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Effect of different serine protease inhibitors in validating the 115 kDa *Leishmania donovani* secretory serine protease as chemotherapeutic target

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Proteases have been considered as an important group of targets for development of antiprotozoal drugs due to their essential roles in host-parasite interactions, parasite immune evasion, life cycle transition and pathogenesis of parasitic diseases. The development of potent and selective serine protease inhibitors targeting *L. donovani* secretory serine protease (pSP) could pave the way to the discovery of potential antileishmanial drugs. Here, we employed different classical serine protease inhibitors (SPIs), such as aprotinin, N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), N-tosyl-lysine chloromethyl ketone (TLCK), benzamidine (Bza) and pSP-antibody to determine the role of the protease in parasitic survival, growth and infectivity. Among the different classical SPIs, aprotinin appeared to be more potent in arresting *L. donovani* promastigotes growth with significant morphological alterations. Furthermore, aprotinin and anti-pSP treated parasites significantly decreased the intracellular parasites and percentage of infected macrophages. These results suggest that SPIs may reduce the infectivity by targeting the serine protease activity and may prove useful to elucidate defined molecular mechanisms of pSP, as well as for the development of novel antileishmanial drugs in future.

Keywords: *Leishmania donovani*, Serine protease, Serine protease inhibitors, Anti-leishmanial activity.

Visceral leishmaniasis (VL) or kala-azar caused by *Leishmania donovani* is a major health problem worldwide and characterized by defective cell-mediated immunity[1]. An estimated annual incidence of 500,000 new cases of VL with more than 90% of cases are centralized to India, Bangladesh, Nepal, Sudan, and Brazil predominantly among the rural poor people[2,3]. In absence of a suitable vaccine, current treatment is based on chemotherapy that depends on drugs with serious limitations like high cost, toxicity and limited efficacy in endemic areas. The problem is compounded by the emergence of drug-resistance parasite[5]. Hence, there is an urgent need for alternative strategies in drug development[4]. During its life cycle, *Leishmania* triggers expression of many molecules, among which proteases play a crucial role to subvert the host immune response thus escaping host defense[6-8]. A number of metalloproteases and cysteine proteases have been well-documented as virulence factors for their essential roles in modulation of host cell signaling to establish *Leishmania* infection and disease progression[9,10]. But, the roles of serine proteases have not been adequately studied and require further investigation.

We have previously reported the presence of an aprotinin-sensitive secretory serine protease (pSP) of molecular mass 115 kDa in the flagellar pocket region of *L. donovani* promastigotes and amastigotes[11,12]. Flow cytometry and confocal immunofluorescence analysis have revealed that the expression of protease diminishes sequentially from virulent to attenuated strains of this species and is associated with the metacyclic stage of *L. donovani* promastigotes[12]. It seems to play essential roles in the infection process and deactivates the macrophages during the initial interaction between the host and parasite[12]. Moreover, the pSP shows strong proteolytic activity against extracellular matrix proteins, such as collagen and fibronectin[11], suggesting that serine protease of *L. donovani* promastigotes might actively participate in host invasion and infection process.

Hence, it could be speculated from the available data that pSP might be a highly promising potential drug target for the development of inhibitors, as well as to design the effective therapeutic strategies against leishmaniasis. Recent studies have demonstrated that

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Abbreviations: Bza, benzamidine; CP, cysteine protease; PBS, phosphate buffer saline; pSP, purified secretory serine protease; SPIs, serine protease inhibitors; L-TAME, α-N-tosyl-l-arginine methyl ester; TLCK, N-tosyl-lysine chloromethyl ketone; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone; VL, visceral leishmaniasis.

in addition to being potential antiparasitic agents, protease inhibitors can also be used as experimental tools to investigate the functions of the enzymes[13-17]. In the present work, four different serine protease inhibitors (SPIs), as well as pSP antibody have been employed to study the survival, growth and infectivity of *L. donovani* promastigotes to correlate the biological functions of pSP in the pathogenesis of VL.

Materials and Methods

Parasites, host cells and culture conditions

Leishmania donovani strains (MHOM/IN/1983/ AG83) were originally isolated from an Indian kala-azar patient. Promastigotes were cultured at 22°C in medium 199 with Hank's salt containing HEPES, L-glutamine, 20% to 10% fetal calf serum, penicillin and streptomycin at 50 Uml^{-1} and 50 $\mu g\ ml^{-1}$ respectively[18]. Macrophages (RAW264.7 cell line) were grown in RPMI medium containing 10% FCS, 2 mM glutamine and penicillin (1 U/ml)-streptomycin (1 µg/ml) at 37°C in a 5% CO_2 atmosphere.

Raising of antibody

Antibody of *L. donovani* aprotinin-sensitive pSP was raised and purified by protein-A agarose affinity chromatography as described previously[11,12].

Protease inhibition assay

Parasites at a concentration of 1×10^7/ml were incubated with the fresh inhibitors in triplicate at different final concentrations of SPIs (aprotinin at 1 µM, TPCK, TLCK at 100 µM and Bza at 1 mM) or anti-pSP polyclonal antiserum (anti-pSP) at 1: 500 dilutions for 6 h at 22°C and then washed five-times with cold PBS in order to remove the unbound inhibitors. The parasites were again allowed to grow in fresh medium at 22°C for four days. The cultured supernatant was collected following centrifugation at 3,000 *g* for 10 min and the supernatant (40 µg protein) was subjected to gelatin SDS-PAGE. The degrees of digestion related to the amount of enzyme present in various samples were quantified using the Bio-Rad Gel-Doc system.

The effect of inhibitors on the proteolytic activity was also measured spectrophotometrically. The cell-free culture supernatant with the inhibitors and pSP antibody was incubated for 30 min at room temperature. The reaction was initiated with the addition of the substrate TAME (250 µM) in 100 mM Tris-HCl buffer (pH 7.5) at 28°C for 30 min, and the absorbance was monitored at 247 nm. Appropriate blanks were prepared for every enzyme-inhibitor. The activity was considered as 100%, where no inhibitor was present.

Immunoblot assay

Western blots were carried out according to the method as described elsewhere[19] with slight modifications. Briefly, cell-free cultured supernatants of SPIs treated virulent promastigotes were resolved in SDS-PAGE under non-reducing conditions and then transferred to nitrocellulose membranes (0.45 µm). The residual binding sites were blocked by incubation with 5% non-fat dry milk in TBS-Tween (50 mM Tris-saline containing 0.05% Tween 20 at pH 7.5). The blots were incubated for 1 h with anti-115 kDa polyclonal antiserum (anti-pSP) at 1: 500 dilutions in TBS at 22°C; then membranes were rinsed four times in TBS-Tween and incubated in horseradish peroxidase conjugated appropriate secondary antibodies. Then, the membrane was washed four-times with TBS-Tween (15 min each) and developed with 0.2 mM 4-chloro-1-naphthol. β-Actin was used as a loading control to normalize the data. Quantity of expression was analyzed using the Bio-Rad Gel-Doc system using pre-quest software.

Viability assay

We determined the parasite viability in presence of protease inhibitors and anti-pSP using subculture in liquid medium. Briefly, promastigotes (1×10^6/well) were incubated with the inhibitor for 5, 10, 15 and 20 h at 22°C, washed twice with PBS and sub-cultured in fresh medium for 48 h at 22°C. The promastigote cells were counted and their viability was determined by quantitative colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay. Briefly, after the inhibitor incubation, cells were stained with MTT (0.5 mg/ml) for 4 h. The media were then removed. The formazan crystals produced in the wells were dissolved by the addition of 200 µl of dimethyl sulfoxide. The absorbance was monitored at 540 nm using an automatic enzyme-linked immunosorbent assay microplate reader (DTX 800 Multimode Detector, Beckman Coulter). Cell viability was defined relative to untreated control cells [i.e., viability (% control) = 100 × (absorbance of treated samples)/ (absorbance of control)][20]. Tests were performed in triplicate and repeated at least three-times.

Antileishmanial activity of SPIs

The effect of the SPIs (aprotinin, TPCK, TLCK and Bza) and anti-pSP on *L. donovani* promastigotes

were tested following the standard method[21]. Briefly, viable promastigotes harvested in the exponential growth phase were resuspended in fresh medium to achieve the final concentration of 1×10^6/ml. The cell viability was assessed by trypan blue exclusion method[22]. Concentration of all the inhibitors was same as used in enzymatic assay. All the SPIs were filter-sterilized in 0.22 µm membranes (Millipore). The parasites were grown at 22°C in presence or in absence of the protease inhibitors for four days. After sequential incubation of 24, 48, 72 and 96 h, the number of the viable motile promastigotes were estimated daily by counting the parasites in a Neubauer chamber. Alternatively, promastigotes grown for 48 h in the presence of each protease inhibitor were washed five-times in cold PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.2) and resuspended in drug-free fresh medium and allowed to grow for 96 h. In separate experiments, concentration-dependent effect of aprotinin or anti-pSP on promastigote growth was also evaluated. The changes in cellular morphology by the effect of aprotinin were observed under optical microscope[23].

Infection of macrophages with *L. donovani* and treatment with SPIs

To investigate the effect of pSP on the host cell invasion, macrophages (RAW264.7 cell line) were plated in 2.6 cm plastic petridishes containing glass coverslips at a concentration of 1×10^6/ml in RPMI 1640 containing 10% FCS and incubated at 37°C in a humidified 5% CO_2 atmosphere. The cells were left to adhere at 37°C overnight. Non-adherent cells were removed by washing with warm RPMI-1640. Prior to infection, *L. donovani* stationary phase promastigotes were incubated in either medium with or without the SPIs or anti-pSP for 2 h at 27°C and then washed thrice with RPMI-1640. Promastigotes (1×10^7cells/ml) were charged to the macrophages and the cells incubated overnight at 37°C in a CO_2 incubator. Excess parasites were removed by washing with warm medium and the coverslips were further incubated for 72 h at 37°C. Slides were stained with Giemsa (Sigma-aldrich). Percentage (%) of infected macrophages and numbers of parasites per infected macrophage were assessed by Giemsa staining[24,25].

Statistical analysis

All experiments were performed at least three-times and values are given as the mean ± SD. Differences between groups for continuous variables were evaluated with analysis of variance (ANOVA) and differences between two groups were analyzed using unpaired Student's t-test. $P<0.05$ was considered statistically significant.

Results

Effect of SPIs on pSP

To study the role of pSP in *L. donovani*, classical serine protease inhibitors (aprotinin, TPCK, TLCK and Bza) along with anti-pSP were employed to evaluate their inhibitory potentiality on the pSP activity (Fig. 1). Initially, the inhibitory activity of SPIs were performed in the gelatine zymogram study (Fig. 1A), where all the inhibitors (SPIs) caused different extent of inhibition of the extracellular protease at molar concentration range (1 µM, 100 µM, 100 µM and 1 mM for aprotinin, TPCK, TLCK and Bza, respectively) selected on the basis of our earlier work[11]. These results demonstrated that extent of inhibition of the 115 kDa pSP by these SPIs was different (Fig. 1A). Among these SPIs, aprotinin appeared to be more specific as pSP activity was completely blocked by aprotinin in the gel assay. In parallel, pSP antibody at 1:500 dilutions abrogated (~91%) proteolytic activity of pSP of *L. donovani* (Fig. 1A). The protease inhibition assays using TAME as a substrate also substantiated these findings (Table 1). The enzymatic activity of culture supernatants were reduced in treated cells with respect to untreated cells. We observed that aprotinin and anti-pSP effectively inhibited the proteolytic activity of pSP at ~100% and ~91%, respectively. Decrease in pSP activity by Bza was ~72%, while TPCK and TLCK reduced its action by ~52% and ~18% respectively.

To test the effects of SPIs and anti-pSP on pSP of *L. donovani*, the promastigotes were pre-treated with the respective inhibitors and after growing the cells in inhibitor-free medium, the proteolytic activity of pSP in culture supernatant (inhibitor free) was measured by immunoblot experiment (Fig. 1B), where the inhibition of extracellular protease secretion correlated with the decrease in proteolytic activity in zymogram assay (Fig. 1A & B). Aprotinin and pSP antibody was found to be most effective (~100%), since among the inhibitors tested, the culture supernatant of aprotinin and anti-pSP treated cells showed least abundance of pSP protein, as observed in the immunoblot study (Fig. 1B), whereas the treatment of TPCK (~53%), TLCK (~22%) and Bza (~71%) did not completely block the protease secretion in the culture supernatant.

Effect of SPIs on the *L. donovani* cellular growth and viability

As a preliminary study, the effect of SPIs on *L. donovani* was evaluated by microscopic counting

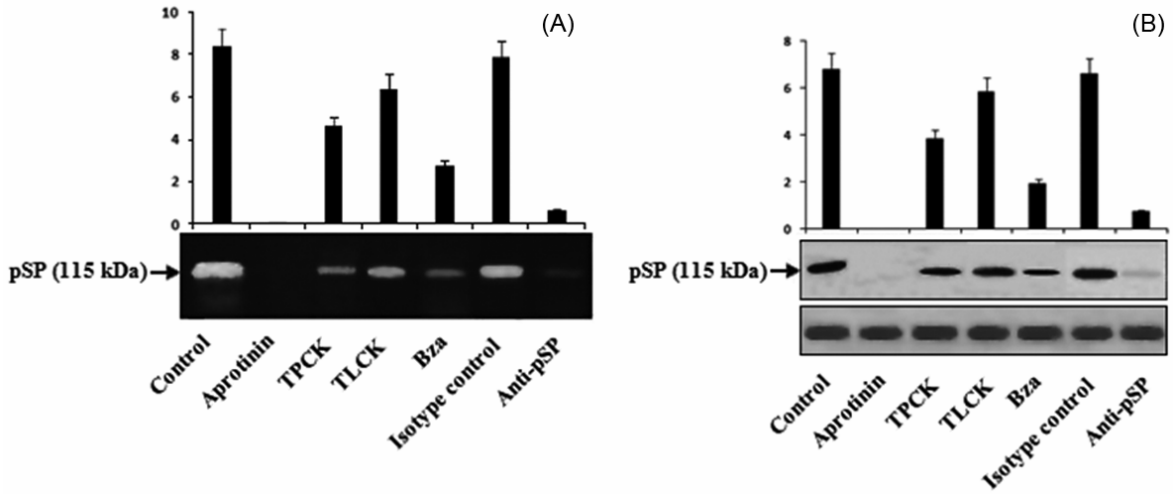

Fig. 1—(A) Effect of serine protease inhibitors (SPIs) or anti-pSP on the secreted 115 kDa serine protease activity (pSP) in living *L. donovani* parasite [The parasites were grown in the absence or in the presence of SPIs or anti-pSP (1:500) for 6 h and then harvested, washed, incubated in fresh inhibitor-free medium for 4 more days and the cultured supernatant was collected, followed by run in gelatine SDS-PAGE. The degrees of digestion related to the amount of enzyme present in various samples were quantified using the Bio-Rad Gel-Doc system and shown at the upper panel; and (B) Effect of SPIs on 115 kDa protein secretion. The 115 kDa protein level of culture supernatant was examined by Western blotting. β-Actin was used as loading control. Semi-quantitative analysis of protease expression by densitometry is shown at the upper panel. The results shown are representative of three individual experiments with similar result performed in triplicate]

Table 1—Assay of proteolytic activity of secreted supernatant of *L. donovani* in presence or absence of inhibitors [Results are mean ± SD (n = 4)]

Culture supernatant	Specific activity (μ mol min^{-1}. mg protein^{-1})
Treated without inhibitors (Control)	46.11 ± 3.08
Treated with aprotinin	1.02 ± 0.18***
Treated with TPCK	26.29 ± 3.01*
Treated with TLCK	36.07 ± 1.35*
Treated with Bza	16.41 ± 1.28**
Isotype control	97.61 ± 1.84
Anti-pSP Ab	4.21 ± 1.76***

p<0.05 **p<0.01 ***p<0.001 compared with control

and MTT assay. There was a time-dependent reduction from 5 to 20 h in parasites viability in presence of the SPIs viz, aprotinin (1 µM), TPCK (100 µM), TLCK (100 µM), Bza (1 mM) and anti-pSP (1:500). Aprotinin as well as pSP antibody exerted a greater growth inhibition response, as compared to TPCK, TLCK or Bza (Fig. 2). The inhibitors employed in this study had also varied effects on the viability of the *L. donovani* infective promastigotes, where aprotinin and pSP antibody caused 10% and 20% reduction of the parasite viability, respectively at 20 h of inhibitor exposure. But, during same exposure time, viable parasites obtained for Bza (~54.3%), TPCK (~49%) and TLCK (~41.5%) seemed less significant (Fig. 2D).

Moreover, TPCK ,TLCK and Bza had a reversible effect on *L. donovani* growth, since parasites that were transferred to a drug-free fresh medium were capable of normal development (Fig. 3A); on the other hand, aprotinin and anti-pSP irreversibly inhibited the growth of *L. donovani* promastigotes as transfer to a drug-free fresh medium did not increase the cell number even after 48 h of cultivation (Fig. 3A). Additionally, we also observed that the strong reduction of *L. donovani* growth induced by aprotinin and anti-pSP was concentration-dependent (Fig. 3B & C). Optical microscopy observations showed loss of flagellum, granulation and round morphology with substantial reduction in size of *L. donovani* promastigote cells. Cellular lysis of promastigote was observed after treatment of the parasites with 1 µM aprotinin for 48 h (Fig. 4). These results suggested that the fate of parasite survival depended to some extent on the metabolic activity of pSP, as treatment with different types of SPIs, especially aprotinin or pSP antibody caused significant cellular damage.

Effect of inhibitors on *L. donovani* infected macrophages

We already found in this study that the SPIs and pSP antibody exhibited strong inhibitory effect on pSP activity and *L. donovani* promastigote propagation. Hence, we investigated the role of pSP on parasite infectivity. Promastigotes were pre-treated

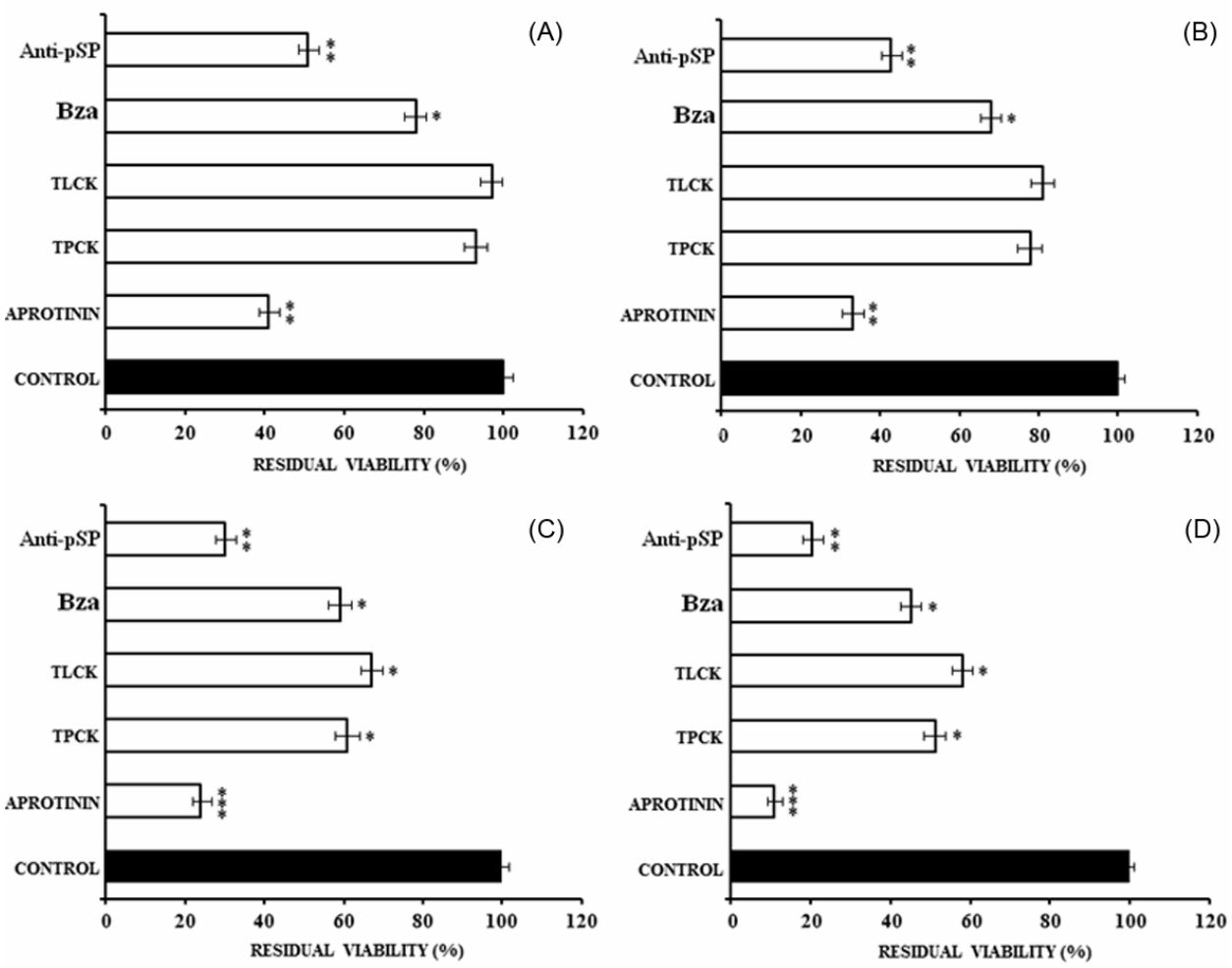

Fig. 2—Assay for the viability of *L. donovani* promastigotes (1×10^6/well) treated with SPIs (Aprotinin, TPCK, TLCK and Bza) or anti-pSP (1:500) [*L. donovani* promastigotes were incubated with or without the SPIs and anti-pSP for 5 h (A), 10 h (B), 15 h (C), or 20 h (D). Values are mean ± SD (n =3, *p<0.05, **p < 0.01, ***p<0.001)]

Table 2—Effect of serine protease inhibitors or anti-pSP on *L. donovani* promastigote infection of macrophages

[Results are representative of three independent experiments and expressed as mean ± SD]

Inhibitors	% Infected macrophage 24 h Post-infection	% Infected macrophage 72 h Post-infection	No of amastigotes/macrophage 48 h Post-infection
Control	22.1 ± 1.1	48 ± 2.3	11 ± 1.7
Aprotinin	3.7 ± 0.8	1.4 ± 0.5**	Not detected
TPCK	18.6 ± 2.4	35 ± 1.8	7.5 ± 1.2
TLCK	19.1 ± 1.2	39.6 ± 2.1	8.6 ± 2.3
Bza	16.3 ± 1.29	29.9 ± 1.9	5.6 ± 1.8
Anti-pSP Ab	11 ± 1.8	6.6 ± 2.4*	2.3 ± 0.7
Isotype control	19.8 ± 3.4	46.2 ± 2.6	ND

*p<0.05, **p<0.01, compared with control [ND: Not done]

with SPIs and pSP antibody prior to infection of host macrophages. The percentage of infected macrophages and the number of amastigotes per infected macrophage were counted microscopically (Table 2). Among the synthetic serine protease inhibitors, marked reduction (~100%) of intracellular amastigotes was observed with aprotinin, followed by TPCK (~32%), TLCK (~22%) and Bza (~49%) after

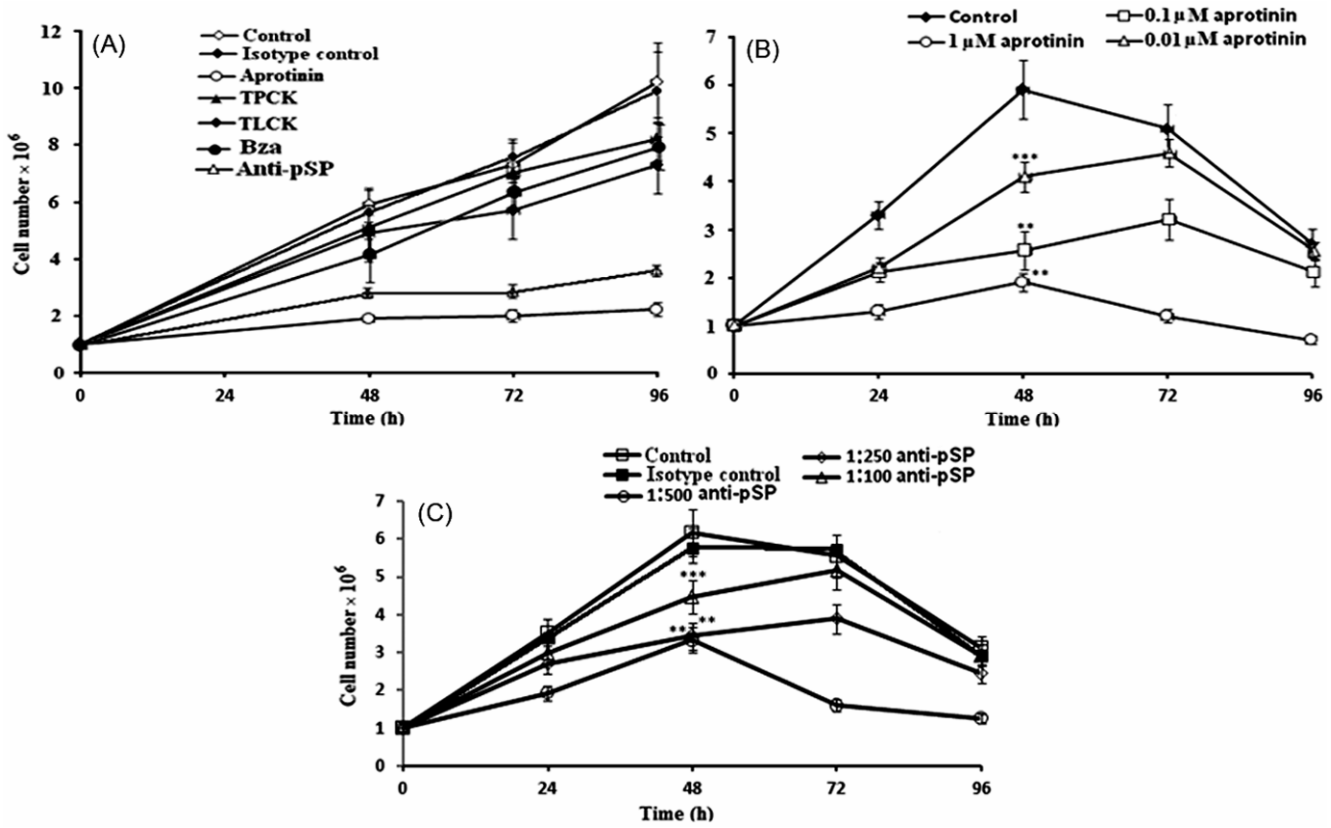

Fig. 3—Effect of different SPIs on the growth of *L. donovani* [(A) Growth pattern of *L. donovani* promastigotes (1×10^6/ml) pre-treated with of SPIs (aprotinin, TPCK, TLCK and Bza); (B) Concentration-dependent effect of aprotinin on *L. donovani* growth rate cultivated for 96 h and C, Effect of anti-pSP with different dilutions on *L. donovani* promastigotes growth. Values are mean ± SD (n = 3). *p<0.05, **p<0.01, ***p<0.001 values are considered as statistically significant with respect to control untreated cells]

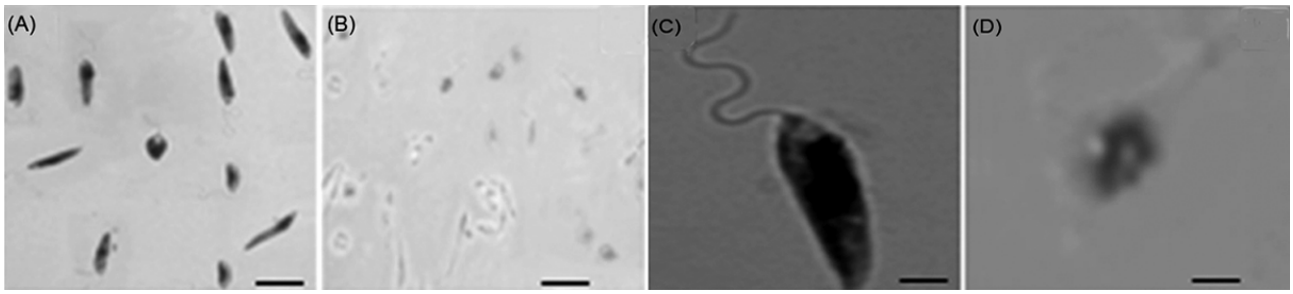

Fig. 4—Effect of aprotinin on *L. donovani* promastigotes morphology observed under phase contrast microscope [(A) Parasites (1×10^6/ml) were cultured without inhibitor (control); (B) parasites were treated with aprotinin at 1 µM of inhibitory concentration for 48 h; (C) Untreated promastigotes; and (D) Aprotinin treated promastigotes, bar = 10 µm]

72 h, compared to control (Table 2). The similar significant reduction was noted with pSP antibody (~79%) in the same experiment, suggesting the role of secreted *L. donovani* serine protease in the infection process. The present experiment also showed the efficacy of inhibitors in the reduction of percentage of infected macrophages as by aprotinin (~97%), anti-pSP (~86%), TPCK (~27%), TLCK (~18%) and Bza (~38%), indicating the possible role of serine proteases in host-parasite interaction.

Discussion

Among the different components characterized as virulence factors, leishmanial proteases are involved in the cell metabolism or in the interaction with the host to assure their survival, proliferation to sustain

the infection[8]. Hence, the selective inhibition of crucial leishmanial proteases is one of the most promising strategies to develop new therapies against leishmaniasis[26]. Unlike metallo and cysteine proteases, there is very little information available concerning *Leishmania* serine proteases in general and particularly for *L. donovani*[8]. An array of *Leishmania* derived proteases have been shown to be clinically important and use of protease inhibitors is relevant in protease targeted therapy[26-28]. Homologous proteases with similar functions can be targeted with a single inhibitor and hence could be used for multiple diseases[29,30]. Therefore, specific protease inhibitor can be employed to regulate protease activity within cell or organisms and at the same time be recommended to understand the biochemical and biological functions of parasite proteases as well. Thus, use of various cysteine and metalloprotease inhibitors makes it possible to comprehend the biological assignment of these two proteases in *Leishmania* pathogenesis e.g., aziridinyl peptides belonging to the family of peptidomimetic cysteine protease (CP) irreversible inhibitors which are active against *Leishmania* CPs[31]. These inhibitors reduce the *in vitro* growth and infectivity of *L. major* promastigotes, modulate cytokine secretion and regulate NO production by infected macrophages[31].

The World Health Organization has classified the leishmaniasis as a major tropical disease. In absence of an effective vaccine against leishmaniasis, chemotherapy is the only effective way to treat all forms of the disease. However, current therapies are inadequate due to serious side effects, cost and drug resistance issue[32]. Hence, there is an urgent need for affordable alternative drugs against leishmaniasis. In this context, application of protease inhibitors to study the relevance of proteases as drug targets and modulators of immune-response is pertinent for protease-targeted treatment[32-36]. Our previous findings of a 115 kDa pSP in Indian strain of *Leishmania donovani* has been found to be localized in the flagellar pocket of promastigotes[11]. But, the actual roles of the *Leishmania* derived pSP in the parasite physiology remains to be elucidated. The present study was undertaken to screen SPIs in order to develop a protease targeted therapy. Principal aim of protease inhibition is of relevance for the development of drugs targeting the proteases for their immense biological importance in parasites' life cycle[37,38]. Here, we demonstrated the role of *L. donovani* pSP on the survival and intracellular transmission of the parasites by means of SPIs and anti-pSP antibody.

Inhibitors of *L. donovani* secretory serine protease were employed to validate prominent role of the pSP in the promastigotes viability and growth in culture. Among the SPIs, aprotinin and anti-pSP were highly effective in reducing promastigote viability and also significantly arresting parasite growth. But, the influence of other SPIs like TPCK, TLCK and Bza were less pronounced on *L. donovani*. However, pretreatment of *L. amazonensis* promastigotes with Bza and TPCK had shown more deleterious effect on parasite viability, whereas the effect of TLCK was less significant[39]. For instance, the TPCK, TLCK and Bza moderately affected viability of *L. donovani* promastigotes, but aprotinin, a Kunitz-type inhibitor and pSP antibody showed pronounced inhibition in a time-dependent manner. Additionally, aprotinin, as well as anti-pSP exhibited prominent effect on the growth of promastigotes and the effect was also found to be irreversible. So, *L. amazonensis* showed difference in inhibitor sensitivity to the classical SPIs (Bza, TPCK and TLCK) rather than to *L. donovani*, where parasite viability could severely be affected by blocking leishmanial serine proteases.

Additionally, *L. donovani* promastigotes underwent morphological alterations with decrease in size, round-shaped cellular bodies devoid of flagellum and granulation after aprotinin treatment. So, effect of aprotinin on the loss of promastigote flagellum indicated that it might be endocyted through the flagellar pocket and interfered with pSP at the flagellar pocket region. Microscopical observation of the lysis of the *L. donovani* promastigotes upon treatment of aprotinin clearly demonstrated that aprotinin caused severe injury to the parasite, but detail mechanisms in these changes await further investigation. These interpretations signified the uniqueness of *L. donovani* pSP to interfere considerably in parasite physiology as well as subsequent infection process and also reinforced the hypothesis that the detrimental effects of SPIs on parasite existence were mainly due to inhibition of *L. donovani* pSP.

Of all the SPIs used, aprotinin, the specific inhibitor of the targeted 115 kDa protease was found to be the most potent one. Pre-treatment of *L.donovani* promastigotes with anti-pSP or aprotinin appreciably decreased the percentage of infected

macrophages and arrested the intracellular parasite growth. Therefore, inhibitory effect of SPIs on cell division of *L. donovani* might indicate the serine proteases-mediated membrane trafficking events in promastigotes and interference with cell proliferation[34,39]. Moreover, exposure to inhibitors or anti-pSP resulted in a remarkable loss of the enzymatic activity of the pSP in promastigotes, which was also corroborated with the findings obtained in the immunoblot analysis of parasite extract.

Importantly, host cell invasion requires several factors, such as parasite motility, formation of a moving junction with the host cell, exocytosis of secretory organelles with alteration of the host cell membrane, and finally the development of the parasitophorous vacuole[40]. Our results suggested that host cell invasion process was affected by these SPIs, probably the moving junction-exocytosis step. In *Toxoplasma gondii* and *Plasmodium falciparum*, the serine proteases serve as potential drug targets due to their role in the processes of egress and invasion at erythrocytic and preerythrocytic stages[41]. In our study, the lack of significant toxicity of SPIs at the concentrations used and their probable access to the site(s) of proteolysis in the intracellular parasites indicated that these inhibitors could be a potentially therapeutic agent. Thus, our data suggested first evidence that parasite proteases are a promising target for chemotherapeutic intervention for visceral leishmaniasis.

Taken together, these results not only provided an important "proof of concept" for the development of SPIs as chemotherapy for a number of disease entities, including leishmaniasis. So, inhibitors provided a useful tool in identifying the target enzyme(s) and unravel its key role in cellular growth, viability and host cell invasion. Thus, serine protease appeared to be crucial for biological functions of *L. donovani* parasite, since its inhibition led to kill the parasites with apparent cellular lysis and reduction in infection rate *in vitro*. However, further research is highly desirable to advance our knowledge about molecular mechanisms of the *L. donovani* secretory serine protease during active *Leishmania* infection and thus could lead to the development of new drug against visceral leishmaniasis.

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