International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.4, pp 595-601, 2015

Effect of inhibitor protein kinase A (PKA) on *Leishmania tropica* promastigotes viability, infectious ability and differentiation

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Abstract: Protein kinase A (PKA) is an important constituent of the signaling pathways involved in various processes including cell proliferation, differentiation and response to environmental changes. Here, we report the effect of inhibitor PKA activity like H89 on viability, infection and differentiation of *Leishmania tropica.* We found that 15 µM of H89 was enough to stop kemptide, substrate of PKA, phosphorylation completely in live *Leishmania*. As for viability, the required concentration of H89 to inhibit cell growth by 50% (IC50) was 13,47 µM. A significant reduce in infection effect on macrophage was observed when *Leishmania* was treated with of H89, whereas, infection percentage was reduced to 56.3 % after treatment with IC50. Cells cycle arrested at G1 phase after 6h exposing promastigotes to differentiation signal (concomitant exposure to 37°C and pH 5.5) then initiated transformation, we observed that arrested cells reduced to 11.5% after treatment with IC-50 of H89, whereas most cells entered in sub G0-G1 phase. According to the previous results, H89 treated parasites (*L. tropica*) could be tested as a probable live attenuated vaccine against *Leishmaniasis.*
Keywords: Cutaneous *Leishmania*sis, *L. tropica*, PKA, H89, Promasigote, Amastigote.

Introduction

Leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, is one of the world's most neglected diseases, affecting mainly very poor people in developing countries. It is prevalent throughout the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe (old world) and South and Central America (new world). Depending largely on the species of the parasite and the immune competence state of the human host, the disease spectrum ranges in severity from cutaneous (CL), to mucocutaneous (MCL) and visceral *leishmaniasis* (VL) [1]. Based on WHO estimates, *Leishmania tropica* is the main species responsible for the cutaneous disease in Syria, and number of *Leishmania*sis have risen steadily over the years despite focal indoor spraying with residual insecticides and other control measures. The disease continues to spread to new areas [2]. *Leishmania* parasites alternate between two different life stages which are important mechanism of adaptation to survive and proliferate, the promastigote and the amastigote. The extracellular, motile promastigotes are transmitted by the sandfly to the mammalian host. Promastigotes are rapidly phagocytized by phagocytic cells at the site of infection, but only differentiate into the obligate intracellular amastigote in response to the temperature and acidic environment of the macrophage phagolysosome [3].

Methods used for prevention or control of *leishmaniasis* have included eradication of the vector or its habitat, treatment of human reservoirs, and vaccination. Technical difficulties such as toxicity of drags, drug resistance, financial constrains, and operational difficulties have impaired progress toward effective control of *leishmaniasis* [4]. The absent of ideal therapy for CL emphasize the need for vaccine development which has proven to be more difficult than bacteria and viruses due to inadequate knowledge of parasite pathogenesis and

the complexity of immune responses needed for protection [5]. Vaccination trials in animal models, human beings, or both have been performed with virulent promastigotes, attenuated-live promastigotes or killed with either gamma irradiation, heat, or mutagen; and specific antigens purified from promastigotes or produced as recombinant proteins [6]. The development of an effective, noninfectious vaccine is problematic.

Many studies focus on the potential of protein kinases as targets for novel drugs whereas, Protein phosphorylation and dephosphorylation is an important mechanism of regulation in the eukaryotic cells. Protein kinases are the mainly regulators of many different cellular processes such as cell cycle progression, differentiation, transcriptional control, and have drawn much attention as potential drug targets to treat a wide range of diseases and syndromes [7].

In *Leishmania*, signal transduction pathways modulated by protein kinases (PK), such as the cAMP dependent protein kinase A (PKA), allow cells to respond to external stimuli and are involved in various processes as cell cycle [8]. PKA is a tetrameric holoenzyme comprised of two catalytic (PKA-C) subunits and two regulatory (PKA-R) subunits. The PKA-R subunits bind cAMP causing a conformational change in the molecule and dissociation of the holoenzyme R and C subunits. Upon dissociation, the now active C subunits phosphorylate specific serine/threonine residues on protein substrates in the cell cytosol and nucleus changing the activity and localization of these molecules [9].

A specific PKA inhibitor H89 is frequently used to block signaling pathways in studies of cellular regulation, to elucidate inhibition mechanisms at atomic resolution and to enable structure-based drug design of potential therapeutic modulators of signaling pathways [10]. In this study, we evaluated the action of PKA on *L. tropica* survival, infectivity and promastigote to Amastigote differentiation using PKA specific inhibitor H89. Our results indicated that PKA might have an interesting role to play in *L. tropica* lifecycle.

Methodology

Cell culture:

We used a *Leishmania tropica* strain isolated previously in Damascus in 2011. Promastigotes were cultivated in RPMI-1640 medium containing penicillin/streptomycin and supplemented with 10 % heat inactivated fetal bovine serum (FBS) (all products from Sigma, USA). The cultures were incubated at 26 °C. To obtain axenic amastigotes, promastigotes in stationary phase in 25 cm^2 ventilated flask were incubated at 37 °C and pH 5.8 with 5 % CO_2. for about 48 hour.

Effect of H89 inhibitor on PKA activity:

Cellular lysates were prepared from treated and untreated *L. tropica*with vary concentrations of H89 (0.1, 1, 5, 10 15µM). Briefly, Live stationary phase parasites were harvested by centrifugation (2500 rpm, 15 min) at room temperature and washed twice by centrifugation with ice-cold PBS (wash buffer). The cells pellet was lysed in lysis buffer [50mM Tris-HCl, 150mM NaCl, 1% Triton X100, 0.1 % sodium deoxycholate, pH:7.5] (Promega, USA) supplemented with protease inhibitors cocktail (Promega, USA) for 10 min on ice. Protein concentration for each sample was assayed using Bradford method. Protein kinas A activity in last cell lysates were assayed using PKA Kinase activity assay kit (Enzo Life Sciences, USA) according to manufacturer's instructions. Briefly, the substrate, which is readily phosphorylated by PKA, is precoated on the wells of the provided PKA Substrate Microtiter Plate. The samples are added to the appropriate wells, followed by the addition of ATP to start the reaction. After that a Phosphospecific Substrate Antibody is added which binds specifically to the phosphorylated peptide substrate. Last antibody is subsequently bound by a peroxidase conjugated secondary antibody. The test is developed with tetramethyl benzidine substrate (TMB) and a color develops in proportion to PKA phosphotransferase activity. The color development is stopped with acid stop solution and the intensity of the color is measured in a microplate reader at 450nm. The PKA activity was calculated by the following equation: kinase activity = (absorbance of sample – absorbance of blank) / quantity of protein.

Viability assay:

Colorimetric assay of MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] measure transference reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable cells (Sigma, USA).Parasites suspension (5×10^5) were treatedto varying concentrations of H89 (10, 12, 14, 16, 18, 20µM) for 24h then transferred to ELISA plate.The formation of formazan was measured by adding MTT and incubating the plate for 3 h at 26 °C. Formazan crystals were dissolved in MTT solvent and absorbance were

measured at 450 nm. The positive control was untreated sample. Results were reported as percentage of viability, determined as: (absorbance of treated sample well/absorbance of positive control well) × 100. IC50, The concentration of sample required to inhibit cell growth by 50% in comparison with the growth of a cell control, was calculated from the dose-response curves. All the assays were done in triplicate and values are means ±SD.

Macrophages infectivity assay:

Circulating white blood cells were obtained by density gradient centifugation with Ficoll then Monocytes were separated from lymphocytes by adherence to tissue culture plastic with medium IMDM supplemented with 10% FBS in the 5% CO2, 37°C incubator, these monocytes differentiate into Macrophage within the first few days of culture [11]. Treated with H89 and untreated stationary phase of promastigotes were washed with PBS, then counted in a Neubauer chamber. Macrophages were infected by addition parasites to cells at ratio 5:1, and incubated for 2 hours at 37C° in a 5% CO2. The free parasites were removed by successive washes. After 24 hour *Leishmania*-infected cells were fixed in methanol, and stained with Giemsa dye. Percentage of infected macrophages and the average of parasites per infected macrophage were counted for 300 macrophages in each of duplicated wells.

Cell cycle and differentiation of *L. tropica*:

The cell cycle of *L. tropica* were analysed by flow cytometry technique for treated and untreated differentiating promastigotes (exposed to pH 5.5, 37C° for 6h) For each assay, cell culture (1×10^6cells) was aliquoted, washed twice with a phosphate buffered saline and then suspended in 90% ice-cold methanol for fixation. Prior to analysis, the cells were stained using propidium iodide supplemented with RNase then analyzed for DNA content using FACS Calibur (Becton–Dickinson, Rockville,MD, USA). In each assay, 10000 cells were counted. The distribution of G1, S and G2/M phases in each experiment was calculated from each histogram using the CellQuest software (BD Biosciences).

Statistical analysis

In this study, all the tests were performed in triplicate and IC50 for H89 on *L. tropica* was determined by logarithmic regression analysisin Excel program. Values are means ± standard deviation (SD).

Results

H89 inhibits PKA activityin vitro:

Leishmanial cell extracts prepared as described in Materialsand Methods were tested for its activity to phosphorylatethe specific peptide substrate (kemptide). The phosphorylation of the peptide by Leishmanial cellextracts is decreased in a dose-dependent manner in the presence of H89. The extent of the peptide phosphorylationwas assayed usingmicroplate reader at 450nm. At low concentration of H89 (0.1 µM,1 µM) the phosphorylation of the peptide was inhibited up to(81 % and 48.8 %respectively) compared to untreated cell lysates. Higher concentrations (5 µM, 10 µM and 15 µM) inhibit the peptide phosphorylation up to (27.9 %, 12.1 % and 0 % respectively) (Fig. 1).

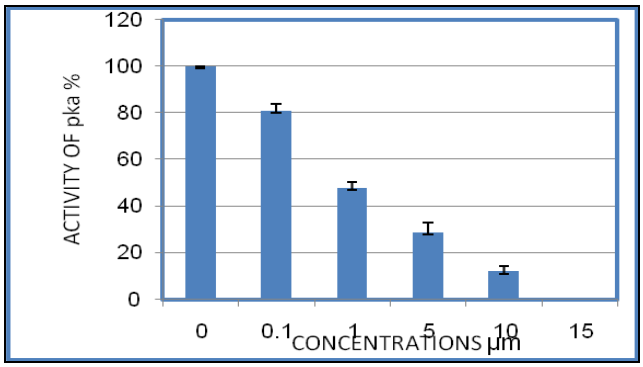

Fig. 1.The effect of PKA inhibitor (H89) on kemptide phosphorylation by treated *L. tropica* lysates. Leishmanial cell lysate was analyzed for PKC activity in the absence of H89 and in the presence 0.1 and 1µM as low concentrations and (5, 10, 15 µM) as high concentrations.

PKA plays important role in viability of *L. tropica*:

Viability test (MTT) was used to investigate the inhibitory effect of the specific PKA inhibitor (H89) on cultured *L. tropica* promastigotes, and to determine the IC50. *L. tropica* promastigotes were treated with increasing concentrations of H89 (10, 12, 14, 16, 18, and 20µM) for 24 h. Afterwards, Promastigote viability was measured by MTT assay. Then, growth percentage of the treated cells was calculated in comparison with untreated control. The viability percentages of treated samples were presented in Fig.2A. Depending on these percentages, IC50 of H89 on *L. tropica* promastigotes was (13.47 µM ±0.07) (Fig. 2B).

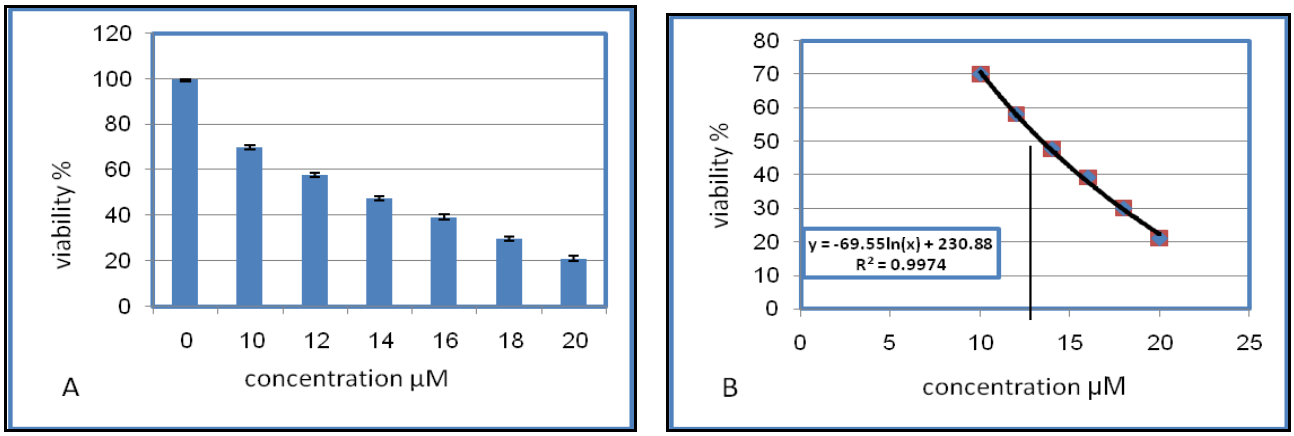

Fig. 2.The effect of inhibitor H89 on viability of *L. tropica* promastigotes (A) percentages of viability after treatment of *L. tropica* with various concentrations of H89 (0, 10, 12, 14, 16, 18 and 20µM). (B) viability curve of treated *L. tropica* by previous concentration and IC50 value.

Effect of H89 on the *Leishmania* promastigotes infectivity

Infection of macrophages derived from human monocytes was used to test the effect of the H89 on *Leishmania* promastigotes infectivity. Promastigotes treated (or not) with H89 (10, IC50, 20µM) were added to the macrophages and the cells incubated for 24h. Results are expressed as the percentage of infected macrophages and the mean number of amastigotes detectable within one infected macrophage (Table 1). Our findings showed decrease in the number of infected macrophages in a dose-dependent manner (Figure 3B-D) compared to macrophages infected with untreated promastigotes (Figure 3A). The greatest decrease was noticed when using 20µM of H89 (table 1. and fig. 3D).

Table 1 showing the median of the percentage of infected macrophages, and the median of the number of amastigotes per 300 macrophage.

Concentration of H89	Infected macrophages	% infection	Amastigotes per infected macrophages	Amastigote per one infected macrophage
0 µM	286	95.3	3203	11.19
10 µM	201	67	1066	5.3
IC50	169	56.3	566	3.34
20 µM	78	26	164	2.1

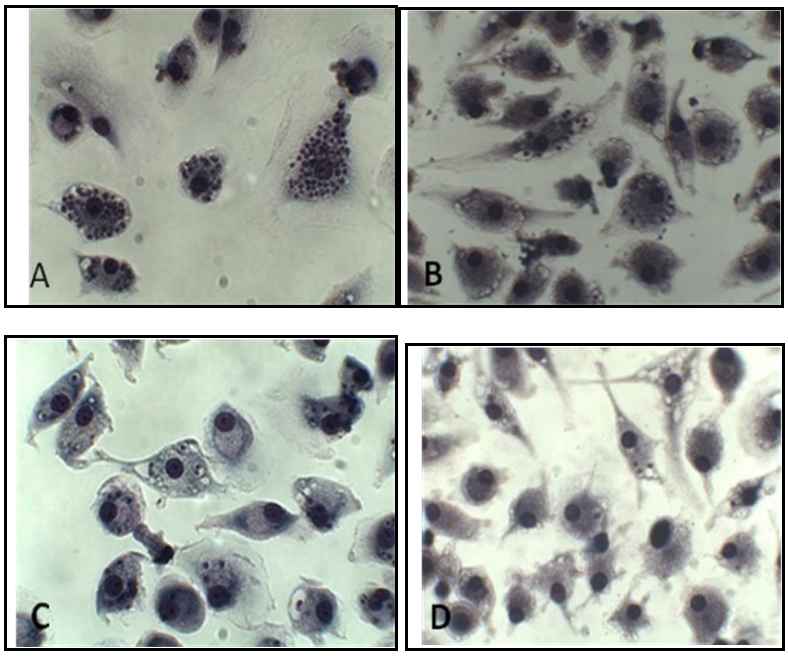

Fig. 3: Microscopic images showing the number of macrophages infected with (10, IC50, and 20µM) H89 treated promastigotes (B, C and D respectively) compared to the number of macrophages infected with untreated promastigotes (A).

Effect of H89 on the process of promastigote to amastigote differentiation

FACS analysis performed by cellular DNA content have demonstreted that morphological transformation of promastigotes to amastigotes by exposure to 37C° and pH 5.5 occurred synchronously, while cells arrested at G1 phase. The percentage of cells arrested in G0/G1 raised to 87% of promastigote population (Fig. 4A). When cells treated by H89 and then exposed to differentiation signal, the precentage of G0/G1 decreased to just 10.3% of promastigote population while the precentage of sub G0/G1 increase to 87.1% (Fig. 4B). These results indicate that H89 inhibited the differentiation process and enhanced the cell death.

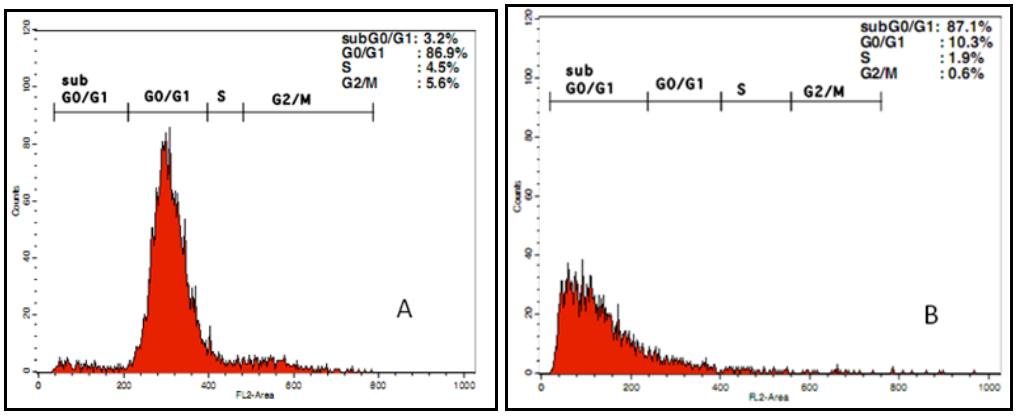

Fig. 4. Flow cytometery analysis during differentiation of promastigote to amastigote. (A) in absence of H89. (B) in presence of H89. 6 h after exposing *L. tropica* promastigotes to differentiation signal (pH 5.5 at 37 C°) amastigotes were stained with propidium iodide and analyzed by flow cytometry. 87 % of untreated cells were arrested at G1 (A) versus 10% of H89 treated cells. In contrast the majority of treated cells is in sub G0/G1 (87 %) versus 3 % of untreated cells.

Discussion:

To test whether kinase A is essential for the viability infectivity and differentiation of *L. tropica* we treated promastigotes with a specific pharmacological inhibitor of PKAc, H89. This inhibitor is frequently used to block signaling pathways in studies of cellular regulation and to enable structure-based drug design of potential therapeutic modulators of signaling pathways [10]. Also H89 is highly specific PKA inhibitor rather

than another inhibitors and has a different effects between Leishmanial species, moreover, H89 is membrane permeable and can be given to live cells [10, 12, 13]. We demonestrated here that PKA activity of L. tropica was inhibited by the treatment with H89, whereas 15µM was enough to total loss of the PKA activity while this activity was decreased to 48.8 % at 1 µM of H89. These results were agreed with previously discriped effect of H89 on PKA activity in lysates of L. major promastigotes [14].

H89 was showed in vitro growth inhibition of L. tropica promastigotes whereas IC50 was 13,47µM. Thus, PKA enzymatic activity is essential for the survival of the parasites. Previous studies were shown similar results on the growth of L. major and L. tropica promastigotes but the concentrations of H89 that have had an effect were considerably higher than that used in our study [12, 14] The contrast between the results obtained by this studies and ours may be due to the methodology used and/or the difference between *Leishmania* species, where the same last study indicated that Growth of promastigotes was measured using the fluorescent viability indicator alamarBlue but this technique is less sensitivity then another methods using tetrazolium salts, such as MTT, which we used [12]. Furthermore, treatment of Plasmodium-infected erythrocytes with the PKA inhibitor H89 blocks parasite growth wheras IC50 was about 3µM also H89 killed 98% of Trypanosoma cruzi epimastigotes at a concentration of 10 during 48h [13, 15].

Our results demonstrated that PKA activity play a role in macrophage infection by metacyclic promastigotes of L. tropica. Parasite treatment with IC50 of PKA inhibitor H89 significantly decreased the percentage of infected macrophages to 56.3% comparison with untreated promastigotes. This is consistent with the results shown by Malki-feldman et al., nevertheless 50nM of H89 was required to reduce macrophages infection at about 40% on day 1 post-infection by L. major [14].

Finally, few previous reports focused on the role of PKA in the differentiation of *Leishmania*. Morphological transformation of L. donovani promastigotes to amastigotes by exposure to 37 ºC and pH 5.5 occurs during cell cycle arrest at G1 phase. Activation of PKA caused a significant G1 phase arrest whereas treatment with PKA inhibitor such DDA, and H89 decreased such arrested condition [16, 17]. In our study we found that about 87% of the logarithmic phase L. tropica promastigotes population reacted to the differentiation signal by synchronizing at G1 within 6 h. While the percentage of cells that arrested at G1 phase after treatment of H89 reduced to11.5%. In contrast that vast majority of these cells accumulated in sub G0-G1 phase. This result indicated the involvement of PKA signaling in G1 arrest of the parasite during differentiation and the inhibition of PKA activity using H89 entered the cells in apoptosis.

Conclusion

The present study underscores the importance of the activity of PKA on *L. tropica* lifecycle. Many studies in animals models have indicated that safe, effective and affordable anti Leishmanial vaccine can be achieved by immunization with defined live attenuated strains of *Leishmania* [5]. Our results suggest that the present inhibitor might be exploited as a target for new drugs against *leishmaniasis*. Furthermore, the promastigotes treated with H89 could be form the basis of a vaccine against *leishmaniasis* consist of alive attenuated strain.

Acknowledgment

We wish to express our gratitude to Dr. S. Al-Okla for their participation in this work. We thank all participants who collaborated in the study, department of animal biology at Damascus university, the atomic energy commission of Syria (AECS) for their kind technical assistance and everyone who help us to accomplish this work.

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