J Incl Phenom Macrocycl Chem (2012) 73:397–404
DOI 10.1007/s10847-011-0077-5

ORIGINAL ARTICLE

Study of benznidazole–cyclodextrin inclusion complexes, cytotoxicity and trypanocidal activity

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Received: 15 July 2011 / Accepted: 24 October 2011 / Published online: 11 December 2011

Abstract The current chemotherapy for Chagas disease is still based on benznidazole, which has low solubility, but complexation with cyclodextrins provides a way of increasing the solubility. The objective of this work was to characterize the inclusion complexes formed between benznidazole (BNZ) and randomly 2-methyled-β-cyclodextrin (RM-β-CD) in aqueous solution and study cytotoxicity and trypanocidal. BNZ:RM-β-CD solution complex systems were prepared and characterized using the phase solubility diagram, nuclear magnetic resonance and a photostability assays, also to investigate the in vitro trypanocidal activity with epimastigote forms of *Trypanossoma cruzi* and the study of cytotoxicity against mammal cells. The phase-solubility diagram displayed an A_L-type feature, providing evidence of the formation of soluble inclusion complexes. The continuous variation method showed the existence of a complex with 1:1 stoichiometry. Toxicity assays demonstrated that inclusion complexes were able to reduce the toxic effects caused by benznidazole alone and that this did not interfere with the trypanocidal activity of the benznidazole. The use of inclusion complexes benznidazole:cyclodextrin is thus a promising alternative for the development of a safe and stable liquid formulation and a new option for the treatment of Chagas disease.

Keywords Inclusion complex · Stability · Solubility

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Introduction

The neglected tropical diseases are chronic and debilitating conditions, caused by parasitic, bacterial, and other infections [1]. According to the World Health Organization at least one billion people, representing one-sixth of the world's population, suffer from one or more neglected tropical diseases. Among them, African (sleeping sickness) and American (Chagas disease) trypanosomiasis are widespread in tropical and subtropical parts of the world [2].

Chagas disease is an endemic illness caused by the protozoan, *Trypanosoma cruzi*, It is considered as a serious medical and social problem in Latin America, affecting approximately 10 million individuals, with an additional 40 million people at risk of infection [3]. The current chemotherapy for Chagas disease is still based on benznidazole (BNZ) (*N*-benzyl-2-nitroimidazole-1-yl acetamide) [4]

(Fig. 1a), which is very toxic and has low solubility [5, 6], but complexation with cyclodextrins provides a way of increasing the solubility of drugs [7].

Cyclodextrins (CD) are a family of natural or synthetically modified cyclic molecules. They have a toroidal shape with a hydrophobic central cavity and a hydrophilic outer surface, on which the hydroxyl groups are located [8].The natural cyclodextrins, in particular β-cyclodextrin (β-CD), are of limited aqueous solubility. Other CD of pharmaceutical interest includes the derivatives of β-CD, such as randomly 2-methylated β-cyclodextrin (RM-β-CD) (Fig. 1b) [9].

This study thus aims to characterize the inclusion complexes (IC) formed between BNZ and RMβCD (Fig. 1c) in aqueous solution using the phase-solubility diagram, nuclear magnetic resonance and photostability assays and investigates its trypanocidal activity and cytotoxicity against mammal cells.

Methods

Materials

The BNZ was donated by Laboratório Farmacêutico de Pernambuco (LAFEPE). The β-CD and RM-β-CD (molar substitution 0.5) was donated by APSEN Pharmaceutica® (Brazil) and Roquette® (Spain). The deuterated water (D_2O) was purchased from Tedia (Brazil). HPLC-grade acetonitrile was obtained from Laboratório de Tecnologia dos Medicamentos (LTM). Other reagents and chemicals were of analytical reagent grade. The solutions were prepared using ultrapure water (MILLI Q) and filtered through 0.22 μm Millipore® nylon and a 0.45 μm cellulose membrane filter (Brazil).

Analytical method

Quantification of the BNZ was measured using UV spectrophotometry and high performance liquid chromatography (HPLC) in accordance with the methodology developed and validated by Soares-Sobrinho et al. [10] and Silva et al. [11]. The equipment used was a HPLC Shimadzu® SCL-10A VP controller pump, a Shimadzu® SIL-10AD VP auto injector, a UV–Vis SPD- M10A VP detector (detection: 316 nm for BNZ), Class-VP 6.14 software, reversed-phase Shimadzu®, C_{18}, 5 μm, 15 × 0.46 cm^2, and the spectrophotometer UV/Vis Micronal® B582 at a wavelength of 324 nm.

Phase-solubility diagram

The study was carried out according to the previously described method [12]. Excess amounts of BNZ (5 mg) were added to 6 mL of solutions containing increasing concentrations of β-CD and RM-β-CD, ranging from 0 to 0.010 M and 0 to 0.050 M respectively. The resulting mixture was equilibrated in a thermostatic shaking water bath for 48 h at 25 °C protected from light. After equilibrium was reached, the suspensions were filtered through a 0.45 μm cellulose membrane filter to remove insoluble solids. Quantification of the BNZ was measured using UV spectrophotometry as described in previous section.

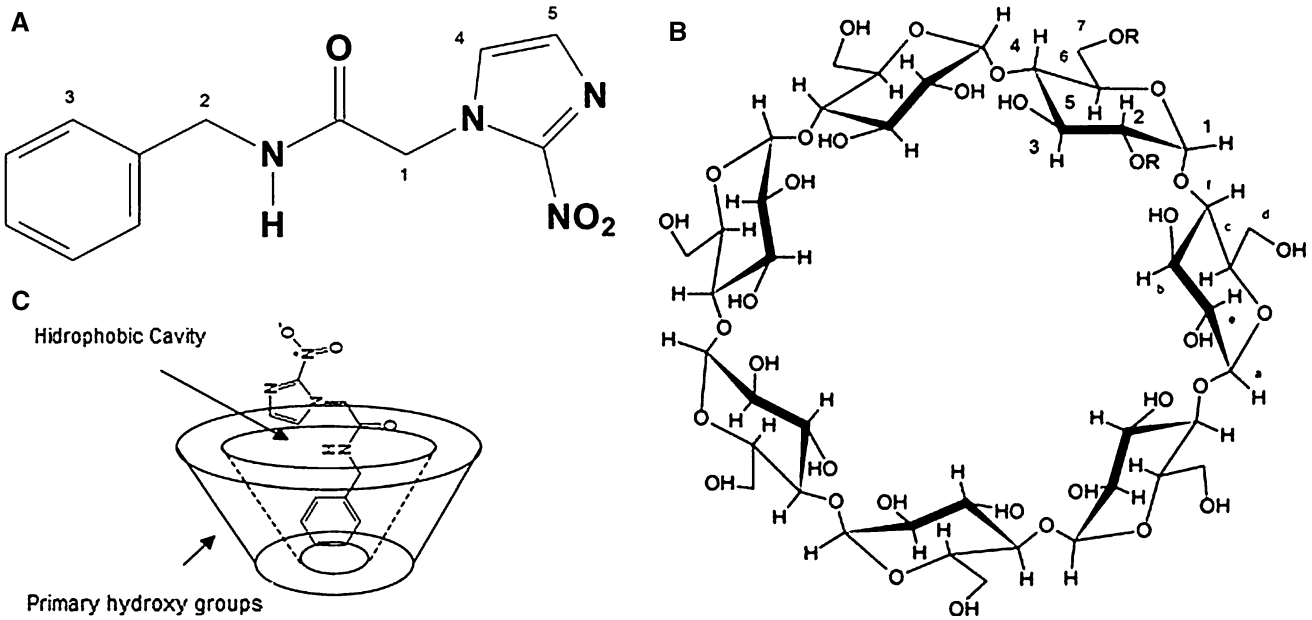

Fig. 1 **a** Chemical structure of BNZ and **b** the natural (β-CD) (R = H) and RM-β-CD (R = CH_3), **c** representation IC of CD with BNZ

The apparent complexation constant (K) was calculated from the phase-solubility diagrams according to the following Eq. 1 [7] from phase solubility slope, where the intercept (S_o) is the intrinsic solubility of BNZ at 25 °C in the absence of CD.

$$K = \frac{\text{slope}}{S_o(1 - \text{slope})} \quad (1)$$

IC were obtained by mixing appropriate amounts of solid BNZ and RM-β-CD in deionized water and D_2O for nucler magnetic resonance analysis, a molar proportion of 1:1. The resulting mixture was magnetically agitated at room temperature (25 °C) for 48 h and protected from light [13].

Study of the complex using nuclear magnetic resonance

One-dimensional ^{1}H NMR spectra were recorded on a Varian UNITY plus 300 MHz spectrometer. The IC were obtained by mixing appropriate amounts of BNZ (0.8 mM) and RM-β-CD in D_2O at a molar ratio of 1:1 as described in the previous section. The solutions were added to 5 mm NMR tubes, to a total sample volume of 600 µL. The probe temperature was set at 20 °C.

The ^{1}H NMR spectra were recorded using a pulse sequence of a single pulse. Typical acquisition parameters consisted of 32 K points covering a sweep width of 5,000 Hz, a pulse width (pw90) of 5 µs and line broadening of 1 Hz was applied to the FID before the FFT. Resonance at 4.72 ppm, which represents the residual water solvent peak, was used as an internal reference.

The shift chemical variations in the formation of IC can provide evidence through Eq. 2:

$$\delta f_{obs} = \chi_f \delta f_f + \chi_b \delta f_b \quad (2)$$

Where δf_{obs}, δf_f, δf_b are the observed chemical shift variation, the chemical shift of the free species and the chemical shift of the bound compounds, respectively. The symbols χ_f e χ_b represent the fractions of free and bound species [14].

Determination of stoichiometry using the continuous variation method (Job's method)

The stoichiometry of inclusion was determined using the method developed by Job [15]. ^{1}H NMR spectra were obtained for a series of BNZ: RM-β-CD mixtures, in which the total initial concentration of both species was kept constant (Equimolar 1.0×10^{-3} M solutions), while the mol fraction of each component varied from 0 to 1.

Photostability assays

The photostability of BNZ (0.8 mM) was assessed in a water solution in the presence and absence of RM-β-CD (0.8 mM). The solutions were positioned 15 cm away from a 22 W UV (Philips–Holland) (254 nm) lamp as a light source. The solutions were placed in an incubator at 28 °C, sampled at specified time intervals (0, 1, 2, 3, 4, 5 and 6 h) and the concentration of BNZ was determined by way of HPLC assay. The mobile phase used for these HPLC assay was acetonitrile: water (50:50, v/v) and pumped at a flow rate of 1.0 mL/min. The chromatographic experiments were carried out at 30 °C and the injection volume was 20 µL for all experiments.

The results were expressed as percentages of the BNZ. Each test was carried out in triplicate. Data analysis was performed using the Student's t test, with a level of significance of 5%.

Trypanocidal activity

Epimastigote forms of *T. cruzi,* DM28c clone, were maintained in a Liver Infusion Triptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) at 28 °C. For the experiments, three-day-old culture forms (5×10^6 parasites/ml) were incubated for 48 h in 24-well microplates at increasing concentrations (0.0125–0.2 mM) of BNZ and BNZ: RM-β-CD complexes with a molar ratio of 1:1 diluted in ultrapure water. Parasites grown in LIT medium were used as a control.

The IC_{50} (the concentration that inhibits 50% parasite growth) was estimated by counting cells using a Neubauer chamber and the data were then submitted to regression analysis using SPSS 8.0 for Windows. Each experiment was carried out in triplicate and in two independent experiments.

Cytotoxicity assays

The cytotoxicity was determined by way of an [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] (MTT) assay. Balb/c mice peritoneal macrophages were obtained and maintained in a Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 120 µg/mL of gentamicin sulfate, in 96-well microplates, in an atmosphere of 5% CO_2, at 37 °C. The assays were performed by incubating cells (10^5 cells/mL) with increasing concentrations (0.025–0.2 mM) of BNZ, RM-β-CD and BNZ:RM-β-CD complexes with a 1:1 molar ratio, for 48 h. Thereafter, the treated and untreated macrophages were incubated for 3 h with 0.5 mg/mL of MTT, at 37 °C. Once the medium had been removed, the number of viable cells was determined by measuring the amount of MTT converted to formazan by mitochondrial dehydrogenases. The formazan crystals formed were dissolved in DMSO and shaken for 1 h at room temperature, and the absorbance was measured using the spectrophotometric microplate reader at 570 nm. Cytotoxicity assay data were

analyzed using one-way analysis of variance (one-way ANOVA).

All experiments involving the use of animals were performed in accordance with ethical standards of Fundação Oswaldo Cruz and were approved by its ethics committee (CEUA- FIOCRUZ L-0001/08).

Results and discussion

Phase-solubility diagram

Determination of the phase-solubility diagram is a widely accepted method for evaluation of the effect of CD complexation the drug solubility [16]. Figure 2 shows the phase solubility diagram for BNZ in the presence of various β-CD and RM-β-CD concentrations. It can be seen that BNZ solubility increases linearly with CD concentration, corresponding to the A_L type profile, as classified by Higuchi and Connors [12], and this may be related to the formation of a water-soluble IC. In the region where a linear increase was observed, a linear regression analysis was performed and the fact that the slope of the diagram is less than 1 suggests the formation of complexes with 1:1 stoichiometry.

According to Jullian et al. [14], the 1:1 drug/CD complex is the most common type of association where a single drug molecule is included in the cavity of one CD molecule, with a K to provide equilibrium between the free and associated species.

The K are listed in Table 1. The K calculated using the phase solubility diagram were 57.73 and 99.31 M^{-1} for BNZ:β-CD and BNZ:RM-β-CD, respectively, suggesting the occurrence of favorable interactions.

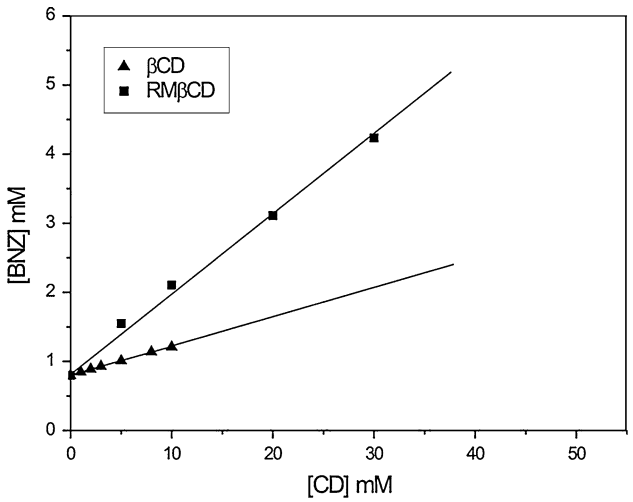

Fig. 2 Phase-solubility diagrams for the BNZ:β-CD and BNZ:RM-β-CD systems in water at 25 °C

Table 1 The $K_{1:1}$ for IC of BNZ with CD as determined using phase-solubility techniques, 25 °C

Inclusion complex	Slope	Intercept (10^{-3})	$K_{1:1}$ (M^{-1})
BNZ:β-CD	0.0497	0.906	57.73 ± 0.8
BNZ:RM-β-CD	0.0993	1.110	99.31 ± 1.2

This diagram indicates that the RM-β-CD IC with BNZ was more stable than the natural CD (β-CD), showing an enhanced complexation and solubility in the presence of methylated groups, since these groups play an important role in enlarging the CD cavity, making its environment more hydrophobic and favoring the adaptation of the CD to a guest, by means of enhanced flexibility [17].

Study of the complex using nuclear magnetic resonance

NMR spectroscopy is the most powerful tool used to study IC formation between CD and a variety of guest molecules, which has been successfully used to confirm the conformations of IC. Chemical shift variations in the host or guest molecule could provide evidence of the formation of IC, since significant changes in microenvironment are known to occur during the transition from the free to the bound state [18].

Figures 3 and 4 shows the NMR spectra of free BNZ (bottom), free RM-β-CD (middle) and the BNZ:RM-β-CD complex (top). As a result of complexation, the BNZ hydrogen resonance H-1(5.15 ppm) e H-5 (7.36 ppm) shows significant changes in chemical shift, as well as the aromatics hydrogen (H-3) occurring between 7.2 and 7.3 ppm. The chemical shift of hydrogens others in the molecule BNZ were smaller, exhibiting a chemical shift variation of <0.010 ppm. This low variation can be explained by the fact that this is a system in fast exchange between the association of BNZ and CD, where the complexes with low binding affinity (K) demonstrate the difficulty of detecting variation in chemical shift as an indicator of free and complexes states [16].

In the hydrogens H-5 e H-6 (Ha) of methyl groups RM-β-CD located inside the cavity of the CD were observed, with based on chemical signals in the β-CD spectrum (Fig. 4), variation chemical shift of IC.

Determination of stoichiometry

The continuous variation method was employed to establish the stoichiometry of the complex using NMR. If a physical parameter directly related to the concentration of the complex is plotted as a function of the mol fraction (r) of BNZ or RM-β-CD, its maximal value occurs at $r_{BNZ} = m/(m + n)$ or $r_{RM\beta CD} = n/(m + n)$, where m and n

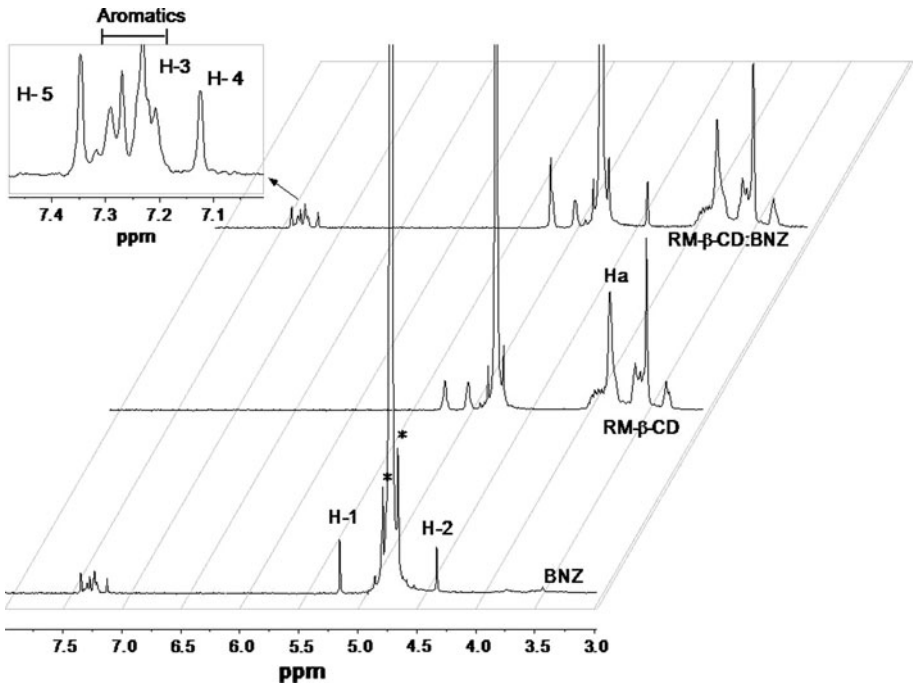

Fig. 3 ^{1}H NMR spectra (300 MHz) of BNZ (0.8 mM), RM-β-CD and BNZ: RM-β-CD (1:1 molar ratio). Samples in D_2O at 25 °C. Peaks marked with *asterisk* (*) are due to the spinner rotation

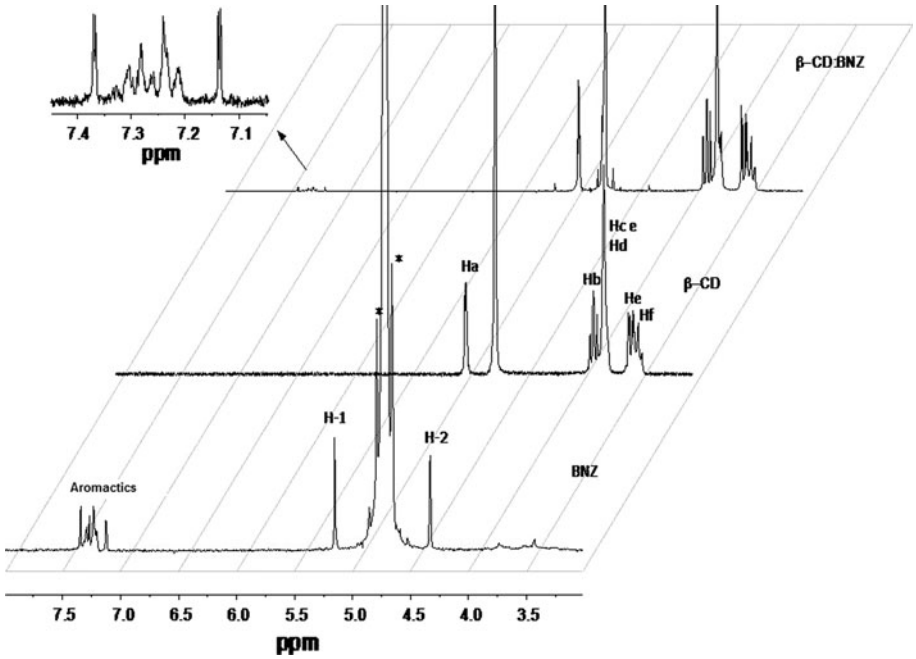

Fig. 4 ^{1}H NMR spectra (300 MHz) of BNZ (0.8 mM), β-CD and BNZ: β-CD (1:1 molar ratio). Samples in D_2O at 25° C. Peaks marked with an *asterisk* (*) are due to the spinner rotation

are the molar ratios of BNZ and RM-β-CD in the complex, respectively [19].

In the NMR spectra, the calculated quantity $\Delta\delta$ [BNZ] will be proportional to the complex concentration and can be plotted against r. The continuous variation method was applied to the hydrogens of the molecules (host and guest) and yielded identical results (Fig. 5). In all cases show a maximum value at $r = 0.5$, indicating the existence of a complex with 1:1 stoichiometry, within the range of the investigated concentrations.

Photostability assays

In this study, the photostability of the BNZ and BNZ:RM-β-CD IC was examined in aqueous solution. A control was carried out using a solution of BNZ in water, in the absence of CD. The data produced indicate that RM-β-CD slowed down the photodegradation of BNZ, making it more stable in the presence of light (Fig. 6). The same effect is described in the literature for other drugs [19].

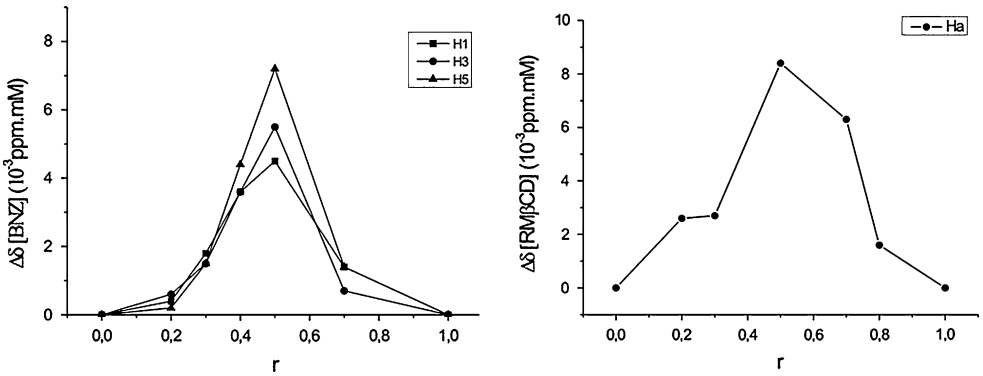

Fig. 5 Job's plots corresponding to the chemical shift displacement of **a** BNZ hydrogens and **b** RM-β-CD (Ha)

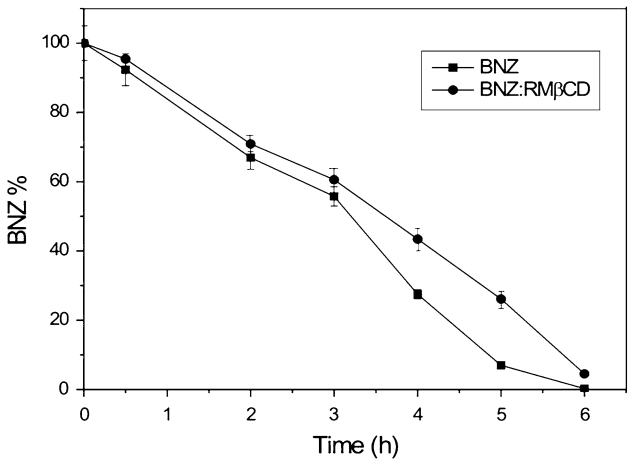

Fig. 6 Photodegradation profiles of BNZ under photoexposure radiation of an aqueous solution containing BNZ free and complexed with BNZ:RM-β-CD

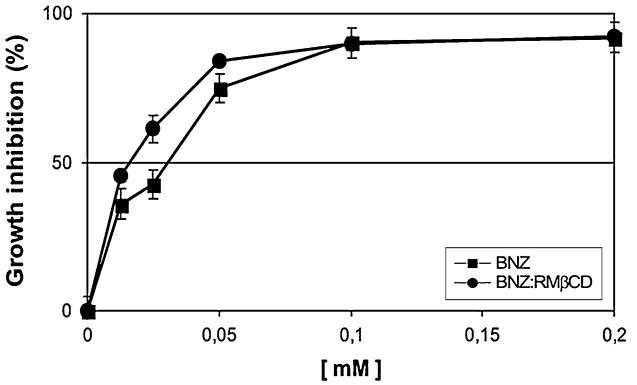

Fig. 7 Growth inhibition percentage after 48 h of BNZ (IC_{50} = 0,037 mM) and BNZ: RM-β-CD (IC_{50} = 0,027 mM) treatment against epimastigote forms of *T. cruzi*. Each point represents the standard deviation of two independent experiments on triplicate

The data reveal that the addition of RM-β-CD was not able to completely inhibit the photochemical decomposition of BNZ: RM-β-CD but decreased the observed apparent-first-order rate of photochemical decomposition of BNZ in a non-linear relationship. This result was consistent with a kinetic system in which a free drug is degraded at higher rates than the drug in the complex form.

Photochemical degradation constants (k_{obs}) for BNZ in the absence and presence of RM-β-CD were determined using the data presented in Fig. 6, by means of first-order kinetics. The values of the *k*obs for BNZ in the absence and presence of RM-β-CD are $9.17 \pm 0.12 \times 10^{-1}$ and $6.49 \pm 0.09 \times 10^{-1}$ min^{-1}, respectively.

The formation of an inclusion complex had a significant stabilizing effect ($p < 0.05$) on BNZ in terms of photodecomposition compared to that of the "free" molecule in solution. However, the observed k_{obs} values are far higher than the expected maximum effect in the free form, indicating the formation of IC.

Trypanocidal activity

Trypanocidal activity was evaluated for free BNZ and the BNZ: RM-β-CD complex. The results obtained are shown in Fig. 7. The values of IC_{50} obtained for free BNZ and for BNZ: RM-β-CD were 0.037 ± 0.2 mM and 0.027 ± 0.4 mM, respectively. The results showed that the growth inhibition performance of both BNZ and BNZ: RM-β-CD was similar and dose- and time-dependent, reaching 92% of growth inhibition for both BNZ and complexes at 0.2 mM (Fig. 7).

These results show that the complexation of BNZ with CD did not interfere with trypanocidal activity, preserving the integrity of the molecule, as observed by other analyses.

Cytotoxic assays

The cytotoxicity of BNZ, BNZ: RM-β-CD and RM-β-CD in mammalian cells was significantly different for BNZ and the BNZ: RM-β-CD complex (Fig. 8). As found by some authors [19, 20] in the case of other drugs, the

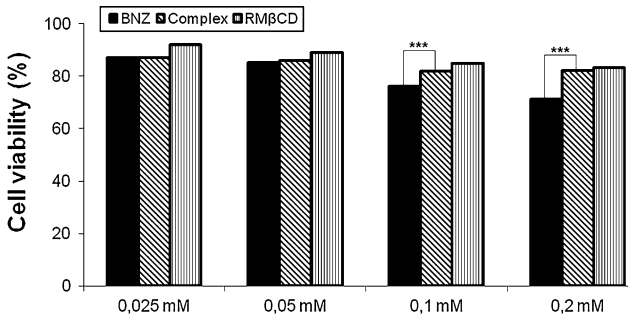

Fig. 8 Cytotoxic effects of BNZ, BNZ: RM-β-CD complex and RM-β-CD at 0,025, 0,05, 0,1 and 0.2 mM on Balb/c mice peritoneal macrophages incubated for 48 h at 37 °C and 5% CO_2 as evaluated by MTT reduction test. Data expressed as % cell viability (Mean ± SD, $n = 4$ experiments). *** Statistical significance $p < 0.05$ (one-way ANOVA)

BNZ:RM-β-CD inclusion complex produced a significant reduction in cytotoxic effects compared with the BNZ, which reduced cell viability by up to 71% at 0.2 mM.

The lower cytotoxicity against macrophages of BNZ:RM-β-CD could be explained by the differences in solubility between the complexes that are more hydrophilic than the pure drug form. These data show that there is a direct relation between cell toxicity and lipophilicity. It has frequently been reported in the literature that lipophilic substances tend to possess stronger cytotoxic properties than hydrophilic compounds, owing to the high degree penetration of these molecules into cell membranes [20].

One of the essential points about this in vitro toxicity model is that, as the observed cytotoxic effects of BNZ are dose-dependent, the protection of cells observed on treatment with the complex could be explained by the sustained release of BNZ free from the CD cavity, as demonstrated by in vitro drug-release assays [21].

Conclusion

In conclusion, our results showed that the formation of IC between BNZ and RM-β-CD considerably increase the BNZ solubilization and decrease the harmful effects of BNZ on mammals' cells and without prejudice to the trypanocidal activity, added a chemical integrity of the BNZ in solution. In this sense, the use of IC (BNZ:CD) is a promising alternative for the development of a liquid formulation, safe and stable, and a new therapeutic option for the treatment of Chagas disease.

Acknowledgments The authors wish to express their gratitude to Laboratory for Pharmaceutical Technology, Laboratory of Microbiology of the group Aggeu Magalhães, Laboratory of Nuclear Magnetic Resonance, Laboratory of Research in Parenteral Nutrition, Laboratory Quality Control of Medicines and Related, Federal University of Pernambuco/Brazil, for providing the necessary facilities to develop the present research. This research was supported by Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco–FACEPE.

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