Etanidazole in pH-sensitive liposomes: Design, characterization and in vitro/in vivo anti-<i>Trypanosoma cruzi</i> activity

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Abstract

In this work, the hydrophilic, low molecular weight and trypanocidal drug etanidazole (ETZ) was loaded in pH-sensitive liposomes (L-ETZ). Liposomes were made of dioleoyl-phosphatidylethanolamine: cholesteryl hemisuccinate (DOPE:CHEMS, 6:4, mol:mol), of 380 nm size at 14% ETZ/total lipid (w/w) ratio. To follow their uptake and intracellular fate by fluorescence microscopy, pH-sensitive liposomes were loaded with the fluorophore/quencher pair HPTS/DPX. A fast and massive delivery of the liposomal aqueous content into the cytosol of murine J774 macrophages was observed. L-ETZ vesicles were phagocytosed by both uninfected and <i>Trypanosoma cruzi</i>-infected macrophages. A 72% of anti-amastigote activity (AA) was demonstrated on L-ETZ-treated J774 cells, whereas the same dose of free ETZ rendered 0% AA. Endovenous administration of L-ETZ at 14 μg/mouse dose provoked significant decrease in parasitemia levels of <i>T. cruzi</i>-infected mice. Conversely, inoculation of a 180-fold higher dose of free ETZ failed in reducing the number of bloodstream trypomastigotes. Hence, these results point to develop systems, such as L-ETZ, designed for selective delivery of drugs to the cytoplasm of phagocytic cells, thus enhancing the efficacy of molecules considered poorly active.

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1. Introduction

Chagas’ disease is widespread throughout Latin America, affecting millions of rural and urban people with a high impact on morbidity and mortality [1]. Current treatment for Chagas’ disease is dependent on
the 2-nitroimidazole benzimidazole (BNZ, Radanil, Roche), the only drug available for human use in Brazil and Argentina. This specific chemotherapy has limitations, such as lack of effectiveness to achieve parasitologic cure, prevention of the chronic phase of disease and emergence of parasite resistance [2–4]. Moreover, the therapeutic dose is very close to the toxic dose and severe side effects have been reported during its clinical use, including polyneuritis, lymphadenopathy, dermatitis and depression of bone marrow [5]. Frequently, as a consequence of the adverse reactions, treatments have to be discontinued. In spite of this, the WHO [6] indicated that every Trypanosoma cruzi-infected individual should be subjected to parasitidal chemotherapy, independently from the stage of infection they undergo.

Undoubtedly, there is a need for developing new compounds or novel strategies to make Chagas’ disease chemotherapy more effective and less toxic. Modulation of biodistribution and/or pharmacokinetics is an important factor to increase the efficacy and lowering the toxic effect of drugs. Particularly, massive and selective drug delivery into the infected cell’s cytoplasm where T. cruzi amastigotes reside, together with a reduction of the dosage, remains as a challenge for Chagas’ disease chemotherapy.

Looking for a new alternative to BNZ, recently it has been demonstrated the in vitro trypanocidal activity of the 2-nitroimidazole etanidazole (ETZ, SR-2508) [7], a well known hypoxia marker and cell radiosensitizer used in cancer diagnosis and chemotherapy that proved to be less toxic in vivo [8]. ETZ displays lethal activity against isolated trypanastigotes as well as amastigotes of T. cruzi (RA strain) grown in Vero cells or murine J774 macrophages without affecting host cell viability. However, ETZ activity was not only time- and parasite-life stage-dependent, but also the LD50 on trypanastigotes and IC50 on amastigotes were substantially lower than those corresponding to BNZ [7]. In addition, ETZ is a hydrophilic drug that diffuses more slowly across both the host and the parasite’s membrane than BNZ, a hydrophobic drug with similar molecular weight [8].

Consequently, its accumulation inside the target cells is probably achieved at lower rate and concentration than that of BNZ. A combination of slower diffusion and less accumulation could account for the lower in vitro activity registered for ETZ, in comparison to BNZ. If this is true, ETZ activity could be increased by forcing its accumulation inside the target cells. This could be achieved by loading ETZ in plain liposomes that, when phagocytosed, force massive amounts of molecules loaded in the inner aqueous phase to rapidly enter the target cells. Ordinarily, liposomes are kept trapped inside the phago-lysosomal system, where both the liposomes and their content are degraded by a combination of low pH and lytic enzymatic activity. Only if the carrier content is able to escape from the endo-lysosomal confinement to the cytosol, a remarkable delivery of intact molecules may be achieved. Hence, pH-sensitive liposomes must be used to get massive delivery of the hydrophilic ETZ inside the cytoplasm. The pH-sensitive mechanism relies on the destabilisation of liposomal membrane and fusion between liposomal and endosome/lysosome membranes, triggered by acidification of the surrounding medium, with subsequent release of hydrophilic molecules loaded in the aqueous phase to the cytoplasm [9–11]. It will be described in this work the design and characterisation of pH-sensitive liposomes loaded with ETZ as a tool to get massive delivery of the drug to the intracellular compartment, with the aim of studying L-ETZ in vitro activity on T. cruzi-infected macrophages and their in vivo efficacy on a murine model of T. cruzi infection.

2. Experimental

2.1. Materials

ETZ (purity: 99.85%) was supplied by the National Institutes of Health (NIH Bethesda, Maryland, USA). Dioleoyl-phosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) were from Avanti Polar Lipids (Alabama, USA). Cholesteryl hemisuccinate (CHEMS) and Tris buffer were from Sigma St. Louis MO, USA. Acetonitrile (ACN) HPLC grade, C18 CH and CH3OH (analytical grade) were purchased by Anedra, Argentina. The pH-sensitive fluorophore 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) and the
quencher p-xylene-bispyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR).

2.2. Liposomal preparation

Liposomes containing etanidazole (L-ETZ) composed of DOPE:CHEMS at 6:4 mol:mol ratio, were prepared by mixing lipids from Cl3CH:CH3OH (9:1, v/v) solutions and rotary evaporated at 50 °C in round bottom flasks until organic solvent elimination. The resulting film was flushed with N2, and rehydrated at 60 °C with 65 mg/ml of ETZ solution in 0.05 M Tris–HCl buffer (pH 8.7) up to a final concentration of around 20 µmol/ml total lipids (TL). The liposomes were extruded through two stacked 0.2 µm pore size filters (Nucleopore, Whatman) using a mini-extruder device from Avanti Polar Lipids. The resulting large unilamellar vesicles (LUV) were submitted to five freeze-thaw cycles between −196 °C and 60 °C in order to increase the drug encapsulation. The free ETZ was separated from liposomal ETZ by gel permeation chromatography in a Sephadex G-50 column.

2.3. Liposomal characterization

Mean particle size was determined by dynamic light scattering at 25 °C with a Nicomp 380 submicron particle sizer. The drug (D)/TL ratio, expressed as % w/w, was determined by lipid and ETZ quantitation. Liposomal lipid was quantified by a colorimetric phosphate micro assay [12] and liposomal ETZ was quantified after complete disruption of one volume of liposomal sample in 10 volumes of ACN, by reverse phase HPLC. HPLC was performed on Beckman System Gold with a Programmable Solvent Module 125 and a Programmable Detector Module 166. The wavelength was set at 324 nm with a response time of 1 s. The analytical column was a reverse phase Kromasil C18 (25×0.4 cm i.d., 100 Å, 5 µm particle size). The mobile phase was acetonitrile–water (ACN:H2O) (10:90, v/v), degassed by vacuum. Elution was performed isocratically at a flow rate of 0.9 ml/min with a sample injection volume of 20 µl. A seven-point calibration curve ranging from 0.3 to 6 µg ETZ/ml, was prepared by adding 10–50 µl of ETZ from a stock solution in deionized water to 500 µl of ACN. Finally, liposomes were visualized by electron microscopy with a JEOL JEM 1200 EX II microscope upon phosphotungstic acid negative staining [13].

2.4. Cell culture and infection

The murine macrophage-like cell line J774 was maintained at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 UI/ml penicillin and 100 µg/ml streptomycin. Cells were plated in 24-well plates at a density of 50,000 cells/well with rounded coverslips on the bottom. After overnight incubation, cells were infected with culture T. cruzi trypomastigotes, RA strain, at a parasite-to-cell ratio of 10:1 for 24 h at 37 °C. Extracellular parasites were removed by six washes with phosphate buffered saline (PBS), pH 7.2.

2.5. Endocytosis and intracellular fate of pH-sensitive liposomes followed by fluorescence microscopy

Liposomes were prepared up to the same final D/TL and TL concentration as stated in 2.2, excepting that the lipid film was hydrated with a solution containing 35 mM HPTS and 50 mM DPX in 0.05 M Tris–HCl buffer (pH 8.7). After extrusion and freeze-thaw cycles, the free HPTS and DPX were eliminated by gel permeation in Sephadex G-50. Non pH-sensitive liposomes made of DOPC:CHEMS (6:4, mol:mol ratio) containing HPTS and DPX were prepared in a similar fashion.

The liposomal suspensions were added to each well of both uninfected and T. cruzi-infected J774 macrophages (72 h post infection) at 640 µg TL/10^6 cells, and the emission of the HPTS was monitored by fluorescence microscopy.

2.6. Anti-amastigote activity of L-ETZ

Murine J774 macrophages infected with T. cruzi were treated with L-ETZ, free ETZ at the same concentration than in liposomes (200 µg ETZ/ml) or empty liposomes, for 2 h at 37 °C. Each treatment was done in duplicate wells. Macrophages were washed and further incubated 72 h in fresh medium at 37 °C. The coverslips were removed, washed with PBS, fixed with methanol and stained with Giemsa. The number of amastigotes/100 cells was counted by
using light microscopy. Untreated infected macrophages were used as control.

Anti-amastigote activity was expressed as: % AA=
\[
\frac{1-(n^c \text{ amast}/100 \text{ cells}) \text{ treated}}{(n^c \text{ amast}/100 \text{ cells}) \text{ control}} \times 100.
\]

2.7. L-ETZ administration to infected mice

Female Balb/c mice (weight, 20 to 22 g) were infected intraperitoneally with 50 T. cruzi RA strain trypomastigotes. Since the fifth day of infection, mice were treated as follows: Group I received L-ETZ at 14 µg ETZ/mouse. Group II received 85 µl of 0.05 M Tris buffer, pH 8.5 (untreated control). Group III received a highly concentrated ETZ solution, at a dose of 2.5 mg ETZ/mouse. Group IV received empty liposomes. All preparations were injected into the tail vein, three times a week over a period of 21 days. Doses used in this experiment were below LD$_{50/2}$ considering that reported LD$_{50/2}$ for ETZ after intravenous administration in mice is 88 mg/mouse [14].

Parasitemia was monitored by daily counting of the number of trypomastigotes per 5 µl of fresh blood [15]. Statistical analyses were carried out with the Prisma 3.0 Software (GraphPad, San Diego, California). Mann–Whitney test was conducted for the data analyses. Differences were considered statistically significant when $P<0.05$.

3. Results and discussion

3.1. Liposomal characterization

The HPLC method was developed ad hoc to quantify small amounts of liposomal ETZ. The isocratic elution with ACN:H$_2$O (10:90, v/v) at a flow rate of 0.9 ml/min resulted in linear response of the peak areas versus the concentration of the compound. The assay was validated using calibration solutions of ETZ in the absence and in the presence of HSPC. The results showed no differences between ETZ chromatograms at both conditions.

The optimisation of L-ETZ preparation was done by tuning three parameters (ETZ concentration in the aqueous buffer of suspension, TL concentration and number of freeze/thaw cycles) until finding that combination that rendered the maximal D/TL ratio. According to that, it was observed that the D/TL increased with the increase of the concentration of ETZ solution, up to the maximal ETZ concentration (65 mg ETZ/ml) above which crystals appeared. The TL concentration was kept as low as possible in 10 mg TL/ml; attempts to increase TL concentration produced a suspension difficult to handle, causing frequent clogging of the gel filtration column and without further increase of D/TL. By keeping constant the ETZ and TL concentration, the D/TL increased with the number of freeze/thaw cycles, resulting optimal 5 cycles beyond that the D/TL ratio remained unchanged. The recovery of TL after exclusion chromatography was around 80%. The concentration of the resulting liposomal suspensions was 1.28 mg TL/ml ± 0.21 at a 14% w/w D/TL ratio with a mean diameter of 379±58 nm. For that condition, the percentage of drug encapsulation corresponded to 0.8–1.2%.

Fig. 1 depicts the electron micrographs of L-ETZ, where the oligolamellar structure of the resulted liposomes after the freeze/thaw cycles is clearly seen. The pH-sensitivity of the oligolamellar vesicles was preliminary evidenced by following the changes of mean size as a function of pH; when the pH of the

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![Fig. 1. Electron micrograph of LUV-ETZ. Liposomes were negatively stained with phosphotungstic acid. Inset: isolated unilamellar vesicle.](image-url)
external medium dropped from 8.7 to nearly 3, the vesicles increased their mean size up to fivefold times (around 2 μm), indicating a fusion process triggered by the acidity of the medium.

3.2. Endocytosis and intracellular fate of pH-sensitive liposomes followed by fluorescence microscopy

This assay was carried out to assess the uptake and intracellular pathway of the fluorescent aqueous content of the liposomes, once captured by phagocytic cells. To this aim, pH-sensitive and non pH-sensitive liposomes containing the fluorophore/quencher pair HPTS/DPX within the aqueous compartment were incubated with J774 macrophages and the course of internalisation was followed by fluorescence microscopy. Upon excitation at 440 nm, a punctual pale emission indicated confinement of the pair HPTS/DPX in acidic compartments. Conversely, homogeneously distributed strong emission upon excitation at 440 nm, indicated release of the pH-sensitive dye HPTS from endosomes/lysosomes towards the cytoplasm [16,17].

Detection of intracellular transit of the fluorescent dye started at least after 15-min incubation. For the pH-sensitive formulation, the initial binding stage was seen as fluorescent dots attached to the cell membrane (Fig. 2a); thereafter, the liposomes entered the endo-lysosomal system and 7 min later an intense fluorescence suddenly filled the cytoplasm (Figs. 2bi,bii), showing release of HPTS to a neutral pH-medium. This indicated that, as a result from membrane fusion between endosomes and vesicles, a massive delivery of the pH-sensitive liposome content into the cytoplasm had occurred, triggered by the pH drop along the endocytic pathway [16]. On the other hand, the events of binding and internalisation for non pH-sensitive formulation followed the same time-course than pH-sensitive liposomes. However, even up to 1.5 h after binding, no release of liposomal content into the cytoplasm was registered, since those vesicles are not affected by the progressive acidification of the phagocytic route [16] (Fig. 2c). Controls done with free HPTS showed that upon incubation cells displayed low levels—compared with pH-sensitive liposomes—of diffuse fluorescence that remained stationary along the time of experiment (data not shown).

Remarkably, phagocytic cells infected with amastigotes of T. cruzi were also capable of entrapping both pH-sensitive and non pH-sensitive liposomes, as seen in Fig. 2d and e. These results demonstrated that the intracellular amastigote nests grown 72 h pi did not impair the functioning of the cell machinery responsible for phagocytosis of particulate material, in agreement with previous reports [18].

3.3. Anti-amastigote activity

Overall, after 2 h of uptake and intracellular transit at 37 °C, no changes in viability of the L-ETZ treated cells, either infected or not, were observed (data not shown). As shown in Table 1, the AA of free ETZ and empty liposomes at 72 h pi was nule (0%); the same dose, however, of L-ETZ had a substantially higher (72%) AA.

In a previous report [7], it was demonstrated that free ETZ displays AA when J774 macrophages were treated with the drug for a much longer interval. Indeed, the time of contact between ETZ and infected cells appears to be central for exerting an effective AA. Free ETZ possesses hydrophilic features that only permit slow drug uptake across the macrophage membrane. Conversely, rapid phagocytosis of massive amounts of L-ETZ would enable fast and full AA in T. cruzi-invaded cells.

Remarkably, a main proportion of the liposomes floated near the surface of the culture medium layering each well. The proportion of floating liposomes was high and although not quantified, the phenomenon should be considered when interpreting these in vitro findings; it is likely that the AA from liposomes resulted from the uptake and delivery of those few vesicles closer to the cell layer.

3.4. Effect of L-ETZ on parasitemia

In the interpretation of the following results, it is necessary to recall background research done with liposomal BNZ. This hydrophobic 2-nitroimidazole is the only drug used in the current treatment against Chagas’ disease. Upon absorption, there is an extensive binding of BNZ to plasma proteins (70%) [19], and results are homogeneously distributed in the body. Therefore, increasing BNZ selectivity for infected tissues should improve its anti-trypanocidal
Fig. 2. Upon incubation with pH-sensitive or non pH-sensitive liposomes for 15 min at 37 °C, cells were washed 4 times and coverslips were mounted on an Olympus BH2-RFGA fluorescence microscope. The emission of HPTS at 510 nm was recorded upon excitation with blue filter (450–490nm). (a) Binding/uptake event: quenched pale points of HPTS fluorescence, observed 7 min post incubation with pH-sensitive liposomes. (bi) Early delivery event: intense dequenched and uniform HPTS fluorescence, corresponding to the first cell where the HPTS was delivered into the cytoplasm, 15 min post incubation with pH-sensitive liposomes. (bii) Late delivery event: cytoplasms showing intense dequenched and uniform HPTS fluorescence, 20 min post incubation with pH-sensitive liposomes loaded with HPTS/DPX. (c) Control: quenched pale points of HPTS fluorescence observed 90 min post incubation with non-pH-sensitive liposomes loaded with HPTS/DPX. (d) HPTS fluorescence observed 7 min post incubation of pH-sensitive liposomes loaded with HPTS/DPX and infected J774 cells; arrows show the amastigotes inside the cytoplasm. Upper left side inset: infected cells stained with Giemsa. (e) Upper image: HPTS fluorescence 7 min post incubation of pH-sensitive liposomes loaded with HPTS/DPX and infected J774 cells. Lower image: time sequence (from left to right): HPTS fluorescence 13, 14 and 15 min post incubation of pH-sensitive liposomes loaded with HPTS/DPX and infected J774 cells.
In a previous work, upon designing a BNZ delivery system for targeting the amastigote nests inside Kupffer cells, the course of parasitemia in T. cruzi-infected mice receiving or not liposomal preparation was followed-up. A single bolus of a 0.7% w/w BNZ/TL multilamellar formulation was i.v. administered three times a week over a 21-day period, at 14 μg BNZ/mouse. It was disappointing to find that, neither the transient and threefold higher accumulation of BNZ in the liver, nor blood levels of BNZ, were sufficient to defeat the infection [20].

The important factor of drug’s ultimate spatial location in the target cells could account for the failure of liposomal BNZ; it is likely that the hydrophobic BNZ remained trapped inside the endolysosomal system instead of being massively delivered to the amastigotes cysts. Since the dose of liposomal BNZ could not be further increased without potentially blocking the reticuloendothelial system, a liposomal form of BNZ represents an inadequate strategy to improve Chagas’ chemotherapy.

On the other hand, as the hydrophilic ETZ, unlike the hydrophobic BNZ, should remain soluble at the inner aqueous phase and unreleased from the membranes upon dilution in the bloodstream [20,21], it is a good candidate to be loaded into liposomes to optimise its anti-T. cruzi activity already demonstrated in vitro.

The results included in the present study show that treatment of infected mice with L-ETZ at 14μg ETZ/mouse (111 μg TL/mouse, nearly 20-fold lower amount of TL than that injected with L-BNZ) provoked a significant decrease in parasitemia levels compared with those recorded in untreated controls (P<0.05 at days 12, 19, 21 and 23 pi) (Fig. 3). Administration of an equivalent amount of empty liposomes had no effect on the parasitemia compared to untreated controls. Remarkably, i.v. administration of free ETZ at 2.5 mg/mouse dose did not show any efficacy. Higher dosages of free ETZ were not assayed, as it was not the aim of this study. A 180-fold lower dose of liposomal ETZ was enough to exert a biological effect in this in vivo model. However, a complete parasitologic cure could not be achieved with L-ETZ treatment.

Even though in the same model a conventional oral therapy with BNZ did lead to whole eradication of infection, it should be considered that the RA strain used along the study displays susceptibility to this drug. However, in vivo induction of BNZ resistance of different isolates of the parasite has been described [22,23].

L-ETZ limitations may be attributed to the fact that, in addition to hepatic and spleen macrophages, along the course of infection T. cruzi also infects non-phagocytic nerve and muscle cells that could remain hardly accessible to this pH-sensitive drug delivery system. Perhaps, L-ETZ should be modified to better reach different cell types and get a more drastic diminution in parasitemia levels.

An important issue to be addressed whenever in vivo studies are pursued is the question about the behaviour of the selected liposomal formulation in biological media. Structural destabilisation has been attributed to interaction with serum lipoproteins as

![Fig. 3. Course of parasitemia in T. cruzi RA strain-infected mice receiving L-ETZ (○), free ETZ (●), empty liposomes (■) or none (■ (dpi: days post infection). ★ P<0.05.](image)
well as uptake by the reticuloendothelial system [24,25]. Previous investigations have proved that pH-sensitive liposomes including anionic stabilizers such as oleic acid in their composition resulted far less stable than those containing CHEMS [26]. On the other hand, recent work from Simões et al. [11] concludes that the fast disappearance of intravenously injected pH-sensitive liposomes made of DOPE:-CHEMS from blood circulation is not due to membrane disruption caused by interaction with plasma proteins, but to their higher tendency to be captured by tissue macrophages, mainly at the liver and the spleen. Therefore, the lack of structural stability exhibited by this kind of liposomes does not prejudice the current strategy, aimed to get a massive delivery of drug over \( T. cruzi \)-infected reticuloendothelial cells.

4. Conclusions

There is an urgent need for developing novel strategies to make Chagas’ disease chemotherapy safer and more effective in eradicating its etiologic agent. We designed a controlled release system capable of delivering ETZ to the cytoplasm of infected cells, tested its anti-\( T. cruzi \) amastigote activity in vitro and studied its in vivo effect on the parasitemia course of infected mice.

The AA of L-ETZ determined in vitro was markedly higher than that displayed by the free drug along 2-h treatment of parasite-invaded cells. The L-ETZ performance in vivo was expected to be limited by a selective uptake by accessible infected phagocytes. Interestingly, L-ETZ trypanocidal effect was sufficient to cause a significant decrease of parasitemia levels in mice infected with the pantrropic/rheiculotropic RA strain of \( T. cruzi \). In addition, when pH-sensitive, ETZ-loaded liposomes were administered at the same dosage than that of liposomal BNZ–useless to diminish parasitemia—a substantial reduction in the concentration of blood parasites was achieved. It is noteworthy that the dose of L-ETZ used was almost 180-fold higher than that of free ETZ which proved ineffective to decrease experimental parasitemia.

Further investigations including evaluation of L-ETZ dose-dependent effect on parasitemia and \( T. cruzi \) killing activity of ETZ when encapsulated in non-pH-sensitive liposomes, remain to be accomplished.

Hence, the preliminary results shown in the present study support the alternative of drug delivery systems, such as L-ETZ, constituted by vesicles designed for selectively delivering massive amounts of hydrophilic drugs to the cytoplasm. This approach could be helpful in improving the potential of a drug initially considered as poorly active and otherwise probably discarded after a conventional drug screening portfolio.

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