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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

(3,3′ Dihexyloxacarbocyanine).

2.5. Studies in vitro of antileishmanial activity

The activity of compounds UBMC1 and UBMC4 was determined on Phorbol 12-myristate 13-acetate-differentiated U-937 cells infected with intracellular amastigotes of *L. panamensis* expressing the green fluorescent protein (GFP) gene (MHOM/CO/87/UA140pIR-GFP) (Pulido et al., 2012). One milliliter of 300.000 cell/mL solution in RPMI 1640 media was dispensed into 24-well plates (RPMI 1640 medium). The plates were incubated at 37 °C, 5% CO_2 for 72 h and then washed twice with phosphate buffered saline (PBS). U-937 cells were then infected with stationary phase promastigotes of *L. panamensis* in a proportion of 15:1 (parasites: cell). The plates were left in an incubator for 3 h at 34 °C, 5% CO_2; after incubation, the cells were washed twice with PBS and incubated again for 24 h at 34 °C, 5% CO_2. Infected cells were exposed during 72 h to four serial concentrations of each chemical compound starting at 50 μM in RPMI 1640 medium, Amphotericin B was used as positive control. Then cells were detached using trypsin/EDTA solution and washed twice with PBS by centrifugation for 10 min at 1100 rpm at 4 °C. Finally, parasite survival was assessed by flow cytometry using an Argon laser flow cytometer (Cytomics FC 500MPL) by reading at 488 nm excitation and 525 nm emission. Ten thousand events were counted from each well. The percentage of infected cells was determined by dot plot analysis while the parasitic load was calculated by the mean fluorescence intensity using histogram analysis. Each concentration was assessed in triplicate in at least two independent experiments.

In vitro antileishmanial activity was determined as percentage of infection (viable parasites inside infected cells) according to the MFI (median fluorescence intensity) units from flow cytometry analysis for each experimental condition using the formula: % infection = (MFI treated infected cells/MFI untreated infected cells) × 100, where MFI of untreated infected cells corresponds to 100% viable parasites. Reduction of infection was calculated using the formula: % inhibition = 100 − % infection. The% inhibition obtained for each experimental condition was used to calculate the effective concentration 50 (EC_{50}) by Probit analysis (Finney, 1978).

Compounds were considered active when the EC_{50} were < 20 μM while EC_{50} values > 20 and < 50 μM were indicative of a moderate antileishmanial activity. The index of selectivity (IS) was calculated by correlating cytotoxicity with antileishmanial activity using the formula: IS = LC_{50}/EC_{50}.

2.6. In vitro cytotoxic studies

The cytotoxicity of both compounds (UBMC1 and UBMC4) was evaluated according to the capacity of killing primary culture of human monocyte-derived macrophages (huMDM) by the MMT method, following the procedure described by others (Montoya et al., 2015). The huMDM were obtained from 50 mL of defibrinated whole blood from healthy donors. These samples were mixed in proportion 1:1 with calcium and magnesium free Dulbeccós phosphate saline (DPBS). This mixture was centrifuged in a Ficoll Hypaque 1077 density gradient in proportion 1:3 (blood-ficoll) for separation of mononuclear cells, by centrifugation at 2000 rpm for 20 min at 22 °C. The corresponding mononuclear phase was separated. Cells were washed twice with DPBS solution, by centrifugation at 13000 rpm for 10 min. After the last wash, the cells were re-suspended in RPMI 1640 medium with 10% of autologous serum at a concentration of 0.3 × 10^6 cells per mL. 1 mL of the cell suspension was dispensed in 24 well cell culture plates and incubated at 37 °C and 5% CO_2 for 72 h to allow the monocyte differentiation to macrophages. For the MTT test huMDM cells were adjusted to a concentration of 0.5 × 10^6 cells/mL in RPMI Medium 1640 and supplemented with 10% fetal bovine serum (FBS) and the corresponding concentration of the compound (four serial diluted concentration starting at 200 μM) was added to the culture. Amphotericin B and Doxorubicin were used as control during cytotoxicity experiments. Each experiment was performed in triplicates in at least two different experiments.

The *in vitro* cytotoxicity was defined as the percentage of viability and growth inhibition obtained from the optical densities (O.D.) for each experimental condition using the formula: viability (%) = (O.D. treated cells/O.D. untreated cells) × 100, where O.D. of untreated cells correspond to 100% viability. In turn, growth inhibition percentage (%) was calculated as 100 − % viability. Growth inhibition% data obtained for each experimental condition was used to calculate the lethal concentration 50 (LC_{50}) by Probit analysis (Finney, 1978).

The cytotoxicity of each compound was graded according to their LC_{50}. Thus, LC_{50} values < 100 μM was considered as high cytotoxicity while LC_{50} > 100μM and < 200μ M was considered as moderate cytotoxicity and LC_{50} > 200 μM was considered as potential non cytotoxic.

2.7. Scanning electron microscopy

Control and treated promastigotes were fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and then adhered for 10 min to coverslips coated with 0.1% poly-L-lysine. The samples were washed with cacodylate buffer and dehydrated with 30%, 50%, 70% and 90% (v/v) acetone for 5 min each step, followed by 3 steps in 100% acetone for 5 min. Following the dehydration steps the cells were critical point dried with CO_2 and adhered to SEM stubs. Finally, the samples were coated with a 20-nm-thick gold layer and observed in a Jeol JSM-6010Plus-LA scanning electron microscope at 20 kV.

2.8. Transmission electron microscopy

Control and treated promastigotes were fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 and for 1 h with 1% OsO_4 diluted in this same buffer. The cells were dehydrated in graded ethanol and embedded in PolyBed-812 resin. Ultra-thin sections were collected on cupper grids and stained with 5% aqueous uranyl acetate and lead citrate. The samples were then examined in a Jeol JEM-1400Plus transmission electron microscope operating at 80 kV.

3. Results

3.1. Sequence analysis and 3D modeling for Akt-like of L. panamensis

The open reading frame for the Akt-*like* of *L. panamensis* is constituted by 1533pb (Gen bank: KP258183.1) and codes for a 510 residues protein, with three predicted structurally conserved domains: the pleckstrin domain (PH), the kinase domain, and the AGC domain. There is also a not structured region that is only present in the Akt-*like* of *L. panamensis*, according to the analysis performed in Prosite® Database of protein domains, families and functional sites (http://prosite.expasy.org/).

The protein Akt-*like* of *L. panamensis* is highly conserved amongst the different species of *Leishmania*, showing a 100% similarity when compared to the Akt of *L. braziliensis,* 93% for Akt of *L. donovani*, and 92% for Akt of *L. infantum*, according to the comparative analysis in Clustal W (Sievers et al., 2011). The inter-domain comparison of the Akt-*like* of *L. panamensis versus* human Akt shows a similarity for the PH domain of 29.8%, the kinase domain showed a similarity of 61.7%, and the AGC domain showed a similarity of 29.6%.

The tridimensional modelling of the Akt-*like* of *L. panamensis* is a first structural approximation for the protein, given that we did not have a crystalized model of the protein yet (Fig. 1a). Along the *in silico* modelling of the Akt-*like* of *L. panamensis,* we made a tridimensional model for human Akt2 (GenBank: M77198.1) (Fig. 1b). Then we designed a docking “box” inside the PH domain of the model of the Akt-

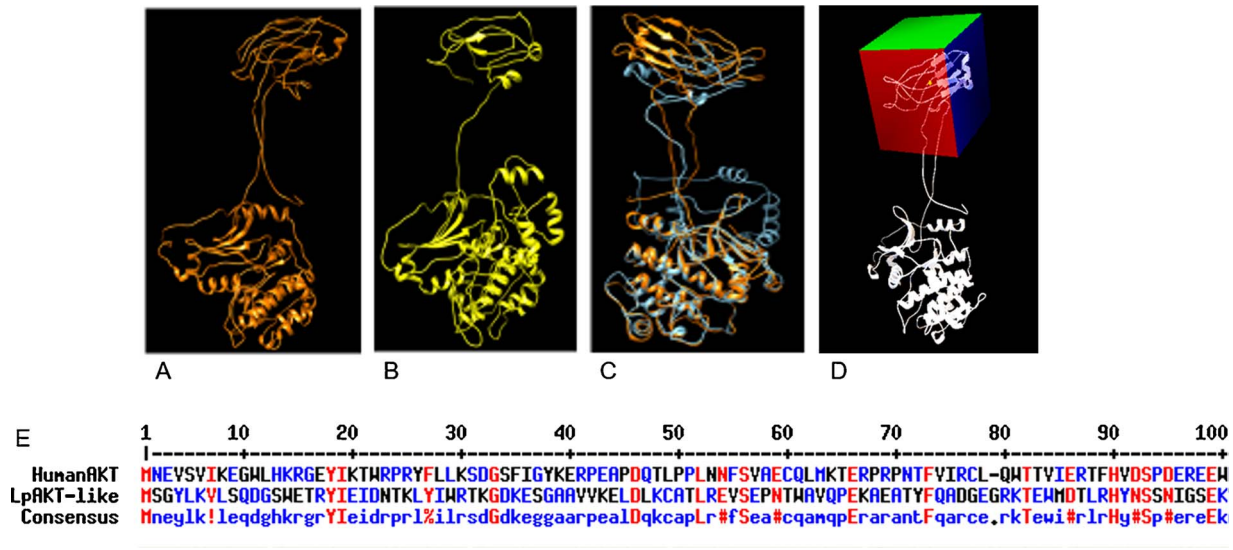

Fig. 1. Tridimensional model of human Akt kinase *versus* Akt-*like* of *L. panamensis*. (A) 3D model of Akt-*like* of *L. panamensis*; (B) 3D model of human Akt; (C) merge of the human Akt and Akt-*like* of *L. panamensis* 3D models; (D) docking box (green, red, and blue cube) on the pleckstrin domain (PH) of Akt-*like* of *L.* panamensis; (E) PH domain alignment for human Akt and Akt-*like* of *L. panamensis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

like of *L. panamensis*, which constrained the possible interactions of the protein with compounds from a sub-library of the Zinc database. The coordinates used in this "box" were x = −31.239, y = −29.864, and z = −14.139; and its dimensions were 26 × 26 × 26 Å respectively (Fig. 1d). Alignment of the PH domains of the human Akt and the Akt-*like* of *L. panamensis* showed a great difference in the sequence between the two domains (Fig. 1e). Molecular docking and molecular dynamics approaches were used in order to screen and refine the above mentioned sub-library allowing us to find two structurally similar compounds, the UBMC1 compound with a free energy of interaction of −10.4 kcal/mol for the Akt-*like* of *L. panamensis* and the UBMC4 compound with a free energy of interaction of −8.2 kcal/mol for the Akt-*like* of *L. panamensis* (Subjected for patenting NC2017/0000871).

3.2. Analysis of the phosphorylation states of the Akt-like L. panamensis under cellular stress

In order to demonstrate that Akt-*like* is activated under cellular stress conditions, a protein phosphorylation analysis was carried out in parasites under two conditions: thermic stress, and nutritional stress. In the phosphorylation assay for Akt-*like,* shown in Fig. 2a, two groups of parasites are observed, one group under nutritional stress depleted of Fetal Bovine Serum (−FBS), and a control group not stressed supplemented with Fetal Bovine Serum (+FBS). It is necessary to take into consideration that while the protein phosphorylation is low for the parasite group +FBS, for the parasites in −FBS the phosphorylation of Akt-*like* is high. During the thermic stress at 37 °C, high phosphorylation is observed for the Akt-*like* as compared with the control at 26 °C. Similar results were achieved in the phosphorylation assay for parasites under nutritional stress. It is evident that the protein was not phosphorylated in parasites at 26 °C, though the total concentration is not diminished, but in parasites stressed at 37 °C, the protein was highly phosphorylated (Fig. 2b).

For parasites under thermic stress at 37 °C and treated for 2 h with 10 μM of the UBMC1 inhibitor phosphorylation of the Akt-*like* was drastically decreased in comparison to the stressed parasites at 37 °C for 2 h without treatment, which showed strong phosphorylation (Fig. 2c). It is noticeable that the commercial rabbit polyclonal antibody (pT 261-AKT, ProteoGenix SAS, France) specifically binds the phosphorylated version of the Akt-*like* of *L. panamensis* since it does not recognize any phosphorylated target in protein extracts from lysate parasites incubated with 100 units of Lambda Protein Phosphatase (Fig. 2d).

To demonstrate that the UBMC1 compound has an inhibitory effect on Akt-*like* of *L. panamensis,* and not on human Akt, the inhibition potential was analyzed for the UBMC1 compound in the phosphorylation for the human Akt. Human breast cancer tumoral cell line MDA-MB-231 ATCC ® HTB-26 is well known for its constitutive activation of Akt. These cells were put under different concentrations of the UBMC1 compound: 5 μM, 10 μM, and 20 μM; it was evident that under our conditions none of the concentrations were capable of inhibiting the phosphorylation of the human Akt in this cell line (Fig. 2e).

3.3. Analysis of apoptosis-like cell death by flow cytometry

The effect of the inhibitors UBMC1 and UBMC4 was evaluated in promastigotes of *L. panamensis* under nutritional stress for 6 h. Each compound was added to a final concentration of 10 μM to the stressed parasites. The cell cycle and mitochondrial membrane potential analyses for the UBMC1 compound showed 7% hypodiploid parasites in the normal +FBS control; however, 3% hypodiploidy was observed in the FBS depleted parasites. Both, +FBS and UBMC1 treated parasites, had a hypodiploidy of 42%, but hypodiploidy was increased to 72.8% in those parasites depleted from FBS and treated with the inhibitor UBMC1. In the case of the UBMC4 inhibitor parasites in presence of FBS and treated with the compound showed 40% hypodiploidy; meanwhile, FBS-depleted parasites and exposed to the compund showed a hypodiploidy of 64% as illustrated in Fig. 3a. Regarding the mitochondrial membrane potential it was found that normal control promastigotes had 5% of positive cells for DIOC6, whereas the −FBS control showed 44.6% of positive cells for DIOC6, indicating a high percentage of membrane-hyperpolarized cells. The +FBS parasites treated with UBMC1 were 25.8% DIOC6 positive, however, the −FBS/UBMC1 parasites showed just 5.5% DIOC6 positivity. On the other hand, the +FBS parasites treated with UBMC4, showed 19.3% positivity for DIOC6, whereas the −FBS group treated with UBMC4 showed a 15.3% of positive cells for DIOC6 label (Fig. 3b).

3.4. Leishmanicidal activity and citotoxicity of the UMBC1 and UMBC4

The UBMC1 compound was capable of eliminate the 50% of the parasites infecting cell at a concentration of 9.2 μM ± 0.8, whereas UBMC4 compound was capable of the same effect at a near half of that concentration (4.6 μM ± 1.6). Regarding the cytotoxicity of the inhibitors on primary huMDM cells, UBMC1 compound was cytotoxic

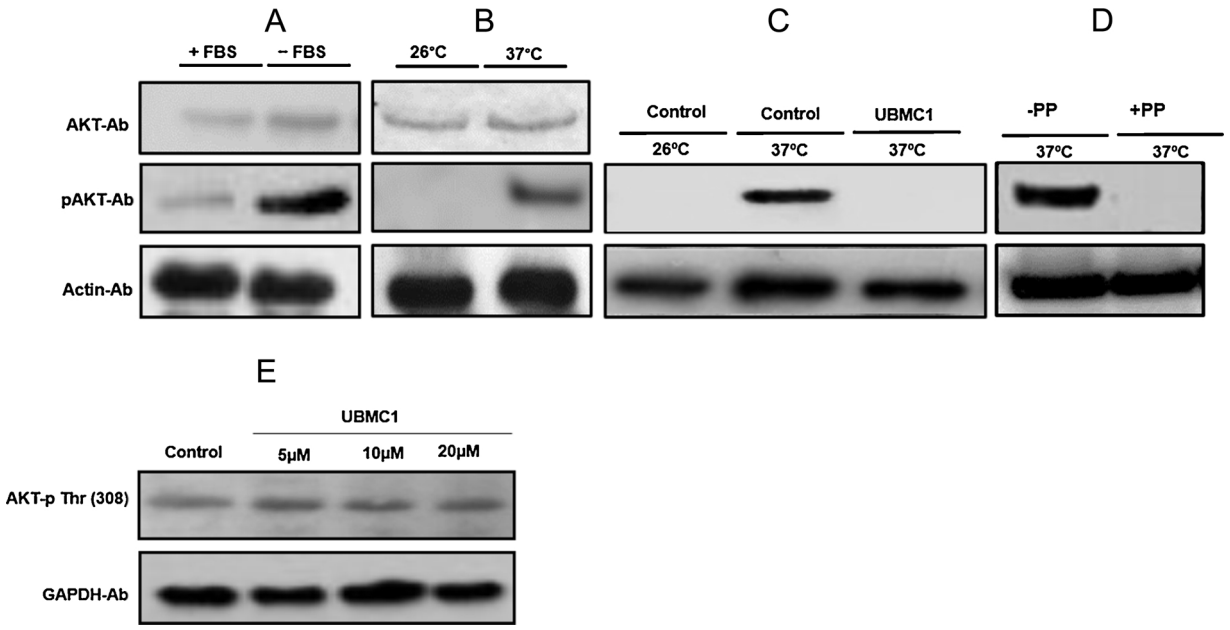

Fig. 2. Activation of the Akt-*like* under stress conditions and upon treatment with UBMC1. (A) Normal culture conditions (+FBS) and nutritional stress (−FBS); (B) Normal culture conditions (26 °C) and thermic stress (37 °C); (C) Inhibition of phosphorylation of AKT-*like* of *L. panamensis,* at 26 °C, 37 °C, and 37 °C for 2 h each condition and 37 °C + UBMC1 10 μM; (D) Specificity test of the p-Akt-*like* antibody in parasites under thermic stress (37 °C) without Lambda Protein Phosphatase treatment (−pp) and with Lambda Protein Phosphatase treatment (+pp); (E) Inhibition of phosphorylation of human Akt on the tumoral cell line MDA-MB-231 ATCC ® HTB-26 treated with 5 μM, 10 μM, and 20 μM of the UBMC1 inhibitor.

at > 29 μM ± 1.2, meanwhile UBMC4 compound cytoxicity was > 40 μM being the latter less toxic than UBMC1 (Table 1).

3.5. Effect of the Akt-like inhibitors on the morphology of L. panamensis promastigotes

Promastigotes of *L. panamensis* under normal culture condition were incubated with the UBMC4 compound at 10 μM and observed by scanning electron microscopy. Loss of flagellum was observed after 1 h of treatment (Fig. 4a–c), while 2 h post-treatment the parasites were completely round, with no flagellum (Fig. 4d).

To observe internal damages in the parasites, promastigotes were treated with UBMC4 inhibitor at 10 μM and compared to control cells kept in RPMI medium supplemented with 10% FBS, observed under transmission electron microscopy (TEM). While control cells showed a round nucleus and normal cytoplasmic structure (Fig. 4e), promastigotes treated with UBMC4 showed changes in their normally-elongated body shape and turned oval, the nucleus structure started to being lost after 1 h post-treatment, with electron-dense lipid vacuoles and a small swelling of the mitochondrion (Fig. 4f). After 2 h post-treatment a very vacuolated cytoplasm could be observed and the nucleus was undistinguishable, with electron-dense lipid droplets, mitochondrial swelling, and complete loss of flagellum (Fig. 4g and h). We carried out the same analysis in parasites under nutritional stress kept in PBS buffer with no FBS. We observed that those promastigotes showed accumulated lipid droplets after one hour treatment, condensed chromatin appeared in the periphery of the nuclear membrane and the kinetoplast lost its compact shape. Treatment for 2 h with UBMC4 resulted in condensed peripheral chromatin, as well as the kinetoplast fractured into small fragments, showing cells in complete degradation and empty cytoplasm with high nuclear fragmentation (data not shown).

Table 1
Antileishmanial activities of UBMC1 and UBMC4 against *L. panamensis* intracellular amastigotes.

Compound	IC_{50}(μM)[a]	LC_{50}(μM)[b] (huMDM)	SI[c]
UBMC1	9.2 ± 0.8	29 ± 1.2	3.1
UBMC4	4.6 ± 1.9	> 40	8.7

Data represent the mean value ± standard deviation.
[a] IC50 Inhibitory concentration of *L. panamensis* amastigote.
[b] LC50 Lethal concentration.
[c] SI selectivity index between (huMDMD) human monocyte-derived macrophages and amastigote.

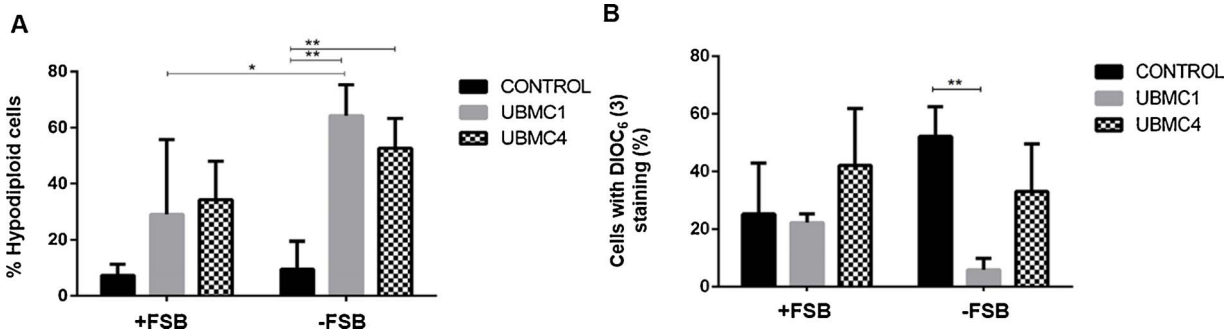

Fig. 3. Analysis of cell death by flow cytometry. (A) % Sub-G0/G1 hypodiploid cells after treatment with 10 μM of UBMC1 +FBS, 10 μM of UBMC1 −FBS, 10 μM of UBMC4 +FBS and 10 μM of UBMC4 −FBS. Controls are cells grown in presence of fetal bovine serum (+FBS) and without fetal bovine serum (−FBS); (B) Δym: Variation on mitochondrial membrane potential (DIOC6) under same conditions as above.

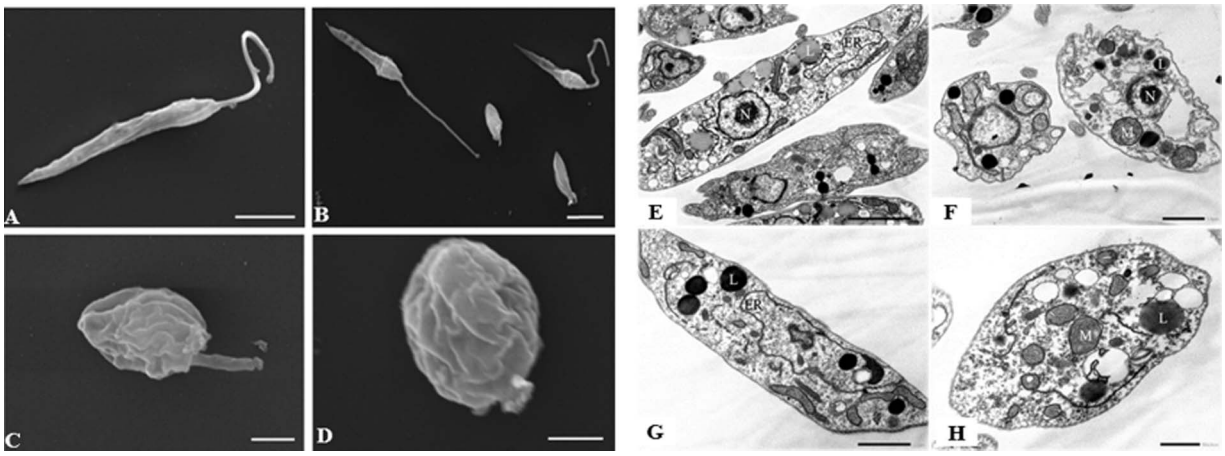

Fig. 4. UBMC4 inhibitor effect on the morphology of promastigotes of *L. panamensis*. Scanning (A–D) and transmission (E–H) electron microscopy of promastigotes of *L. panamensis* incubated in RMPI +SFB and treated with UBMC4 inhibitor at 10 μM. (A) Normal parasite morphology in non-treated cells; (B, C) parasite with progressive loss of flagellum after 1 h treatment; (D) complete lost of flagella and round shape after prolonged treatment. Scale bars in A and B are 5 μm, and for C and D 1 μm; (E) Non-treated control cells show round nucleus (N) with peripheral chromatin, compact kinetoplastid DNA (K) and some lipid vacuoles (L); ER: endoplasmic reticulum; (F) Cells treated for 1 h with UBMC4 inhibitor at 10 μM: lipid vacuoles (L) appear more electron-dense, with small swelling of the mitochondrion with cellular vacuolation; (G, H) Cells treated for 2 h: electron-dense lipid droplets (L), mitochondrial swelling (M). Some cells showed empty cytoplasm (D), with several vacuoles.

4. Discussion

The present study shows the inhibitory effect of two new compounds over the PH domain of the Akt-*like* de *L. panamensis* enzyme, aiming to interfere the natural binding of the protein to its cognate ligand (3,4,5)-trisphosphate (PIP3) in the parasite membrane during cell signaling processes, therefore the proposed compounds are possible inhibitors of the "*PI3K-Akt-mTOR-like*" signaling pathway, which is involved in the cell survival in the parasites (Graphical abstract).

The Akt-*like* protein is conserved in all *Leishmania* species with > 90% similarity between them, indicating that this protein may be essential for the parasites. Although the molecular mechanisms of adaptation in *Leishmania* parasites are widely unknown, it has been established *in vitro* that during infection the parasites are subjected to nutrient deprivation (−FBS), temperature elevation (37 °C) and pH reduction (pH 5.5) inside the parasitophorous vacuole. Such stimuli could give insights into the cellular processes experienced by the parasite during infection (Tsigankov et al., 2014). Transcriptomic and proteomic analysis have showed that parasite differentiation from promastigotes to amastigotes is a coordinated and dynamic process whose result is the adaptation to the hostile environment of the phagolysosome. Phospho-proteomic analysis has revealed significative differences in the protein phosphorylation profile between promastigotes and amastigotes, giving a paramount participation to the kinases in the mediation of the physiological and morphological changes experienced by the parasite during adaptative events through the selective activation of signaling pathways inside the parasite (Tsigankov et al., 2014). Our results have shown that the Akt-*like* de *L. panamensis* may be a kinase involved in the cell stress response. We proved as well that the enzyme is specifically phosphorylated at the residue Thr 261 during stress conditions. Altogether the presented evidence strongly supports the participation of the Akt-*like* de *L. panamensis* in adaptative processes and cytoplasmic cell signaling pathways.

By using computational approaches, we performed an *in silico* screening of a sub-library of compounds using a model of the PH domain of the Akt-*like* of *L. panamensis*. These analyses allowed us to identify two molecules with high affinity for the PH domain as predicted by docking and molecular dynamics. The compounds, named here UMBC1 and UMBC4, showed strong inhibitory activity against the *in vivo* phosphorylation of the Akt-*like* of *L. panamensis*. Additionally, we proved that while the enzyme is naturally phosphorylated under stress conditions, such phosphorylation is inhibited by UMBC1 and UMBC4 at concentrations below 10 μM. The phosphorylation profile of the treated parasites is comparable with the one from non-stressed parasites.

Most of the kinase inhibitors are considered promiscuous or unspecific at high concentrations (Wang et al., 1999); previous to the docking analysis performed here we made a comparison between the human Akt isoforms and the Akt-*like* of *L. panamensis*, those analyses showed that it is possible to inhibit specifically the leishmanial enzyme by directly targeting the PH domain, which presents a similarity lower that 30% with respect to the human counterparts (Fig. 1e). In the present work we showed that the compound UMBC1 is highly specific for the Akt-*like* of *L. panamensis* at low concentrations, and that it does not exert inhibitory effects over the human counterparts Akt 1/2/3 at concentration between 3 μM and 20 μM in tumoral cell line MDA-321, which has a constitutive activation of the Akt (Chavez et al., 2010; Nicholson et al., 2003), evidencing a high selectivity of the described molecules towards the Akt-*like* of *L. panamensis*.

The above mentioned experiments were complemented with *in vitro* evaluation of the inhibitory activity of UMBC1 and UMBC4 over the phosphorylation profiles of the *Leishmania* parasites under stress conditions as well as its leishmanicidal activity *in vitro*. Both compounds showed deleterious effects over parasites in short periods of time (< 2 h). In a frame between 2 and 6 h parasites presented high hyplodiploidity, loss of mitochondrial membrane potential, loss of cell membrane integrity, loss of flagella and consequentially lower motility and several morphological changes associated to cytoskeletal rearrangement similar to shapes of apoptotic bodies and cytoplasmic vacuolation and DNA fragmentation. Such phenotypic observations are tightly associated with cell death processes by apoptosis-*like* mechanisms in the parasite, mainly those related to DNA alterations as observed by electron microscopy and genotoxicity assays (data not shown). Regardless apoptosis may appear under several cellular circumstances, in this particular case the induction of such process is associated with the presence of toxic agents as the cell signaling inhibitors studies here (Ouyang et al., 2012). The afore-mentioned evidence supports the hypothesis that the parasites experience an apoptosis-*like* cell death process that compromises the promastigote viability through the inhibition of the Akt-*like* protein which is essential for cell survival. Apoptosis in human cells can be induced by two pathways: the intrinsic pathway which involves the mitochondria and the extrinsic pathway that involves the so called cell death receptors which generates intracellular signaling upon stimulation (Cascales, 2003).

Since the published genomes of *Leishmania* spp. to the date do not

present any caspases or cell death receptors, it is plausible to propose that other proteins may be responsible for the apoptosis-*like* mechanisms (Castanys-Muñoz et al., 2012). Our data strongly supports the idea that the treatment of *L. panamensis* parasites with the UMBC1 and UMB4 compounds experience an apoptosis-*like* process through the intrinsic pathway; however, it is necessary to make further studies that allow us to elucidate the signaling pathways, kinases and mechanisms involved in the activation of the apoptosis-*like* process in *Leishmania* spp.

The evaluation of the leishmanicidal effect of the compounds over intracellular amastigotes of *L. panamensis* allowed to establish that UBMC1 and UBMC4 inhibits 50% of the cell growth of the amastigotes at a concentration < 10 μM; while the cytotoxicity is 3–9 times above the observed antileishmanial concentration. Such results strongly support the candidacy of these compounds as potential therapeutic alternatives for the treatment of cutaneous leishmaniasis, additionally the exploratory studies of its drugability showed that they do meet at least five of rules of Lipinski (2004); nevertheless it would be necessary to perform *in vivo* studies in order to test the behavior of the compounds beyond the *in vitro* systems. Selectivity index demands values above 10 in order to prove that the activity of a compound is exerted over intracellular amastigotes without affecting mammalian host cells; from the two evaluated compounds only UBMC4 meets this criterion (selectivity index > 8.7), this observation indicates as well that the biological efficiency of the compound as leishmanicidal agent is not due to its cytotoxicity over the host cell (Weniger et al., 2001).

The increasing published evidence about kinomes suggest that the super family of the protein kinases are potential therapeutic targets for trypanosomatids such as *Leishmania* spp. and therefore, it is paramount to recognize that these enzymes play important roles in the proliferation of parasites and the maintenance of their life cycle during their stages specially those clinically relevant. Considering that protein kinases of trypanosomatids exhibit important biochemical differences when compared to their homologues in mammals, it can be arguable that they can be selectively exploited in the search of potential chemical inhibitors (Ward and Goldberg, 2012). The experimental and computational evidences that support this study first demonstrates to certain extent that the Akt-*like* de *L. panamensis* constitutes an important element during cellular stress conditions in the parasites, and that its specific inhibition is associated with the parasite death by apoptosis-*like* mechanisms most likely through the intrinsic pathway which involves the mitochondria.

Competing interest

The authors declare that they have no competing interest.

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Authors' contributions

DTD: Masters Student, development of experiments, article writing.

MMV: Project writing, master's student tutoring, data analysis, article writing.

R.O: Project writing, bioinformatic analysis of data, article writing.

GBF: Development of experiments.

MJS: Scanning and transmission electron microscopy, data analysis, article writing.

SMR: Development of EC50 and LC50 experiments, data analysis, article writing.

REVM: Project writing, master's student tutoring, data analysis, article writing.

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