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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].