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Resveratrol inhibits *Trypanosoma cruzi* arginine kinase and exerts a trypanocidal activity

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ABSTRACT

Arginine kinase catalyzes the reversible transphosphorylation between ADP and phosphoarginine which plays a critical role in the maintenance of cellular energy homeostasis. Arginine kinase from the protozoan parasite *Trypanosoma cruzi*, the etiologic agent of Chagas disease, meets the requirements to be considered as a potential therapeutic target for rational drug design including being absent in its mammalian hosts. In this study a group of polyphenolic compounds was evaluated as potential inhibitors of arginine kinase using molecular docking techniques. Among the analyzed compounds with the lowest free binding energy to the arginine kinase active site (<−6.96 kcal/mol), resveratrol was chosen for subsequent assays. Resveratrol inhibits 50% of recombinant arginine kinase activity at 325 μM. The trypanocidal effect of resveratrol was evaluated on the *T. cruzi* trypomastigotes bursting from infected CHO K1 cells, with IC_{50} = 77 μM. Additionally epimastigotes overexpressing arginine kinase were 5 times more resistant to resveratrol compared to controls. Taking into account that: (1) resveratrol is considered as completely nontoxic; (2) is easily accessible due to its low market price; and (3) has as a well-defined target enzyme which is absent in the mammalian host, it is a promising compound as a trypanocidal drug for Chagas disease.

1. Introduction

The order Kinetoplastidae comprises flagellated protozoan organisms which include many human pathogens from the genera *Trypanosoma* and *Leishmania*. *Trypanosoma cruzi* is the causative agent of Chagas disease, a parasitic zoonosis affecting approximately 10 million people in the Americas [1]. Nowadays, only two drugs are approved as treatment for Chagas disease, the nitroimidazole benznidazole and the nitrofuran nifurtimox, both discovered over 40 years ago [2]. These data highlight the need for development of new therapeutic alternatives and the identification of novel drug targets. One promising technique to rational drug design is molecular docking using a validated protein target with known structural features and a library of small compounds [3].

Arginine kinase catalyzes the reversible transphosphorylation between phosphoarginine and ADP. Phosphoarginine is found in several organisms, ranging from yeasts and protozoa to invertebrates, and plays a critical role as energy reserve because the high energy phosphate can be transferred to ADP when renewal of ATP is needed [4]. It has been proposed that phosphoarginine supports bursts of cellular activity until metabolic events such as glycogenolysis, glycolysis, and oxidative phosphorylation are switched on [5]. In the last decade, the molecular and biochemical characterization of arginine kinases from trypanosomatid organisms has been reported [6,7]. In *T. cruzi* parasites treated with hydrogen peroxide an increase in arginine kinase expression up to 10-fold was observed. In addition, among other oxidative stress generating compounds tested, the trypanocidal drug nifurtimox also produced an upregulation of arginine kinase expression. Moreover, transgenic parasites overexpressing arginine kinase showed an improved tolerance to hydrogen peroxide exposure [8,9]. In *T. brucei*, increased arginine kinase activity increases the cell proliferation in procyclic forms during oxidative challenges with hydrogen peroxide. Elimination of arginine kinase activity by RNA interfer-

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ence decreased growth under standard culture conditions and was completely lethal under oxidative stress conditions [10]. In spite of having been determined the crystal structure of ligand free arginine kinase from *T. cruzi* (*Tc*AK) by molecular replacement methods and refined at 1.9 Å resolution [11], until today no outstanding arginine kinase inhibitors have been found. Only a few compounds have been reported exerting a partial inhibition of this protein. For example, *T. cruzi* recombinant arginine kinase was moderately inhibited by the green tea polyphenols catechingallate and gallocatechingallate [12]. In silico docking studies predicted that the polyphenol rutin (quercetin-3-*O*-rutinoside) is an arginine kinase non-competitive inhibitor, interacting mainly by a hydrophobic force forming an intermolecular complex with the enzyme [13]. On the other hand, arginine kinase was also inhibited by many arginine analogs, which also presented a slight trypanocidal activity [14].

A potential antitrypanosomal therapeutic target has to meets at least two requirements: (1) being absent in mammal hosts; and (2) be essential for the survival of the parasite [15]. Since *T. cruzi* arginine kinase complies with these conditions and its three dimensional structure is available, in this work, a group of polyphenols was evaluated as putative enzyme inhibitors using molecular docking and further in vivo assays.

2. Materials and methods

2.1. Molecular docking studies

3D structures from 24 polyphenolic compounds and 18 arginine analogues were downloaded from ZINC database (http://zinc.docking.org/) of commercially available compounds for virtual screening. The compounds analyzed and the corresponding ZINC IDs were: agmatine (1532560), apigenin (3871576), D-arginine (1532749), L-arginine (1532525), L-aspartate (895032), canavanine (3869452), capsaicin (1530575), L-citrulline (1532614), curcumin (899824), cyanidin (3775158), daidzein (18847034), delphinidin (3777403), diosmin (4098512), eriocitrin (98246397), eriodictyol (58117), genistein (18825330), gingerol (1531846), L-glutamate (1482113), glycine (4658552), glycitein (5999205), guanidine (8101126), hesperitin (39092), L-histidine (6661227), L-homoarginine (1529320), L-isoleucine (3581355), isoquercitrin (4096845), kaempferol (3869768), lutein (8221225), luteolin (18185774), L-lysine (1532522), N-methyl-L-arginine (1529776), methylguanidine (4658576), naringenin (156701), nitroarginine (19796052), L-ornithine (1532530), pelargonidin (391840), peonidin (897727), petunidin (3954302), putrescine (1532552), quercetin (3869685), resveratrol (6787), and rutin (59764511).

Further preparation of the PDBQT files were performed using AutoDock Tools 1.5.6 [16]. The published crystal structure of *Tc*AK (PDB ID: 2J1Q) lacks amino acid residues from 309 to 321, some of which are involved in arginine binding [17]. In order to consider these residues, as to obtain a closed conformation model, three dimensional structure was obtained by homology modeling of the protein sequence (GenBank ID: AAC82390.1) with the *Limulus polyphemus* arginine kinase (*Lp*AK) (PDB ID: 1BG0) (http://swissmodel.expasy.org/). From this model, residues SER63, ILE65, TYR68, GLU225, CYS271, GLU314 and HIS315 were taken as flexible using AutoDock Tools 1.5.6.

The grid parameter file was generated with Autogrid 4.2.6 so as to surround the flexible residues, being the dimensions of each grid map 40 grid points in each dimension, with spacing of 0.0375 nm and centered on position X = 40.149, Y = 9.807, and Z = 31.899.

AutoDock 4.2.6 was used for calculation of optimal energy conformations for the ligands interacting with the protein active site, running the Lamarckian Genetic Algorithm 100 times for each ligand, with a population size of 300, and 2.7×10^4 as maximum number of generations. For each ligand, bound conformations were clustered and two criteria for selection of the preferred model were followed, taking the lowest free binding energy conformation of all and the lowest binding energy conformation from the most populated cluster.

Two "Receiver Operating Characteristic" (ROC) curves were carried on the control compounds, one using the global lowest binding energy for each molecule, another using the lowest binding energy of the most populated cluster for each control compound, and classifying them as true positives or negatives according to previous publication [18]. According to ROC analysis results, selection criterion with an area under the curve more proximate to 1.0 was taken as representative for docking results.

2.2. Protein expression and purification

The full length *Tc*AK gene was obtained by PCR amplification using genomic *T. cruzi* DNA as template. The PCR product was cloned into the pRSET-A expression vector (Invitrogen). Expression of recombinant arginine kinase was performed in *E. coli* strain BL21 (DE3) pLysE. Recombinant protein was purified by affinity chromatography using a Ni-NTA Superflow resin (QIAGEN). Purity of the protein was checked by SDS-PAGE followed by Coomassie blue staining.

2.3. Arginine kinase inhibition assays

Enzyme activity was measured by a spectrophotometric coupled-enzymes method [14]. Reaction buffer consisted of 100 mM Tris-HCl buffer pH 8.2, 1.5 mM MgCl, 0.5 mM DTT, 1.5 mM phosphoenol-pyruvic acid, 0.3 mM NADH, 5 units of lactate dehydrogenase (Sigma Chemical Company), 5 units of pyruvate kinase (Sigma Chemical Company). The enzyme source was affinity purified recombinant *Tc*AK. Reaction was started by addition of 1.5 mM ATP. Different concentrations of resveratrol (0–1 mM) were added to the reaction buffer and incubated 6 min at 37 °C prior ATP addition. NADH oxidation was measured using a spectrophotometer at $\lambda = 360$ nm. IC_{50} was determined by nonlinear regression from three independent measurements, by using GraphPad Prism 6.01 for Windows (GraphPad Software). Arginine kinase activity was also assayed using a second spectrophotometric method, a modified protocol for colorimetric determination of phosphate [19], in presence of resveratrol (1–1000 μM). Recombinant enzyme was incubated with arginine (10 mM), ATP (1.5 mM), and $MgCl_2$ (3 mM) in Tris-HCl (100 mM, pH 8.4) in a final volume of 170 μl. After 5 min reaction was stopped by addition of 180 μl trichloroacetic acid (2.5% w/v), heated for 2 min in boiling water in order to fully hydrolyze phosphoarginine, plunged in ice 2 min for quick cooling and left at room temperature for 5 min. Liberated Pi was detected by addition of 100 μl from a 1:4 solution of ascorbic acid (9% w/v) and ammonium heptamolybdate (5.2 mM). After 5 min of color development D.O was measured using a spectrophotometer at $\lambda = 700$ nm.

2.4. Cells and parasites

CHO-K1 cells were cultured in RPMI medium supplemented with 10% heat inactivated Fetal Calf Serum (FCS), 0.15% (w/v) $NaCO_3$, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in 5% CO_2. Epimastigotes of *T. cruzi* (Y strain) were maintained in exponential growth phase by subculturing every other day in Brain-Heart Infusion Tryptose (BHT) medium supplemented with 10% FCS at 28 °C without shaking. Trypomastigotes were obtained from the extracellular medium of CHO-K1 infected cells as previously described [20].

2.5. Arginine kinase overexpressing parasites

The full-length arginine kinase gene was cloned into the pTREX expression plasmid [21]. The construction was transfected into *T. cruzi* epimastigotes as follows. 10^8 parasites grown at 28 °C in BHT medium were harvested by centrifugation, washed with PBS, and resuspended in 0.35 mL of electroporation buffer (PBS containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). This cell suspension was mixed with 50 μg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories). The parasites were electroporated using a single pulse of 400 V, 500 μF with a time constant of about 5 ms. Transfected parasites were cultured in BHT medium containing 500 μg mL^{-1} G418.

2.6. Evaluation of inhibition of epimastigotes proliferation

Epimastigotes of *T. cruzi* (Y strain) were cultured (as in Section 2.4) in 24 wells plate by inoculation with 1.5×10^6 cells/mL in 1.5 mL of BHT medium and were tested with increasing concentrations of resveratrol 0–1 mM. As resveratrol was previously diluted in DMSO 100%, the later was added in such volumes that equaled the concentration of DMSO in every culture (up to 0.5% DMSO). Parasite proliferation was determined after 3 days by cell counting in a Neubauer chamber.

2.7. Inhibition of trypomastigote bursting from infected cells

CHO-K1 cells (5×10^4 per well) were infected with trypomastigote forms (2.5×10^6 per well) for four hours. After this period, the infected cells were washed twice with PBS, the RPMI medium was replaced, and the cells were kept in culture in the presence of different concentrations of resveratrol (1–100 μM). After infection, plates were then incubated at 33 °C. Trypomastigotes were collected from the extracellular medium on the fifth day and counted using a Neubauer chamber.

2.8. Induction of apoptosis in epimastigote cells

Analysis of apoptosis by TUNEL on epimastigotes was performed using the "In situ cell death detection Kit" (Roche) according to the instructions of manufacturer.

2.9. Statistics and data analysis

All the experiments were made at least in triplicates and results presented here are representative of three independent assays. IC_{50} values were obtained from nonlinear regressions to dose-response logistic functions, by using GraphPad Prism 6.01 for Windows.

3. Results

3.1. Molecular docking studies

To take the first step in identifying polyphenol molecules that can bind the *Tc*AK arginine binding residues, a computer-assisted method was applied. Molecular docking analyses were performed using a small group of twenty four polyphenols, selected on the bases of their structural diversity. The structural coordinates of the *Tc*AK was completed by homology modeling from *Lp*AK [11] and used as protein target (Fig. 1A). The AutoDock 4.0 software was used to calculate possible conformations of the ligands that bind to *Tc*AK. Predicted free energy, ligand efficiency, and the clustering based on the measure of the average distance between the atoms were used as criteria for selection of the best ligand efficiency.

Two criteria were used to analyze docking results: lowest global binding score, and lowest binding score from the most populated cluster for each compound. Here, we observed that in many cases the lowest binding energy was not in the most populated cluster, and some indeed belonged to the less populated group. A ROC analysis (Supplementary Fig. 1) was performed for both classification methods in 19 control molecules. For this the rutin was taken as true positive [13], while from the 18 arginine analogues those that showed *Tc*AK inhibition were also taken as true positives, while the rest as negatives [14]. This analysis describes the tradeoff between specificity and sensibility, which means the ability to avoid false positives while detecting true positives. The value under the ROC curve points out the quality of the classification, a value near 1.0 means a good classifier, while 0.5 indicates a random ordering.

From Supplementary Fig. 1 it is noted that classification based on lowest binding energy resulted being random, while taking binding energies from the most populated cluster showed a better ROC value, making it the best classifier of the both tested. Also, ROC analysis suggested a free binding energy cutoff value <−6.74 kcal/mol, taking the 18 molecules with smaller binding

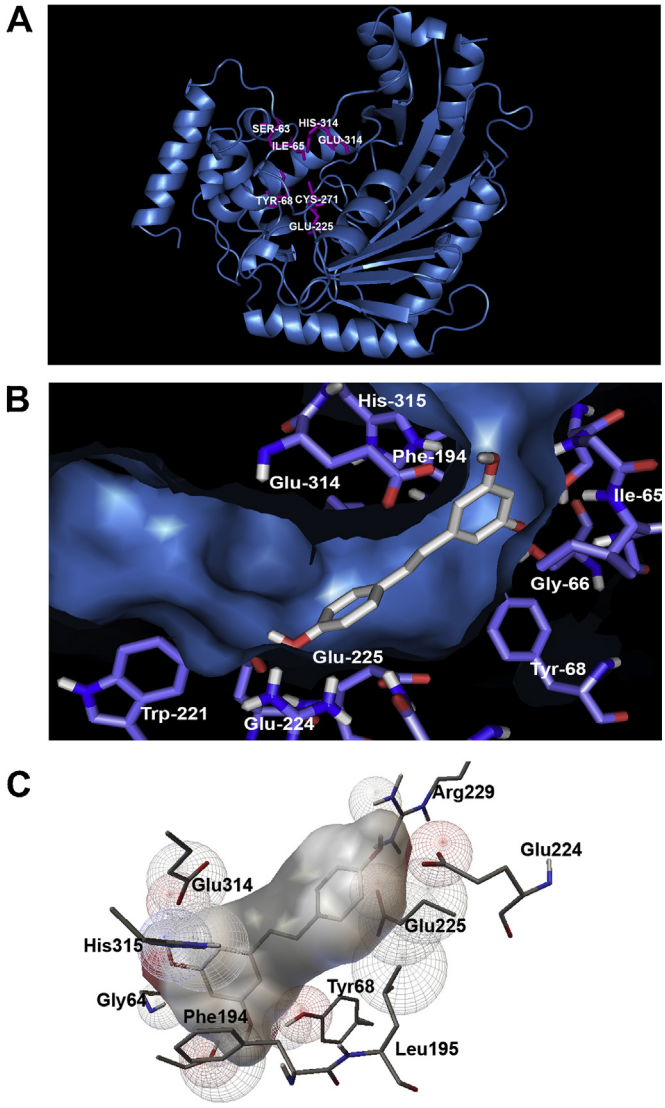

Fig. 1. Predicted binding between resveratrol and arginine kinase. (A) Three dimensional model of *T. cruzi* arginine kinase used in docking assays, obtained by homology modeling between arginine kinases of *Limulus poliphemus* and *T. cruzi* (PDB ID: 1BG0 and 2J1Q, respectively), showing the residues taken as flexible for docking. (B) Docked conformation of resveratrol into homology modeled *T. cruzi* arginine kinase showing the molecular surface of the active site pocket and (C) the close contacts formed between resveratrol and the protein represented as meshed spheres.

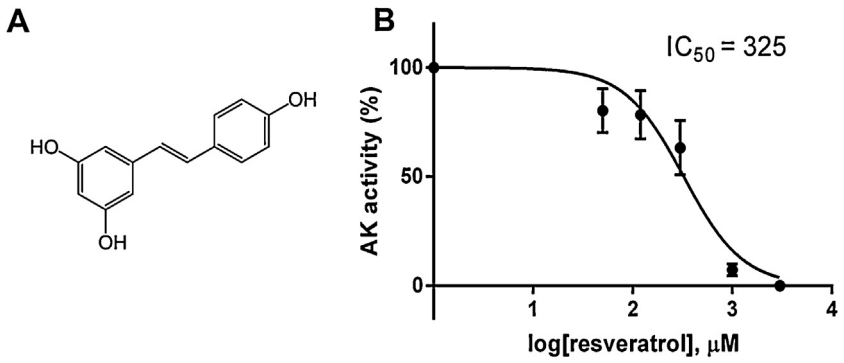

Fig. 2. Effect of resveratrol on arginine kinase activity. (A) Two dimensional representation of the chemical structure of resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol). (B) Effect of resveratrol upon arginine kinase activity. Recombinant *Tc*AK was produced and Ni-affinity purified and relative activity was measured by coupled enzymes method in presence of 0–1 mM resveratrol (plotted in log scale), IC_{50} value was calculated by nonlinear regression.

Table 1
Predicted binding energies for the best 18 docked molecules (in alphabetical order) with predicted inhibition constant (K_i) and the corresponding cluster population (families of similar conformations) of each docking result.

Ligand	Binding Energy (kcal/mol)	Ki (μM)	Cluster Population
Apigein	−7.03	7.02	29
Capsaicin	−8.56	5.84	6
Curcumin	−8.13	1.1	13
Cyanidin	−9.04	0.23	63
Daidzein	−7.36	4.03	74
Delphinidin	−9.3	0.15	40
Eriocitrin	−7.18	5.42	13
Eriodictyol	−7.09	6.31	25
Genistein	−7.97	1.44	60
Glycitein	−7.91	1.59	62
Hesperetin	−7.34	4.2	32
Kaempferol	−7.42	3.64	27
Luteolin	−7.83	1.82	44
Malvidin	−6.9	8.81	84
Pelargonidin	−9.37	0.13	34
Peonidin	−7.74	2.13	20
Quercetin	−7.67	2.4	28
Resveratrol	−6.96	7.93	59

energy values as the best candidates (in alphabetical order): apigein, capsaicin, curcumin, cyaniding, daidzein, delphinidin, eriocitrin, eriodictyol, genistein, glycitein, hesperetin, kaempferol, luteolin, malvidin, pelargonidin, peonidin, quercetin, and resveratrol (Table 1). *Ki* values from the docking were not used as criteria for evaluation because they are directly related (and calculated from) binding energies, so they vary accordingly.

Among the polyphenols that present the best binding parameters, resveratrol was selected for further studies. Resveratrol is a phenolic phytoalexin found in grapes and other plants. According to docking model, it binds within the hydrophobic pocket of the enzyme, and forms no hydrogen bonds with the protein (Fig. 1B and C). Resveratrol possess a ligand efficiency of −0.41 kcal/mol for the arginine binding site of arginine kinase, three structures in the cluster with the lowest global free binding score (−7.48 kcal/mol), and being the second cluster the most populated, with 59 poses and a free docking energy of −6.59 kcal/mol.

3.2. Arginine kinase inhibition assays

Inhibition studies were performed using recombinant arginine kinase at different concentration of resveratrol (Fig. 2A). Enzyme activity was measured by an indirect assay that detects the conversion of ATP to ADP using an enzyme coupled system [22]. Resveratrol was assessed in the range 0–1.5 mM and it showed a dose dependent inhibition of arginine kinase activity. Resveratrol concentration where half arginine kinase inhibition was achieved

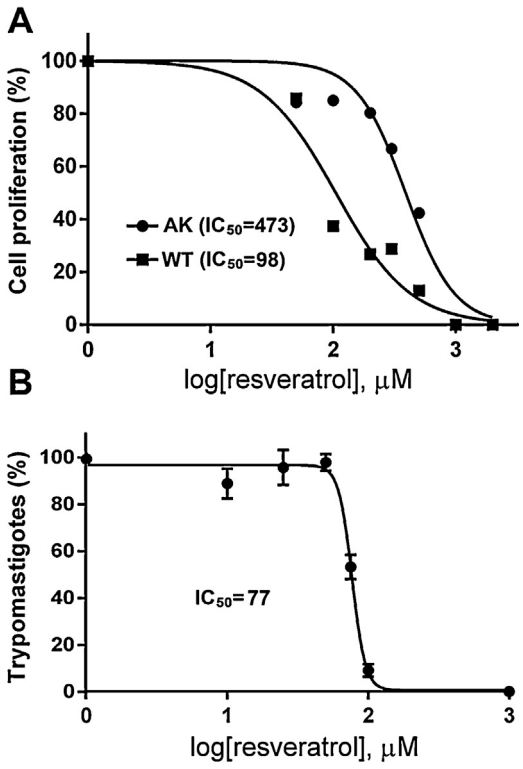

Fig. 3. Effect of resveratrol upon two *T. cruzi* life cycle forms. (A) 7.5×10^6 exponential phase epimastigotes (overexpressing *Tc*AK, circles, and wild type, squares) were treated with different concentrations of resveratrol (0–1 mM) during 72 h, when relative cell proliferation (%) was measured by absorbance at 560 nm. (B) Relative amount (%) of trypomastigotes obtained from the first burst of infected CHO-K1 cells in presence of 0–1 mM resveratrol. In both cases IC_{50} values were calculated by non-linear regression.

(IC_{50}) was 325 μM (Fig. 2B). To further validate these results, a different technique of measuring enzyme activity was used. Results obtained using a colorimetric method for phosphate determination [19] confirmed the inhibiting effect of arginine kinase by resveratrol treatment (Supplementary Fig. 2).

3.3. Evaluation of trypanocidal activity

To assess the effect of resveratrol on the viability of the replicative non infective forms of *T. cruzi*, epimastigotes from Y strains were exposed to resveratrol for 72 h in the concentrations range of 0–1 mM. Resveratrol inhibited the epimastigotes growth with an IC_{50} of 98 μM (Fig. 3A). To test the hypothesis that the observed trypanocidal effect of resveratrol was mostly mediated by inhi-

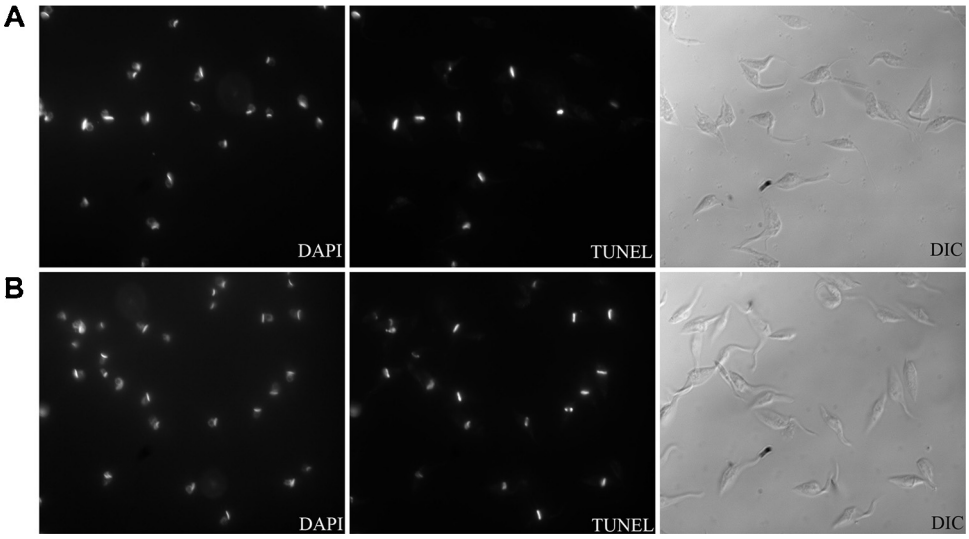

Fig. 4. TUNEL assay of resveratrol treated epimastigotes. 7.5×10^6 exponential phase epimastigotes were cultures over 24 h in presence (A) or absence (B) of resveratrol 0.6 mM. Images acquired by fluorescence microscopy under 100× objective show negative TUNEL signal in nuclei of control and treated conditions. DIC (Differential interference contrast) images showed no differences in cell morphology after 24 h resveratrol exposure.

bition of arginine kinase, transgenic parasites overexpressing arginine kinase were treated with resveratrol and the obtained IC_{50} values were compared with control parasites. The results showed that the resistance to resveratrol increases about 5 times in arginine kinase overexpressing parasites (IC_{50} = 473 μM) compared to the wild-type controls (Fig. 3A).

The effect of resveratrol was also assessed in trypomastigotes, the mammal infective form of *T. cruzi*, using a model of in vitro infection in CHO-K1 cells (Chinese Hamster Ovary). Trypomastigotes were exposed to resveratrol for 24 h in the concentrations range of 0–100 μM. Interestingly, resveratrol inhibited the trypomastigotes burst of infected cells at lower concentration than epimastigotes with an IC_{50} of 77 μM (Fig. 3B). These concentrations had no effect on the growth of uninfected CHO-K1cells. In addition, analysis of apoptosis was performed using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. Negative staining of treated epimastigotes suggests that another cell death mechanism different from apoptosis occurs (Fig. 4).

4. Discussion

Arginine kinase, a phosphotransferase involved in energy metabolism, is an essential enzyme in trypanosomatids under oxidative stress conditions [10]. In this work we explored polyphenolic enzyme inhibitors using in silico and in vivo approaches. We identified by molecular docking studies several polyphenols that might be able to bind the active site of *Tc*AK. Between the candidates, resveratrol was chosen for further studies since this compound is widely used in humans, it is completely nontoxic and is easily accessible due to its low market price [23]. Resveratrol is present in several plants species, such as grapes [24] and has a variety of biological effects, including anti-inflammatory properties, hypoglycemic and hypolipidemic effects, cancer prevention, cardiovascular protection, and prolongation of life span [25]. Enzyme inhibition of arginine kinase, as well as activity in epimastigotes growth and trypomastigotes burst of infected CHO-K1 cells were observed, with IC_{50} values in the micromolar range (325, 98, 77 μM, respectively). These data are in accordance with recent reports showing that resveratrol possesses both, anti promastigote and anti amastigote effects in *L. amazonensis* [26]. Trypanocidal effects of resveratrol through arginine kinase inhibition was confirmed by the model of transgenic parasites that overexpress the enzyme, which were significantly more resistant to resveratrol than wild type parasites.

The successful development of resveratrol therapies for human patients depends on the bioavailability, arising from a combination of several limiting factors, including poor water solubility, limited chemical stability and high metabolism. Recently, a formulation of resveratrol as a self-emulsifying drug delivery system significantly improved its cellular uptake and potentiated its antioxidant properties on bovine aortic endothelial cells [27]. This novel strategy could be tested in order to reduce the trypanocidal effective concentrations.

It cannot be ruled out that the trypanocidal effect of resveratrol could be exerted due to its action over enzymes other than arginine kinase, such as sirtuins, since resveratrol was described as a potent activator of this family of deacetylases [28].

The urgent need for new drugs to treat Chagas disease encourages further studies on the trypanocidal activity of resveratrol, mainly oriented to develop variants that may decrease the effective concentrations. Drug repositioning using novel molecular targets for known compounds could be an alternative experimental approach for tropical diseases treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.03.014.

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