difficult, due to the larger number of factors influencing the outcome, such as solvent removal, polymer concentration, surfactant concentration, stirring speed, stirring time, and others. The effect of each these parameters could be determined empirically (Herrmann and Bodmeier 1998).

The microparticles formed presented narrow distribution size and a mean diameter of 0.862 μm, with PDI of 0.072 nm (Fig. 2a). Scanning electron micrographs of PLGA microparticles obtained showed that HNK loaded microparticles presented, smooth and spherical surface (Fig. 2b, c). Due to their small diameter (approximately 1 μm), the HNK microparticles obtained are better suited for parenteral delivery (Cegnar et al. 2005).

The encapsulation efficiency of HNK-loaded microparticles with a drug to polymer weight ratio of (1:10) was 72.46±2.92% and resulted in a sustained release profile as showed in Fig. 3. In vitro dissolution tests results showed that 70% of the drug was released from PLGA microparticles during the first 12 h, and 100% within a total of 96 h. The drug release from microparticles seems to consist of two phases: an initial rapid release followed by a slower exponential stage.

Burst release from PLGA microparticles has been attributed to the dissolution of surface-associated drug. The rate and degree of drug diffusion from microparticles that characterizes burst release is affected by intrinsic factors like drug molecular weight, particle size, and the partition coefficient of the drug between the polymer and release medium environments. Diffusion also depends on the mode of drug dispersion within the particle, which is determined by solubility of the drug in the organic solvents commonly used for microparticle fabrication, such as dichloromethane. Small, hydrophobic drugs like steroids and HNK are typically soluble in these organic solvents and are distributed in microparticles as molecular dispersions within the amorphous polymer matrix. For this broad category of drug, the affinity of the drug for the polymer may affect release to varying degrees (Allison 2008).

In a biodegradable system, drug release from the polymer matrix is very complex, and follows erosion-diffusion kinetics. When the active agent is dispersed in the polymeric matrix, the release is governed by a mechanism

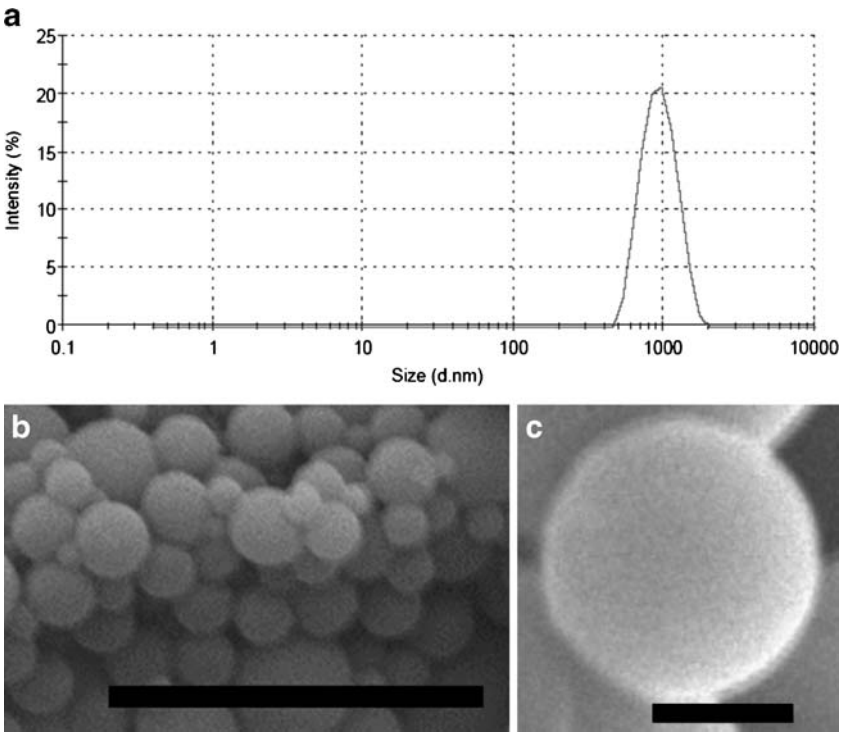

Fig. 2 Size distribution of (−)−hinokinin-loaded microparticles (**a**) and scanning electronic photomicrographs of (−)−hinokinin-loaded microparticles: (**b**) panoramic view of (−)−hinokinin loaded microparticles (bar = 10 μm), (**c**) isolated magnified particle (bar = 1 μm)

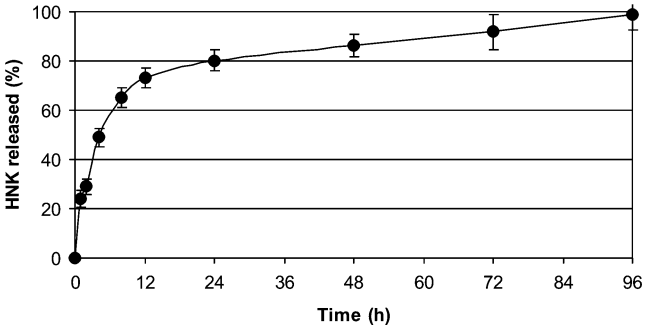

Fig. 3 In vitro release profiles of (−)−hinokinin (HNK)-loaded microspheres (*filled circles*)

that can be explained by Higuchi model (Higuchi 1961, 1963), which is fundamental by the first lei of Fick.

Comparing the coefficient of determination for each kinetic model proposed, Higuchi model presented the higher R^2, with residual variance constant and mean zero, thus it was selected to describe the kinetics release of HNK from microparticles (Table 1). According to Higuchi model theory, the HNK released from the microparticles studied in this work is mainly controlled by micropores diffusion.

Typanocidal activity in vivo

Sustained release microparticles can be good candidates for improving the in vivo activity of HNK. The delivery system developed and evaluated facilitates dosage administration and improve the efficacy of HNK for experimental Chagas disease (Fig. 4).

The treatment of infected mice with 40 mg/kg of HNK-loaded microparticles each 2 days was able to provoke a significant decrease in parasitemia levels compared with those recorded in untreated controls ($P<0.05$ at days 12, 14, 16, 19, and 21 post-infection with *T. cruzi*). The treatment with an equivalent amount of empty micropartricles had no effect on the parasitemia compared to untreated controls. Moreover, administration of HNK-loaded microparticles was able to reduce the number of parasites more than the treatment with 20 mg/kg/day of HNK not only in the parasitemic peak, but also in the course of infection ($P<0.05$ at days 14, 16, 19, and 21 post-infection with *T. cruzi*).

Table 1 Kinetic assessment of release data of poly(D,L-lactide-co-glycolide) microparticles contains (−)−hinokinin (HNK) in the dissolution medium

Kinetic model	R^2
Zero order	0.8995
First order	0.7385
Higuchi	0.9589
Hixon–Crowel	0.7414

R^2: Coefficient of determination

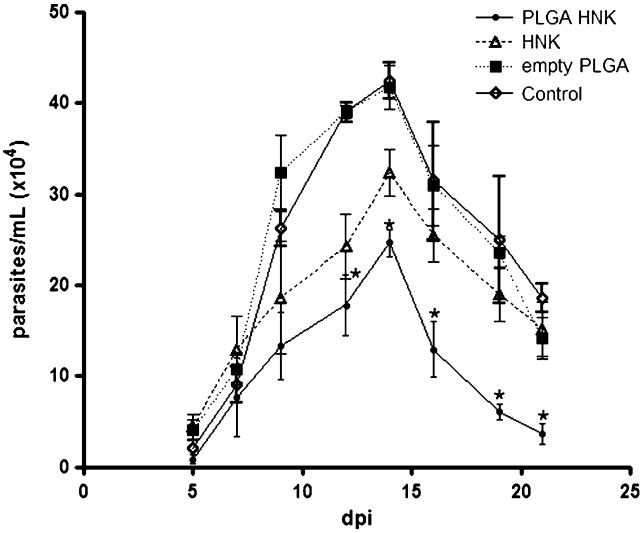

Fig. 4 Course of parasitemia in *T. cruzi* CL B5 clone-infected mice receiving: (−)−hinokinin-loaded microparticles (PLGA HNK, *filled circles*), (−)−hinokinin (HNK, Δ), empty PLGA (*filled sqaures*) or none (control, *empty diamonds*). dpi: days post infection. *$P<0.05$

The use of PLGA microparticles as vehicle for HNK delivery could improve HNK trypanocidal activity. It may be attributed to the fact that it can protect HNK of biological interactions and promote its sustained release, with maintenance of its plasmatic concentration in therapeutic levels. Additionally, the system obtained can become possible the administration of HNK by different routes and increase the time intervals between doses, reducing the discomfort of daily administration.

Conclusion

The Chagas disease chemotherapy is still insufficient and need more efficient and less toxic drugs to act specifically against the pathogen. Administration of drugs using biodegradable PLGA polymers has generated great interest due to its excellent biocompatibility and biodegradability. We demonstrated the development of a delivery system that improves trypanocidal activity of HNK. The microparticles preparation method used to be suitable, since the morphological characteristics, encapsulation efficiency, and in vitro release profile were satisfactory.

Hence, the HNK-loaded microparticles developed are a promising system for sustained release of HNK for therapeutic use and could be used in future clinical studies. Also, it is very important to point out that other in vivo

assays, under different therapeutic protocols, will be developed by our group in order to evaluate the parasitological cure of infection and the activity of this delivery system coating HNK against other strains of *T. cruzi*.

Acknowledgments The authors would like to thank "Centro Nacional de Desenvolvimento Científico e Tecnológico" (Cnpq, Brazil) and "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP, Brazil) for financial support.

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