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The Akt-*like* kinase of *Leishmania panamensis*: As a new molecular target for drug discovery

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ABSTRACT

The Akt-*like* kinase of *Leishmania* spp. is a cytoplasmic orthologous protein of the serine/threonine kinase B-PKB/human-Akt group, which is involved in the cellular survival of these parasites. By the application of a computational strategy we obtained two specific inhibitors of the Akt-*like* protein of *L. panamensis* (UBMC1 and UBMC4), which are predicted to bind specifically to the pleckstrin domain (PH) of the enzyme. We show that the Akt-*like* of *Leishmania panamensis* is phospho-activated in parasites under nutritional and thermic stress, this phosphorylation is blocked by the UBMC1 and UMBC2 and such inhibition leads to cell death. Amongst the effects caused by the inhibitors on the parasites we found high percentage of hypodiploidy and loss of mitochondrial membrane potential. Ultrastructural studies showed highly vacuolated cytoplasm, as well as shortening of the flagellum, loss of nuclear membrane integrity and DNA fragmentation. Altogether the presented results suggest that the cell death caused by UMBC1 and UMBC4 may be associated to an apoptosis-*like* process. The compounds present an inhibitory concentration (IC_{50}) over intracellular amastigotes of *L. panamensis* of 9.2 ± 0.8 μM for UBMC1 and 4.6 ± 1.9 μM for UBMC4. The cytotoxic activity for UBMC1 and UBMC4 in human macrophages derived from monocytes (huMDM) was 29 ± 1.2 μM and > 40 μM respectively. Our findings strongly support that the presented compounds can be plausible candidates as a new therapeutic alternative for the inhibition of specific kinases of the parasite.

1. Introduction

Leishmaniasis is a complex disease caused by the protozoan *Leishmania*, classified by the World Health Organization (WHO) as one of the most neglected tropical diseases. Leishmaniasis is a major health problem in many countries, affecting 12 million people worldwide (WHO, 2010; Alvar et al., 2012). This illness has three main clinical presentations: cutaneous, mucocutaneous, and visceral. Cutaneous leishmaniasis is the most problematic in Colombia and is caused by *L. panamensis*, *L. braziliensis*, and *L. guyanensis* species (WHO, 2010). The lack of a vaccine makes chemotherapy the main alternative to combat the disease. Classical chemotherapy includes a rather small number of drugs (pentavalent antimoniate or meglumine antimoniate, miltefosine, pentamidine, amphotericin B). Besides the limited number of alternatives the above-mentioned drugs present several disadvantages like high toxicity, adverse side-effects, high costs, and development of drug resistance (Aït-Oudhia et al., 2011; Rojas et al., 2006; Grogl et al., 1992).

The availability of the complete genome and kinome sequence of various species of *Leishmania*, including *L. major*, *L. infantum*, *L. braziliensis*, *L. donovani* and *L. mexicana* (Ivens et al., 2005; Peacock et al., 2007; Downing et al., 2011; Rogers et al., 2011) is an extraordinary tool to seek out new molecular targets on which new molecules can be selected by bioinformatics techniques.

Protein kinases are involved in several essential biological processes, including metabolism, gene expression, cell proliferation, motility, differentiation, and death. Recently, protein kinases became one of the most explored therapeutic targets in cancer by the pharmaceutical industry, with focus on the discovery of non-ATP competitive kinase inhibitors, given that modulators targeting allosteric sites can regulate specific protein kinases without affecting other protein kinases in normal physiological conditions. The trypanosomatid kinomes, which are about one third the size of the human one, differ in numerous ways from the kinome of their mammalian hosts. For comparative

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purposes the members of the kinomes of three trypanosomatid species were classified into the seven major groups of eukaryotic protein kinases (ePKs) (defined on the basis of sequence similarity of the catalytic domains: AGC, CAMK, CMGC, TK, TKL, STE, Other), according to the nomenclature of Manning (Manning et al., 2002; Gomase and Tagore, 2008; Naula et al., 2005).

Within the group AGC, the serine/threonine kinase protein kinase B (PKB) or Akt, identified by Stephen Staal in 1987, is an important regulator of cell proliferation and survival in mammalian cells. Data accumulated in the last decade established that Akt also plays a major role in cancer development and progression, prompting the development of drugs targeting this survival pathway in cancer therapy (Falasca, 2010). Akt is a serine/threonine kinase with three conserved domains, namely: pleckstrin homology domain (PH), which binds phosphoinositides with high affinity and induces a conformational change (Akt is normally maintained in an inactive state through an intramolecular interaction in the PH domain), as well as catalytic and regulatory domains (Brazil and Hemmings, 2001).

Akt has a wide range of cellular substrates and the oncogenicity of Akt arises from activation of both proliferative and anti-apoptotic signaling pathways making this kinase an attractive target for cancer therapy. Activation of mammalian Akt depends on its recruitment to membranes upon binding of phosphatidylinositol-3,4,5-trisphosphate (PIP3) to the PH domain, and subsequent phosphorylation at two key residues, Thr308 and Ser473, located at the catalytic domain and C-terminal regulatory domain, respectively (Franke, 2008). The physiological action of Akt kinase is mediated through the phosphorylation of a wide variety of downstream substrates (Brazil and Hemmings, 2001; Franke, 2008; LoPiccolo et al., 2008; Hers et al., 2011). One of the best known Akt substrates is glycogen synthase kinase-3 (GSK-3) which has been recently identified in *L. major* and *Trypanosoma brucei* (Ojo et al., 2011, 2008). A subgroup of Ser/Thr protein kinases, related to protein kinases A and C (RAC) or PKB/Akt have been identified in a number of mammalian cells (Song et al., 2005), *Drosophila melanogaster* (Scanga et al., 2000), *Caenorhabditis elegans* (Paradis and Ruvkun, 1998), *Dyctiostelium discoideum* (Meili et al., 2000), *Entamoeba histolytica* (Que and Reed, 1994), *Giardia intestinalis* (Kim et al., 2005), and *T. cruzi* (Pascuccelli et al., 1999). So far, none of these proteins have been reported in *Leishmania panamensis*.

In this work, we show that the *L. panamensis* genome codifies for an Akt-*like* gen, which we have cloned, sequenced and name it as Lp-RAC/Akt-*like* (Genbank:KP258183.1). The cloned gene encodes a protein closely related to previously reported RAC serine-threonine kinases from other *Leishmania* and *Trypanosoma* species. Our data suggest that Lp-RAC/Akt-*like* protein may behave as a survival molecule in *Leishmania* parasites and we propose it as a novel target in leishmaniasis therapy through its allosteric inhibition in the PH homologous domain. We also show that such inhibition can achieved by a new kind of molecules discovered by bioinformatics means. The putative Akt inhibitors UBMC1 and UBMC4 exert specific leishmanicidal activity and low cytotoxic effects against human cells. The discovery and description of new allosteric inhibitors of the Akt will allow the *in vitro* study of the signaling pathways where the Akt participates, which are a biological process widely unknown in *Leishmania* parasites. Last but not least, Akt inhibitors might represent a new source for the development of therapeutic alternatives for leishmaniasis treatment, in this regard it is necessary to perform further assays in order to assess its therapeutic potential *in vivo* and the drugability of such molecules.

2. Methodology

2.1. Sequence analysis

The sequence of the *L. panamensis* Akt-*like* protein was compared with homologue proteins from *L. braziliensis, L. donovani* and *L. infantum*. For that purpose, a multiple alignment was carried out using Clustal Omega web server (Sievers et al., 2011). Similarly, the sequence was pair-aligned using BLAST (Altschul et al., 1990) against reported Akt protein sequences from other organisms, including human. Identity percentages, query coverage and the identification of conserved motifs were analyzed.

2.2. In silico discovery of the Akt-like inhibitors

In order to find putative inhibitors for the Akt-*like* of *Leishmania panamensis* we performed molecular docking-based strategy for the virtual screening of a sub library of 600.000 compounds from the ZINC database (Irwin and Shoichet 2005). We first generated a 3D model of the Akt-*like* protein for *L. panamensis* using the I-Tasser web server (Roy et al., 2010), then we performed the molecular docking just with the pleckstrin domain (PH) of the Akt-*like* model using the AutoDock Vina software (Trott and Olson, 2009). Finally, the best docked complexes obtained for the PH domain-compound pairs (UBMC1 and UBMC4) were tested through a Molecular Dynamics (MD) analysis using the software GROMACS package (Hess et al., 2008).

2.3. Western blot

Total protein from 1.5×10^7 promastigotes was extracted in 180 µL of lysis buffer (150 mM NaCl, 10 mM HEPES, 1% CHAPS and 0.1 mM sodium orthovanadate), supplemented with a protease inhibitor and 40–60 µg of protein extract were separated on a 12% SDS-PAGE. The proteins were then transferred to a PVDF membrane (Thermo Scientific 0.45 µm), blocked with skim milk powder 5% (w/v) in TBST buffer (50 mM TRIS, pH 7.6, 150 mM NaCl, and 0.1% (% v/v) Tween 20) overnight at 4 °C. The PVDF membrane was incubated at 4 °C for 12 h with the following rabbit polyclonal antibodies made by ProteoGenix (France) on request: anti-Akt, which recognizes the peptide sequence comprised between Ser 418 and Glu 432 (SEQEKSPSHSPTIAE) in Akt-*like* protein, and anti p-Akt, which recognizes the peptide sequence comprised between Val254 and Tyr268 when the residue Thr261 is phosphorylated (VHEPNAV[$_P$T]YCGTNEY); primary antibodies were used at a 1:1000 dilution in TBST 1X with 5% skim milk. As a load control we used *Leishmania* spp. anti-actin antibody (Kalb et al., 2013). For human Akt blots we used anti-human phospho-AKT 1/2/3 (Thr308) (Cells signaling®), and human load control GAPDH at a 1:1000 dilution in TBST 1X with 5% skim milk (Reis-Sobreiro et al., 2013). In order to test the specificity of the anti p-Akt-*like* antibody, we treated 60 µg of total cell lysate with 100 units of the Lambda Protein Phosphatase (Lambda PP) for 3 h a 30 °C previous to the blotting. In all experiments we used an anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) as a secondary antibody (Sigma Aldrich), and Super signal pico chemiluminescent substrate (Thermo Scientific). Developments was done by exposure to x-ray photographic films.

2.4. Analysis of cell death by flow cytometry

L. panamensis promastigotes (MHOM/UA140) were grown at 26 °C in Schneider culture medium pH 6.9 supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin. 2×10^6 promastigotes were used for nutrition and thermic stress assays in 2 mL of the above-described media. Nutrition stress was induced by FBS depletion during six hours. Meanwhile, thermic stress was induced by incubation of the parasites at 37 °C for three hours in FBS supplemented media. Parasite cultures were treated with 10 µM UBMC1 and UBMC4, separately. Flow cytometry analysis were done with 2×10^6 promastigotes to test the inhibitors effect, using a BD FACSCanto II 4/2/2 Sys IVD cytometer. Labeling with propidium iodide was used in order to follow up of the parasite cell cycle. Apoptotic cells were quantified by finding the proportion of cells in sub-G0/G1 region of the cell cycle (hypodiploid cells). Loss of mitochondrial membrane potential was assayed using the probe DIOC6