Contents lists available at ScienceDirect

Experimental Parasitology

Trypanosoma cruzi: Insights into naphthoquinone effects on growth and proteinase activity

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ARTICLE INFO

ABSTRACT

In this study we compared the effects of naphthoquinones (α-lapachone, β-lapachone, nor-β-lapachone and Epoxy-α-lap) on growth of *Trypanosoma cruzi* epimastigotes forms, and on viability of VERO cells. In addition we also experimentally analyzed the most active compounds inhibitory profile against *T. cruzi* serine- and cysteine-proteinases activity and theoretically evaluated them against cruzain, the major *T. cruzi* cysteine proteinase by using a molecular docking approach. Our results confirmed β-lapachone and Epoxy-α-lap with a high trypanocidal activity in contrast to α-lapachone and nor-β-lapachone whereas Epoxy-α-lap presented the safest toxicity profile against VERO cells. Interestingly the evaluation of the active compounds effects against *T. cruzi* cysteine- and serine-proteinases activities revealed different targets for these molecules. β-Lapachone is able to inhibit the cysteine-proteinase activity of *T. cruzi* proteic whole extract and of cruzain, similar to E-64, a classical cysteine-proteinase inhibitor. Differently, Epoxy-α-lap inhibited the *T. cruzi* serine-proteinase activity, similar to PMSF, a classical serine-proteinase inhibitor. In agreement to these biological profiles in the enzymatic assays, our theoretical analysis showed that E-64 and β-lapachone interact with the cruzain specific S2 pocket and active site whereas Epoxy-α-lap showed no important interactions. Overall, our results infer that β-lapachone and Epoxy-α-lap compounds may inhibit *T. cruzi* epimastigotes growth by affecting *T. cruzi* different proteinases. Thus the present data shows the potential of these compounds as prototype of protease inhibitors on drug design studies for developing new antichagasic compounds.

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1. Introduction

Chagas disease is a public health problem that affects approximately 16 to 18 million people in Latin America (World Health Organization, 2008). It is caused by *Trypanosoma cruzi* and leads to an irreversible cardiomyopathy. The current treatment of Chagas disease involves the use of a nitroheterocyclic compound (i.e., benznidazole) that produces severe side effects (Brener, 1979; Schmuñis et al., 1980). In addition, the efficiency of the treatment depends on the susceptibility of different *T. cruzi* strains.

Despite the progress in the study of *T. cruzi* biochemistry and physiology, Chagas disease remains part of a group of neglected diseases, to which chemotherapy needs to be developed (Hotez et al., 2004). Thus, the search for new active compounds against *T. cruzi* with low toxicity to mammalian cells and high efficacy during the chronic phase of Chagas disease still remains.

Quinones derivatives have been developed in drug design studies for development of new trypanocidal compounds against Chagas disease (Silva et al., 1992; Pinto et al., 2000; Menna-Barreto et al., 2005). β-Lapachone is a naphtoquinone present in the heartwood of *Tabebuia* sp., whose extract has been used in popular medicine for centuries against several diseases (Pinto et al., 1977). Studies on β-lapachone focused on cancer chemotherapy pointed topoisomerases I and II involved in the apoptosis as biochemical targets (Frydman et al., 1997; Pardee et al., 2002; Ravelo et al., 2004). In trypanosomatids, topoisomerases play key functions in the replication and organization of kinetoplast DNA (kDNA) and are potential targets for anti-parasite drugs (Cavalcanti et al.,

2004). Since β-lapachone has been discarded as a potential trypanocidal drug due to its high toxicity, several studies have addressed chemical modifications at quinonoid center of these molecules to generate pharmacologically effective derivatives (i.e., Epoxy-α-lap) (Ferreira et al., 2006).

Literature has described trypanocidal effects of oxyranes targeting some specific proteinases (Cazzulo, 2002). Interestingly, L-trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E-64) is a natural cysteine-proteinase inhibitor with an oxyran ring that displays potent trypanocidal activity (Roush et al., 1998). In the present study we compared the trypanocidal and cytotoxic profiles of naphtoquinones compounds (α-lapachone, β-lapachone, nor-β-lapachone) including an oxyran-naphtoquinone derivative (Epoxy-α-lap). Then we evaluated the effects of the most active compounds on T. cruzi proteic whole extract different proteinases groups (cysteine- and serine-proteinases) and against cruzain, the T. cruzi major cysteine-proteinase. We also compared their biological profile with classical protease inhibitors E-64 and phenylmethyl-sulphonyl-fluoride (PMSF). Finally we used a molecular modeling approach to theoretically investigate the mechanism of inhibition of these compounds against cruzain.

2. Materials and methods

2.1. Chemicals and culture reagents

3-[(3-Cholamidopropyl)-dimethylammonium]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), proteinases substrates including pGlu-Phe-Leu-p Nitroanilide (pEFLpNan) for cysteine-proteinases ($\Delta E = 10.500 \, M^{-1} \, cm^{-1}$; $\lambda = 405 \, nm$) and Nα-p-Tosyl-l-Arg-methyl ester (TAME) for serine-proteinase ($\Delta E = 409 \, M^{-1} \, cm^{-1}$; $\lambda = 247 \, nm$), proteinases inhibitors including L-trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E-64) for cysteine-proteinases and phenyl-methyl-sulphonyl-fluoride (PMSF) for serine-proteinases. PMSF stock solution was prepared in alcohol. Hexamethyl pararosaniline (crystal violet), Dulbecco's Modified Eagle's Medium (DMEM), dimethyl sulfoxide (DMSO), brilliant blue R-250 (CBBR-250; $\lambda = 595 \, nm$), sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was purchased from Cultilab S/A (Brazil). Brain Heart Infusion (BHI) purchased from Oxoid Australia (West Heidelberg, Vic., Australia). Naphthoquinones derivatives included β-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b] pyran-5,6-dione), nor-β-lapachone, α-lapachone and Epoxy-α-lap (2,3-dihydro-3,3-dimethyl-espiro[1H-4-oxantracen-5,2′-oxyran]-10(5H)-one)] were synthesized by Organic Chemistry Department from Federal Fluminense University.

Sepharose 4B and E-64-Sepharose resin were purchased from Calbiochem–Novabiochem (UK) Ltd. t-octylphenoxypolyethoxyethanol (Triton X-100) and phenyl-methyl-sulphonyl-fluoride (PMSF) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Trypanosoma cruzi epimastigotes

2.2.1. Cell culture

Epimastigotes forms of Trypanosoma cruzi (Dm 28c clone) were obtained from late log phase of cultures (3×10^7 cells/mL) in BHI medium supplemented with 10% heat-inactivated FCS at 26 °C.

2.2.2. Trypanosoma cruzi proteic whole extract

The parasites were washed three times (3000g, 15 min, 5 °C) in phosphate buffer saline (PBS), pH 7.2 and lysed in 10 mM Tris–HCl, pH 6.8, containing 1% CHAPS (40 min, 4 °C), as previously described (Alves et al., 2005). The solubilized proteins were recovered by centrifugation (10,000g, 20 min, 4C) and stored at 70 °C. Protein concentration was determined as previously described (Lowry et al., 1951), using BSA as standard.

2.2.3. Trypanosoma cruzi cysteine-proteinases

The parasites (2.5×10^9 parasites) were washed three times with PBS, pH 7.2 and submitted to 10 cycles of the freezing–thawing in equilibrium buffer 20 mM Tris–HCl pH 7.4 containing 150 mM NaCl and 100 μM PMSF), which allows to assess T. cruzi proteinase activity. This methodology was used due to the rigidity of the trypanosomatid protozoa surface that presents a row of subpellicular microtubules beneath the cell membrane (Soares, 2006). The soluble proteins (1 mL) were obtained by ultracentrifugation (33,000g, 60 min, 4 °C), and incubated (60 min, 4 °C) with 25 mg of swelled Sepharose 4B resin under agitation. Later, non-binding proteins were recovered from the supernatant by new centrifugation (1000g, 3 min, 4 °C), which was incubated with 25 mg of swelled E-64-Sepharose resin previously equilibrated in the same buffer. The column was washed with the equilibrium buffer and the retained proteins were recovered in the supernatant by centrifugation (1000g, 3 min, 4 °C) using the same buffer containing 2.0 M NaCl. The eluted fraction was concentrated and used in the proteinase assays.

2.3. Cytotoxic effects in mammalian VERO cells

Cytotoxic effects of the compounds were evaluated on VERO cells (ATCC, CRL-1586TM), as previously described (Ferreira et al., 2006; Jorqueira et al., 2006). Briefly, cells (10^5 cells/well) were maintained at 37 °C in DMEM supplemented with 10% FCS and Hepes buffer in 24-well plates during 12 h, in a 5% CO_2 atmosphere. Then, the cells were incubated (37 °C, 72 h) with different concentrations of naphthoquinones derivatives (α-lapachone, β-lapachone, nor-β-lapachone and Epoxy-α-lap) diluted in DMSO. We also included negative control wells containing medium only or 1% DMSO crystal violet diluted in PBS and positive control. Subsequently, treated and untreated cells were washed twice with PBS and fixed with 0.2% formaldehyde in PBS for 15 min. Cells were stained for 1 h at room temperature with 0.2% CBBR-250 solution and then the staining was eluted from the cells with 1.0 ml of 1% SDS for 1 h. Quantification was performed using the correlation (r) between cell number and the CBBR-250 absorbance. Compounds toxicity was expressed as percentage of VERO cells death, compared to controls. Cytotoxic effect was expressed by percentage of live VERO cells after 24 h of incubation with the compounds in three different concentrations (3.1, 12.5 and 50 μM). The values represent the media and standard deviation (±) of three independent experiments.

2.4. Evaluation of the compounds effects on in vitro growth epimastigotes

The epimastigotes were incubated with different concentrations of naphthoquinones derivatives (α-lapachone, β-lapachone, nor-β-lapachone and Epoxy-α-lap) and crystal violet, used as positive trypanocidal control. The assays were performed in 96-wells microplates with 10^4 epimastigotes/well, at a final volume of 200 μL/well of BHI medium supplemented with 10% FCS at 26 °C. After 72 h, the cultures were analyzed by optical microscopy for quantification of resistant parasites, using Neubauers hemocytometer. Non-treated epimastigotes cultures were used as negative control. The results are mean (±) standard deviation of three independent experiments.

2.5. Determination of the naphtoquinones inhibitory effect on T. cruzi proteolytic activity

2.5.1. Enzymatic activity assays

The tests were performed according to Alves and co-workers (2005). Briefly, 5 μg of crude extract resuspended in activation buffer (final volume of 400 μL, 25 °C, 30 min) was incubated with pEFLpNan, a cysteine-proteinase chromogenic substrate, diluted in 10 mM Tris–HCl pH 5.5 containing 1 mM DTT, or TAME, a serine proteinase substrate diluted in 10 mM Tris–HCl pH 7.5. The inhibition assays were performed by adding the proteinases classical inhibitor as E-64 and PMSF (50 μM each) prepared in bidestilated water and naphthoquinones derivatives as α-lapachone, β-lapachone and oxyran 10 (50 μM each) prepared in DMSO monitoring the reaction during 30 min. The velocity of the reaction was defined using the formula $v = [s - s_0]/(t - t_0)$, where v = velocity, $[s - s_0]$ = final substrate concentration subtracted from the initial substrate concentration, and $(t - t_0)$ = final time subtracted from the initial time. The enzymatic activity was expressed in μmoles - min^{-1} mg of $protein^{-1}$, and represented by the mean (±) standard deviation of three independent experiments.

2.5.2. Zymographic assays

Cysteine-proteinase activity was determined using SDS–PAGE with gelatin copolymerized in the gel (substrate-SDS–PAGE) as previously described (Heussen and Dowdle, 1980; Alves et al., 1993). Briefly, T. cruzi soluble proteins were submitted onto electrophoresis under reducing conditions (in sample buffer) using 12% acrylamide gels copolymerized with 0.1% gelatin. Following electrophoresis, the gel was washed (1 h, 25 °C) with 2.5% Triton X-100 and then incubated (16 h, 37 °C) with 10 mM sodium acetate pH 5.5 containing 1 mM DTT. Hydrolysis of gelatin was detected by staining the gels with 0.1% (w/v) amide black, prepared in a methanol:acetic acid:water (3:1:6, v/v/v) solution.

2.6. Docking

In order to investigate the binding mode of E-64, β-lapachone and Epoxy-α-lap, we docked them into cruzain using Autodock 4.0 program running on a Windows based PC. The 3D structures of ligand molecules were built and minimized to the AM1 level in the molecular modeling program Spartan'08 (Wavefunction Inc.). Cruzain crystal structure was obtained from Protein Data Bank (PDB code 1U9Q) and then the inhibitor was docked by superposition with Cathepsin-K-E-64 complex (PDB code 1ATK). This complex is covalently bound and the enzyme was selected due to its high degree of identity with cruzain (ID = 41%) and its similar active site (RMSD = 0.91 Å) whereas E-64 is one of the most known cruzain inhibitor.

The cruzain–E-64 complex constructed herein was used as the orienting template for preparing the naphthoquinones complexes. Briefly the native ligand was embedded in the cruzain region that is comprised by cruzain catalytic site represented by the cubic grid box of $50 \times 50 \times 50$ Å^3 with a spacing of 0.375 Å.

Docking studies were carried out using the empirical free energy function and the Lamarckian genetic algorithm applying a standard protocol, with an initial population of 150 randomly placed individuals and a maximum number of 2.5×10^6 energy evaluations. A total of 50 independent docking runs were carried out for each compound. Structures differing by less than 0.5 Å in positional root-mean-square deviation (RMSD) were clustered together and the results of the most favorable free energy of binding were selected as the resultant complex structures. The inhibition constants were calculated based on the dissociation of the enzyme inhibitor complex and the thermodynamics formula of $\Delta G = RT \ln K_i$ (Morris et al., 1998) leading to the 'Estimated Free Energy of Binding' (kcal/mol). In case of Epoxy-α-lapachone, docking runs were divided in 2 clusters using 2.0A of RMSD for each conformation. Each cluster containing 40 and 10 conformations, with −4.8 and −5.18 kcal/mol, respectively. For β-lapachone, docking runs were divided in 3 clusters using 2.0A of RMS with each cluster containing 1, 26 and 24 conformations with −4.65, −4.95 and −4.75 kcal/mol, respectively. Despite their differences, β-lapachone and Epoxy-α-lap have rigid structures with no rotatable bonds and all clusters of low energy conformations for both ligands were almost identical. Therefore, we selected the docking structure with the lowest energy to perform the comparison of the binding mode. We performed and analyzed docking calculations for the cruzain-ligand (E-64, β-lapachone and Epoxy-α-lap) complexes to validate the docking accuracy. We also performed a re-docking of the ligand [1-(1-methyl-4,5-dioxo-pent-2-enylcarbamoyl)-2-phenyl-ethyl]-carbamic acid benzyl ester named 186 into the crystal structure of the native cruzain (PDB ID = 1U9Q) that generated 50 conformations. The re-docking of 186 into cruzain binding site was performed in duplicate and the comparison with the co-crystallized form revealed five structures similar to the crystallographic conformation with a root-mean-square deviation (RMSD) of 0.89 Å. Meanwhile another four conformations presented a RMSD between 2 and 4 Å when compared to the crystal structure. It is important to consider that 186 has 10 rotatable bonds, which leads to a more flexible structure than our compound. Anyway, in both experiments, we obtained the crystallographic conformation as the lowest conformation ($\Delta G = -8$ kcal/mol).

3. Results

3.1. Cytotoxic effects of naphthoquinones derivatives in mammalian VERO cells

The cytotoxicity assays against VERO cells showed that both β-lapachone and nor-β-lapachone present a significant cytotoxicity higher than the positive control (crystal violet) that showed a dose-dependent profile (Table 1). On the other hand, the experiments with the Epoxy-α-lap demonstrated a lower toxic effect

Table 1

Comparison of trypanocidal and cytotoxicity effects of naphtoquinones against T. cruzi epimastigotes forms and VERO cells, respectively. The values represent the media and standard deviation (±) of three independent experiments.

Compounds	Trypanocidal effect*			Cytotoxicity**		
	3.1 μM	12.5 μM	50 μM	3.1 μM	12.5 μM	50 μM
β-Lapachone	00 ± 0.0	00 ± 0.0	00 ± 0.0	03 ± 0.1	02 ± 0.1	00 ± 0
Nor-β-lapachone	100 ± 5.0	100 ± 3.0	100 ± 2.0	34 ± 1.0	06 ± 0.1	00 ± 0
α-Lapachone	100 ± 3.0	100 ± 3.0	100 ± 4.0	100 ± 5.0	94 ± 2.0	87 ± 4.0
Epoxy-α-lap	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100 ± 6.0	100 ± 8.0	96 ± 3.0
Crystal violet	22 ± 4.0	9.0 ± 1.0	0.0 ± 0.0	96 ± 3.0	16 ± 1.0	09 ± 2.0

Expressed by percentage of live (*) parasites or (**) VERO cells as described on Section 2.

and safer profile with almost 100% of live cells in the three concentrations assayed. The α-lapachone presented no cytotoxicity on the cells at 3.1 μM, and a discrete profile at higher levels (94% and 87% of live cells at 12.5 and 50 μM, respectively).

3.2. Trypanocidal profile of naphtoquinones derivatives

The evaluation of the trypanocidal activity showed that whereas crystal violet presented a dose-dependent trypanocidal effect similar to its cytotoxic profile on VERO cells, β-lapachone and Epoxy-α-lap were active in all concentrations tested, killing 100% of parasites. In contrast α-lapachone and nor-β-lapachone showed no effect even after 72 h in culture (Table 2).

3.3. Effects of naphthoquinones derivatives on Trypanosoma cruzi Dm 28c proteinases activities

The analysis of naphthoquinones derivatives inhibitory effects on *T. cruzi* serine-proteinases showed that Epoxy-α-lap inhibited approximately 80% of the parasite enzymatic activity on TAME hydrolysis (122.0 ± 20 μmoles min^{-1} mg de protein^{-1}) in a slightly better profile than PMSF, a classical serine-proteinase inhibitor (160.0 ± 10 μmoles min^{-1} mg de protein^{-1}) (Table 2). Contrastingly, both β-lapachone and α-lapachone (639.2 ± 25 and 520.0 ± 30 μmoles min^{-1} mg of protein^{-1}, respectively) did not affect the *T. cruzi* serine-proteinase activity.

We also evaluated the naphthoquinones derivatives effects on *T. cruzi* proteic whole extract using a cysteine-proteinase substrate (pEFLNan) (Table 2). Our results showed that α-lapachone and Epoxy-α-lap presented no effect on the *T. cruzi* cysteine-proteinase activity (13.0 ± 2.0 and 12.9 ± 0.9 μmoles min^{-1} mg of protein^{-1}, respectively) whereas β-lapachone showed a significant active profile (4.9 ± 1.0 μmoles min^{-1} mg of protein^{-1}). Interestingly, β-lapachone inhibitory activity was higher than E-64, a specific inhibitor of cysteine-proteinases (7.9 ± 0.2 μmoles min^{-1} mg of protein^{-1}) compared to non-treated control (14.9 ± 0.8 μmoles - min^{-1} mg of protein^{-1}). In accord E-64 and β-lapachone were also able to inhibit the 60 kDa-band corresponding to cruzain (Cazzulo et al., 1990) obtained from *T. cruzi* by using an E-64 column affinity chromatography. The isolated cruzain was able to hydrolyze pEFLpNan substrate (4.0 ± 0.2 × 10^3 μmoles min^{-1} mg of protein^{-1}) and was strongly inhibited by β-lapachone and E-64 (95% and 97%, respectively), in contrast to Epoxy-α-lap (<17%) (Fig. 1).

Table 2
Effects of naphtoquinones on activity of *Trypanosoma cruzi* proteic whole extract against cysteine-proteinase (pEFLpNan) and serine-proteinase (TAME) substrates.

Serine proteinase substrate[a] (TAME)		Cysteine proteinase substrate[b] (pEFLpNan)	
Non-treated control	644.1 ± 30.0 (100%)	Non-treated control	14.9 ± 0.8 (100%)
PMSF	160.0 ± 10.0 (25.8%)	E-64	7.9 ± 0.2 (53.0%)
Epoxy-α-lap	122.0 ± 20.0 (18.9%)	Epoxy-α-lap	12.9 ± 0.9 (89.6%)
α-Lapachone	520.0 ± 30.0 (80.7%)	α-Lapachone	13.0 ± 2.0 (87.2%)
β-Lapachone	639.2 ± 25.0 (99.2%)	β-Lapachone	4.9 ± 1.0 (32.8%)

[a,b] The enzymatic activity was expressed in μmol min^{-1} mg of protein^{-1} and residual activity (%). The values represent the media and the standard deviation (±) of three independent experiments.
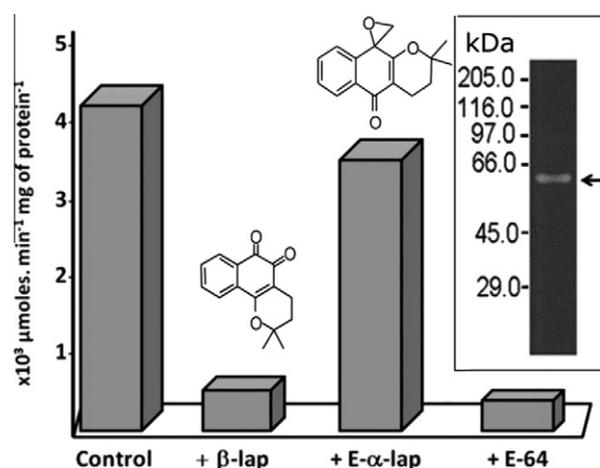
Fig. 1. Effects of naphthoquinone compounds (β-lapachone, β-lap; Epoxi-α-lap, E-α-lap) and trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E-64) (50 μM) on cruzain activity against pEFLpNan substrate. The inset shows the zymography gel of cruzain—the 60 kDa-band of the *T. cruzi* eluted material from E-64-affinity column. Molecular mass markers are indicated (kDa). These results are representative of three independent experiments.

3.4. Docking

The initial analysis of the docking results of E-64 into cruzain showed a RMSD of 1 Å when aligned with its original co-crystallized conformation, pointing the methodology as theoretically reliable to show ligands bound conformations. In addition we performed a re-docking of another ligand, an alpha-ketoester called 186, into cruzain and compared with the co-crystallized form (not shown) which revealed a similar conformation (RMSD = 0.89 Å and binding free energy = −8 kcal/mol).

In order to theoretically analyze the binding mode of the trypanocidal compounds (β-lapachone and Epoxy-α-lap), we docked these molecules into cruzain crystal structure and compared to E-64. Interesting the comparison of the compounds docking complexes with that of E-64 revealed several differences (Fig. 2).

The cruzain–E-64 docking complex conserved the main hydrogen bonds as well as hydrophobic interactions that are observed in the cathepsin K–E-64 complex crystal structure with no steric hindrances (Zhao et al., 1997). The carboxylate group of E-64 interacted through hydrogen bonds with the Hε22 of Gln19 and NH group of Cys25 of cruzain whereas the oxygen atom of its carbonyl group interacted with the NH group of Gly66 of this enzyme (Zhao et al., 1997). In addition, the isobutyl moiety of this inhibitor lies at cruzain S2 pocket, indicating a hydrophobic interaction in this region involving mainly Leu67 and Leu157 residues, also similar to the cathepsin K–E-64 crystal structure (Fig. 2).

The comparison of E-64 and β-lapachone docking complexes revealed that both inhibitors are placed in cruzain S2 pocket, a hydrophobic region (Leu67, Met68, Ala133, Leu157, Gly160 and Glu205) (Fig. 2) essential for the enzyme specificity (Lecaille et al., 2001). In β-lapachone–cruzain complex, the compound interacts with not only S2 pocket (Leu67, Met68) but also with Ser64, Gly65, Gly66 and Asp158. Additionally, hydrogen bonds were also observed between the oxygen from β-lapachone pyran ring and oxygen carbonyl of Gly66 and the oxygen carbonyl of the lapachone derivative and Ser64. Considering the mechanism of inhibition of cysteine proteinases in which the thiolate ion makes a nucleophilic attack to the carbonyl of the inhibitor (Babine and Bender, 1997), our theoretical study pointed a distance between the thiolate of the enzyme and the carbonyl group of β-lapachone (∼3.8 Å), which suggests this reaction with the enzyme. This is reinforced by the experimental assays data that revealed the ability of β-lapachone of inhibiting *T. cruzi* cysteine-proteinase activity.
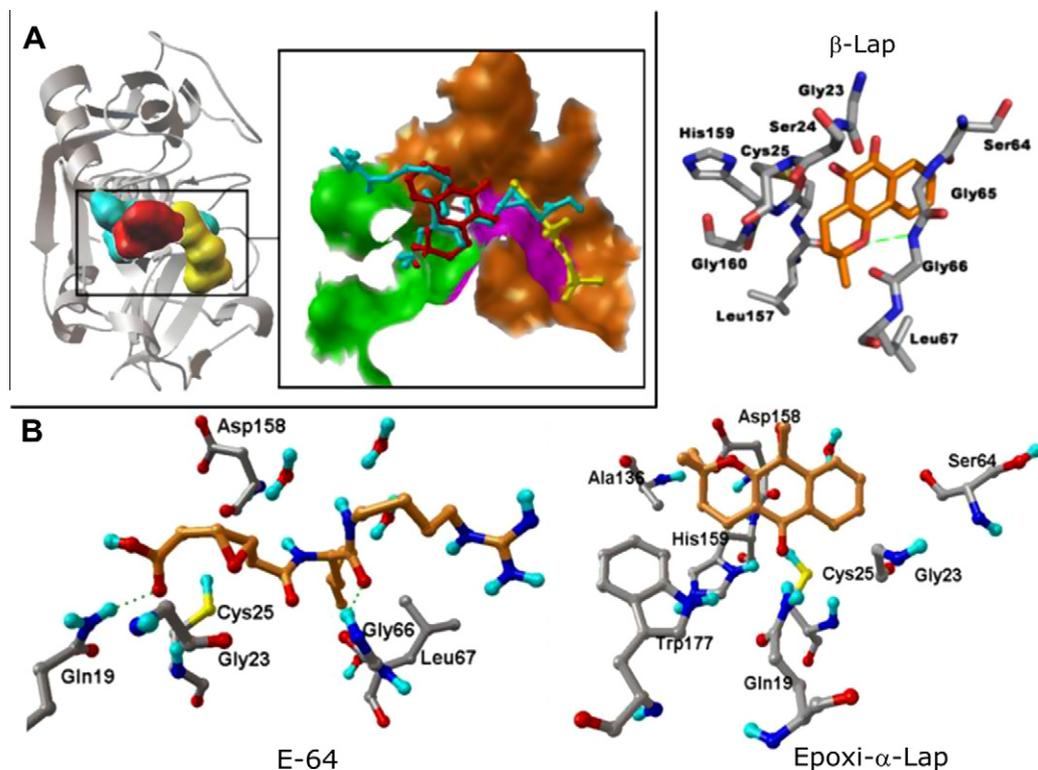
Fig. 2. Comparison of docking complexes of β-lapachone, Epoxy-α-lap and E-64 with cruzain. (A) Structural alignment of the three docking complexes revealing the occupied region by β-lapachone (red), E-64 (blue) and Epoxy-α-lap (yellow) within cruzain structure. The whole enzyme is in secondary structure representation (light gray) and inhibitors are on CPK (left) whereas the S2 pocket (green), the catalytic triad (pink) and other interactive residues regions (orange) are zoomed with inhibitors in ball-stick representation (right)—β-lapachone (red), Epoxy-α-lap (yellow) and E-64 (blue). (B) Residues involved on the interactions between the compounds E-64, β-lapachone and Epoxy-α-lap (orange) and cruzain (gray) including the S2 pocket (Leu67, Met68, Ala133, Leu157, Gly160 and Glu205), the catalytic triad (Cys25, His159 and Asn175) and others interactive residues. Nitrogen atom is in blue, Carbon in gray, Oxygen in red and Hydrogen in light blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Different from E-64 and β-lapachone, docking showed that oxyran ring of Epoxy-α-lap pointed out of the enzyme catalytic site (Fig. 2). This feature is in agreement to the dipole vector of Epoxy-α-lap that also points to opposite direction of β-lapachone (Ferreira et al., 2006). Furthermore, Epoxy-α-lap interacts only with residues located in the S1' pocket (i.e., Trp177 and Ala136) with low energy interactions (i.e., hydrophobic interactions) presenting no hydrogen bonds. Finally, Epoxy-α-lap lies in the opposite side of cruzain active site, which probably compromised its inhibitory profile against cysteine-proteinases and oriented it for targeting serine-proteinases.

4. Discussion

Chagas disease represents a major impact on Latin America population and still remains without an efficient treatment (World Health Organization, 2008). As the current antichagasic drugs cause severe side effects, the development of different chemotherapeutic agents has been proposed, including plant-derived compounds such as naphtoquinones compounds (Ferreira et al., 2006; Paulino et al., 2005; Silva et al., 2006). In this context, our purpose was to compare the trypanocidal profile of isomeric forms of quinones, and to identify *T. cruzi* proteinase activity as feasible targets for these molecules.

Initially we compared the cytotoxicity profile of these compounds against VERO cells and their trypanocidal effects, which revealed that β-lapachone is active against *T. cruzi* and more toxic compared to α-lapachone, its natural isomer that presented a lower dose-dependent cytotoxic effect on VERO cells and no trypanocidal effect. Nor-β-lapachone was also a non-promising molecule

since it showed no safe profile or trypanocidal activity in this work. Differently Epoxy-α-lap showed a significant trypanocidal activity with no cytotoxicity against VERO cells similar to that observed by Ferreira and co-workers (2006).

Overall our cellular results reinforced that β-lapachone does not present the necessary features to be used as a feasible chemotherapeutic agent against Chagas disease. However, this molecule may help on designing new derivatives, such as naphthoimidazole, a β-lapachone derivative that was recently reported more ten times more active against the three *T. cruzi* forms than its precursor (Menna-Barreto et al., 2005). Meanwhile, the main structural difference between Epoxy-α-lap and α-lapachone, the addition of an oxyran ring, probably determines the structural and stereo-electronic features responsible for the significant trypanocidal profile with low cytotoxicity against mammalian cells displayed by this compound.

Due to the structural similarity of the Epoxy-α-lap with some natural proteinases inhibitors containing an oxyran ring, such as E-64, we decided to characterize the potential of the trypanocidal naphthoquinones derived compounds as proteinase inhibitors. Therefore, we evaluated the inhibitory profile of Epoxy-α-lap and β-lapachone against *T. cruzi* cysteine- and serine-proteinase activity using specific chromogenic substrates.

Initially we verified the effects of these naphthoquinones on the esterase activity of the *T. cruzi* proteic whole extract against TAME. Interestingly, we observed that Epoxy-α-lap strongly affected *T. cruzi* enzymatic activity on TAME, similar to PMSF, a classical inhibitor of serine-proteinase. Differently no significant inhibitory effects were detected when using β-lapachone or α-lapachone. Trypanosomes present an oligopeptidase B, a member of the

prolyl-oligopeptidase family of serine proteinase involved in their cell invasion processes (Polgar, 1999). In fact *T. cruzi* have a prolyl oligopeptidase 80 kDa (Tc80) secreted by the infective trypomastigotes that hydrolyzes native collagens and might be involved in infection by degrading extracellular matrix components. Apparently this enzyme is related with the invasion of nonphagocytic cells (Grellier et al., 2001). Data presented here reinforced the hypothesis that serine proteases are essential for *T. cruzi* survival and are feasible targets for developing new inhibitors. Thus *T. cruzi* serine proteinases such as Tc80 may be a feasible target for Epoxy-α-lap.

In addition, we tested the *T. cruzi* whole proteic extract on pEFLpNan searching for the catalytic activity of the parasite cysteine-proteinases. Importantly the *T. cruzi* whole extract was able to hydrolyze this specific substrate commonly used in assays for classical cysteine-proteinases such as papain (*Carica papaya*), ficine (*Ficus carica*), bromeline (*Ananas comosus*) and cruzain (*T. cruzi*) (Filippova et al., 1984). This substrate presents a phenylalanine residue at P2 position compatible with the specificity of cysteine proteinases that contains a thiol group at the active center. Cysteine proteinases are involved in several aspects of host–parasite interactions and are the most abundant proteinases in most parasitic protozoa (North et al., 1990). Thus these enzymes are of great interest, since they may be specific targets for chemotherapeutic drugs (McKerrow, 1989).

Based on the trypanocidal activity of the naphthoquinone compounds, we evaluated them against *T. cruzi* cysteine-proteinase activity. Interestingly the activity of the *T. cruzi* whole extract on pEFLNan was strongly inhibited by β-lapachone, similar to E-64, which is a classic inhibitor of cysteine proteinases. In contrast no inhibitory effects were observed when using Epoxy-α-lap and α-lapachone compounds.

Due to their mechanism of action, the use of enzymatic inhibitors, reversible or not, is among of the several modern strategies of molecular design for new active prototypes against many pathologies (Wermuth, 1996; Klinghofer et al., 2001; Combs et al., 2005). Since proteinases are potential targets for killing *T. cruzi*, new strategies and drugs that affect this parasite through these enzymes are currently of interest. Despite the high activity of Epoxy-α-lap against the serine-proteinase profile of *T. cruzi* whole extract, the absence of a crystal structure of these enzymes restrains the molecular docking studies since target-based virtual screening methods depend on the availability of the target structural information (Klebe, 2006). On that matter the further use of homology modeling techniques for studying the *T. cruzi* serine-proteinases 3D structures may corroborates on that purpose (De Matos Guedes et al., 2007).

Cruzain is the most abundant cysteine proteinase in *T. cruzi* and is a promising target for the development of new chemotherapy of Chagas disease (Brak et al., 2008). This enzyme is one of the few parasites molecules with the crystal structure currently available in the Protein Data Bank (Scharfstein et al., 1986). Therefore, we experimentally and theoretically tested the ability of these compounds on inhibiting this enzyme. Interestingly and in agreement to the *T. cruzi* whole extract results, both E-64 and β-lapachone were able to experimentally inhibit cruzain isolated from *T. cruzi* by using an affinity column. In contrast, Epoxy-α-lap was not able to significantly inhibit cruzain.

Our theoretical evaluation of the docking complexes of β-lapachone and Epoxy-α-lap with cruzain and their comparison with a classical inhibitor (E-64), revealed that β-lapachone presented hydrophobic interactions with residues of the main specificity S2 pocket, and several hydrogen bonds with Ser64 and Gly66 residues of cruzain, with a distance of 2.93–3.8 Å, similar to E-64. Thus these interactions may contribute to the stabilization and maintenance of β-lapachone into cruzain active site. In addi-

tion β-lapachone presents an electrophilic moiety susceptible to a nucleophilic attack by the activated catalytic cysteine in a distance that suggested this reaction.

Despite of its electrophilic carbonyl, docking studies revealed that Epoxy-α-lap did not form any hydrogen bond with cruzain. The small number of hydrophobic interactions and the lack of hydrogen bonds with the active site residues may be due a different orientation inside the enzyme, which may not corroborate to its maintenance into the cruzain catalytic cleft. These features apparently justify the experimental inactive profile of Epoxy-α-lap against cruzain and the cysteine-proteinase activity of *T. cruzi* whole protein extract.

Recently in our previous structure–activity relationship (SAR) analysis of lapachone derivatives, we suggested that β-lapachone and Epoxy-α-lap could interact with different parasite targets due to their specific chemical structural features [13]. In this work, our experimental and theoretical data confirmed this prediction as both E-64 and β-lapachone inhibited *T. cruzi* cysteine-proteinase activity whereas interacted with residues from cruzain S2 pocket (so the enzymatic reaction cannot occur). Moreover, Epoxy-α-lap showed no high energy interactions with this enzyme, which probably avoided significant effects on the cysteine proteinases assays. Far from stablishing absolute results, the molecular modeling data obtained in this work may help to orientate the design of new compounds based on these naphthoquinones compounds targeting *T. cruzi* key enzymes.

In summary, despite the high trypanocidal profiles, our results indicated that β-lapachone and Epoxy-α-lap affects *T. cruzi* through different pathways since they may act as inhibitors of different proteinases groups. β-Lapachone inhibits thiol-containing proteinases and probably affects *T. cruzi* during infection of mammal cells as well as in biosynthesis of proinflammatory prostaglandins reducing clotting efficiency (Livio et al., 1978; Taussig, 1980). Meanwhile Epoxy-α-lap affects serine-proteinases and interferes on the mammalian host cell invasion and infection establishment (Caler et al., 1998).

References

Alves, C.R., Marzochi, M.C.A., Giovanni-de-Simone, S., 1993. Heterogeneity of cysteine proteinases in *Leishmania brasiliensis* and *Leishmania major*. Brazilian Journal of Medical and Biological Research 26, 167–171.

Alves, C.R., Corte-Real, S., Bourguignon, S.C., Chaves, C.S., Saraiva, E.M., 2005. *Leishmania amazonensis*: early proteinase activities during promastigote-amastigote differentiation in vitro. Experimental Parasitology 109, 38–48.

Babine, R.E., Bender, S.L., 1997. Molecular recognition of protein–ligand complexes: applications to drug design. Chemical Reviews 97, 1359–1472.

Brak, K., Doyle, P.S., McKerrow, J.H., Ellman, J.A., 2008. Identification of a new class of nonpeptidic inhibitors of cruzain. Journal of the American Chemical Society 130, 6404–6410.

Brener, Z., 1979. Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Western Hemisphere. Pharmacology & Therapeutics 7, 71–90.

Caler, E.V., Avalos, S.V., Haynes, P.A., Andrews, N.W., Burleigh, B.A., 1998. Oligopeptidase B-dependent signaling mediates host cell by *Trypanosoma cruzi*. The European Molecular Biology Organization Journal 17, 4975–4986.

Cavalcanti, P.C., Fragoso, S.P., Goldenberg, S., Souza, W., Motta, M.C.M., 2004. The effect of topoisomerase II inhibitors on the kinetoplast ultrastructure. Parasitology Research 94, 439–448.

Cazzulo, J.J., 2002. Proteinases of *Trypanosoma cruzi*: patential targets for the chemotherapy of Chagas disease. Current Topics in Medicinal Chemistry 2, 1261–1271.

Cazzulo, J.J., Franke, M.C.C., Martinez, J., Decazzulo, B.M.F., 1990. Some kinetic-properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. Biochimica et Biophysica Acta 2, 186–191.

Combs, A.P., Yue, E.W., Bower, M., Ala, P.J., Wayland, B., Douty, B., Takvorian, A., Polam, P., Wasserman, Z., Zhu, W., Crawley, M.L., Pruitt, J., Sparks, R., Glass, B., Modi, D., McLaughlin, E., Bostrom, L., Li, M., Galya, L., Blom, K., Hillman, M., Gonneville, L., Reid, B.G., Wei, M., Becker-Pasha, M., Klabe, R., Huber, R., Li, Y., Hollis, G., Burn, T.C., Wynn, R., Liu, P., Metcalf, B., 2005. Structure-based design and discovery of protein tyrosine phosphatase inhibitors incorporating novel isothiazolidinone heterocyclic phosphotyrosine mimetics. Journal of Medicinal Chemistry 48, 544–6548.

De Matos Guedes, H.L., Duarte Carneiro, M.P., de Oliveira Gomes, D.C., Rossi-Bergmann, B., Giovanni-De-Simone, S., 2007. Oligopeptidase B from *Leishmania amazonensis*: molecular cloning, gene expression analysis and molecular model. Parasitology Research 101, 865–875.

Ferreira, V.F., Jorqueira, A., Souza, A.M., da Silva, M.N., de Souza, M.C., Gouvêa, R.M., Rodrigues, C.R., Pinto, A.V., Castro, H.C., Santos, D.O., Araújo, H.P., Bourguignon, S.C., 2006. Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives. Bioorganic & Medicinal Chemistry 14, 5459–5466.

Filippova, I.Yu., Lysogorskaya, E.N., Oksenoit, E.S., Rudenskaya, G.N., Stepanov, V.M., 1984. ʟ-Pyroglutamyl-ʟ-phenylalanyl-ʟ-leucine-p-nitroanilidea chromogenic substrate for thiol proteinase assay. Analytical Biochemistry 143, 293–297.

Frydman, B., Marton, L.J., Sun, S.J., Neder, K., Witiak, T.D., Liu, A.A., Wang, H.M., Mao, Y., Wu, H.Y., Sanders, M.M., Liu, L.F., 1997. Induction of DNA topoisomerase II-mediated DNA cleavage by beta-lapachone and related naphthoquinones. Cancer Research 57, 620–627.

Grellier, P., Vendeville, S., Joyeau, R., Bastos, I.M., Drobecq, H., Frappier, F., Teixeira, A.R., Schrével, J., Davioud-Charvet, E., Sergheraert, C., Santana, J.M., 2001. *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes. The Journal of Biological Chemistry 276, 47078–47086.

Heussen, C., Dowdle, E.B., 1980. Electrophoretic analysis of plasminogen activators in polyacrilamide gels containing sodium dodecyl-sulfate and copolymerized substrates. Analytical Biochemistry 102, 196–202.

Hotez, P.J., Remme, J.H., Buss, P., Alleyne, G., Morel, C., Breman, J.G., 2004. Combating tropical infectious diseases: report of the disease control priorities in developing countries project. Clinical Infectious Diseases 38, 871–878.

Jorqueira, A., Gouvêa, R.M., Ferreira, V.F., Silva, M.N., Souza, M.C.B.V., Zuma, A.A., Cavalcanti, D.F.B., Araújo, H.P., Santos, D.O., Bourguignon, S.C., 2006. Oxyran derivative of α-lapachone is potent growth inhibitor of *Trypanosoma cruzi* epimastigote forms. Parasitology Research 99, 429–433.

Klebe, G., 2006. Virtual ligand screening: strategies, perspectives and limitations. Drug Discovery Today 11, 580–594.

Klinghofer, V., Stewart, K., McGonigal, T., Smith, R., Sarthy, A., Nienaber, V., Butler, C., Dorwin, S., Richardson, P., Weitzberg, M., Wendt, M., Rockway, T., Zhao, X., Hulkower, K.I., Giranda, V.L., 2001. Species specificity of amidine-based urokinase inhibitors. Biochemistry 40, 9125–9131.

Lecaille, F., Authié, E., Moreau, T., Serveau, C., Gauthier, F., Lalmanach, G., 2001. Subsite specificity of trypanosomal cathepsin L-like cysteine proteases. Probing the S2 pocket with phenylalanine-derived amino acids. European Journal of Biochemistry 268, 2733–2741.

Livio, M., Bertoni, M.P., De Gaetano, G., Donati, M.B., 1978. Effect of bromelain on fibrinogen level, prothrombin complex factors and platelet aggregation in Prostaglandin and in the rat. Drugs under Experimental and Clinical Research 4, 49–53.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

McKerrow, J.H., 1989. Molecular and biochemical studies of the cercarial proteinase of *Schistosoma mansoni*. Memórias do Instituto Oswaldo Cruz 84, 209–210.

Menna-Barreto, R.F., Henriques-Pons, A., Pinto, A.V., Morgado-Diaz, J.A., Soares, M.J., De Castro, S.L., 2005. Effect of a beta-lapachone-derived naphthoimidazole on *Trypanosoma cruzi*: identification of target organelles. The Journal of Antimicrobial Chemotherapy 6, 1034–1041.

Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J., 1998. Automated docking using Lamarckian genetic algorithm and an empirical binding free energy function. Journal of Computational Chemistry 19, 1639–1662.

North, M.J., Mottram, J.C., Coombs, G.H., 1990. Cysteine proteinases of parasitic protozoa. Parasitology Today 6, 270–275.

Pardee, A.B., Li, Y.Z., Li, C.J., 2002. Cancer therapy with beta-lapachone. Current Cancer Drug Targets 2, 227–242.

Paulino, M., Iribarne, F., Dubin, M., Aguilera-Morales, S., Tapia, O., Stoppani, A.O., 2005. The chemotherapy of Chagas' disease: an overview. Mini Reviews in Medicinal Chemistry 5, 499–519.

Pinto, A.V., Pinto, M.C.R., Gilbert, B., Pellegrino, J., Mello, R.T., 1977. Schistosomiasis mansoni: blockage of cercarial skin penetration by chemical agents: i. Naphthoquinones and derivatives. Transactions of the Royal Society of Tropical Medicine and Hygiene 71, 133–135.

Pinto, C.N., Dantas, A.P., De Moura, K.C., Emery, F.S., Polequevitch, P.F., Pinto, M.C., de Castro, S.L., Pinto, A.V., 2000. Chemical reactivity studies with naphthoquinones from Tabebuia with anti-trypanosomal efficacy. Arzneimittelforschung 50, 1120–1128.

Polgar, L., 1999. Oligopeptidase B: a new type of serine peptidase with unique substrate-dependent temperature sensitivity. Biochemistry 38, 5548–15555.

Ravelo, A.G., Estévez-Braun, A., Chávez-Orellana, H., Pérez-Sacau, E., Mesa-Siverio, D., 2004. Recent studies on natural products as anticancer agents. Current Topics in Medicinal Chemistry 4, 241–265.

Roush, W.R., González, F.V., McKerrow, J.H., Hansell, E., 1998. Design and synthesis of dipeptidyl alpha', beta'-epoxy ketones, potent irreversible inhibitors of the cysteine protease cruzain. Bioorganic & Medicinal Chemistry 8, 2809–2812.

Scharfstein, J., Schechter, M., Senna, M., Peralta, J.M., Mendonça-Previato, L., Miles, M.A., 1986. *Trypanosoma cruzi*: characterization and isolation of a 57/51, 000 m.w. surface glycoprotein (GP57/51) expressed by epimastigotes and bloodstream trypomastigotes. Journal of Immunology 137, 1336–1341.

Schmuñis, G.A., Szarfman, A., Coarasa, L., Guilleron, C., Peralta, J.M., 1980. Anti-*Trypanosoma cruzi* agglutinins in acute human Chagas' disease. The American Journal of Tropical Medicine and Hygiene 29, 170–178.

Silva, J.S., Ferrioli-Filho, F., Kanesiro, M.M., Ferreira, V.F., Santos, S.C., Pinto, C.N., Fonseca, J.L., Mizrahy, H.E., Gilbert, B., Pinto, M.C., 1992. Evaluation of some organic compounds on bloodstream forms of *Trypanosoma cruzi*. Memórias do Instituto Oswaldo Cruz 87, 345–351.

Silva, R.S., Costa, E.M., Trindade, U.L., Teixeira, D.V., Pinto, M.de C., Santos, G.L., Malta, V.R, De Simone, C.A., Pinto, A.V., de Castro, S.L., 2006. Synthesis of naphthofuranquinones with activity against *Trypanosoma cruzi*. European Journal of Medicinal Chemistry 41, 526–530.

Taussig, S.J., 1980. The mechanism of the physiological action of bromelain. Medical Hypotheses 6, 99–104.

Wermuth, C.G., 1996. In: Wermuth, C.G. (Ed.), The Practice of Medicinal Chemistry. Academic Press, New York. p. 3.

World Health Organization, 2008. Report by the secretariat–Chagas disease: control and elimination, World Health Organization EXECUTIVE BOARD 124th Session, Provision agenda item 4.14. EB124/17.

Zhao, B., Janson, C.A., Amegadzie, B.Y., D'Alessio, K., Griffin, C., Hanning, C.R., Jones, C., Kurdyla, J., McQueney, M., Qiu, X., Smith, W.W., Abdel-Meguid, S.S., 1997. Crystal structure of human osteoclast cathepsin K complex with E-64. Nature Structural Biology 4, 109–111.