Src and PI3 K inhibitors affect the virulence factors of *Entamoeba histolytica*

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SUMMARY

Protein kinases (PKs) of parasitic protozoa are being evaluated as drug targets. A large number of protein kinases within the protein kinome of *Entamoeba histolytica* strongly suggest that protein phosphorylation is a key component of pathogenesis regulation by this parasite. PI3 K and Src are kinases previously described in this parasite, but their role is poorly understood. Here, the effect of Src-1-inhibitor and PI3 K inhibitor (Wortmannin) on the virulence factors of *E. histolytica* was evaluated. Results show that both inhibitors affect the actin cytoskeleton and the amoebic movement. Also, the proteolytic activity is diminished by Wortmannin, but not by Src-inhibitor-1; however, the phagocytic capacity is diminished by Wortmannin and Src-1-inhibitor. Finally, we found that the virulence *in vivo* of *E. histolytica* is affected by Wortmannin but not by Src-1-inhibitor. This study opens the way for the design of anti-amoebic drugs based on kinase inhibition.

Key words: *Entamoeba histolytica*, PI3 K, Src, virulence factors.

INTRODUCTION

Entamoeba histolytica, the protozoan parasite capable of invading the intestinal mucosa and spreading to other organs, mainly the liver, is a significant source of morbidity and mortality in developing countries (Haque *et al*. 2003; World Health Organization, 2011).

In humans, invasion starts when trophozoites residing in the colon deplete the mucus, interact with vulnerable enterocytes, dismantle cell junctions, and lyse host cells. Once the epithelial layer is disrupted, amoebae cross the basal lamina and the extracellular matrix (ECM); this is a process involving parasite motility and cytotoxicity towards host cells by proteolytic activity and phagocytic capacity (Meza, 2000). Some trophozoites, which reside in the colon, penetrate the portal system and follow the bloodstream to the hepatic portal venule and sinusoids (Tsutsumi *et al*. 1984). The latter are the main structures where amoebae cross the endothelium to reach the parenchyma, with a concomitant initiation of inflammatory foci and abscesses. The classical amoebic liver abscess (ALA) is due to necrotic lysis of the liver tissue, which varies in size from a few centimetres to a large lesion (Que and Reed, 2000; Labruyère and Guillén, 2006; Campos-Rodríguez *et al*. 2009; Santi-Roca *et al*. 2009).

It is not yet clear what factors or signals, in virulent trophozoites, are particularly involved in tissue invasion; therefore, studies of signalling proteins and pathways may help us to understand the processes that may have an impact on the invasive disease. Recently, the protein kinome of *E. histolytica* has been described; however, the role of the identified kinases is unknown on this eukaryotic parasite (Loftus *et al*. 2005; Anamika *et al*. 2008).

Src and PI3 K kinases are two proteins implicated in cell adhesion, motility and invasion (Heinz *et al*. 1999; Thamilselvan *et al*. 2007; Sanchez *et al*. 2010). These processes are essential for *E. histolytica* invasiveness and, interestingly, these two kinases have also been described in this parasite; however, their function is poorly characterized. There is evidence that PI3 K signalling pathways regulate phagocytosis and migration in *E. histolytica* trophozoites (Batista and De Souza, 2004; Blazquez *et al*. 2008). In the case of Src, only its presence has been reported on the protein kinome of *E. histolytica* by *in silico* analysis (Anamika *et al*. 2008). Previous work in our laboratory described the involvement of Src during trophozoite adherence to fibronectin, suggesting a possible role for this protein in the parasitic adhesion process (Flores-Robles *et al*. 2003).

In this work, we decided to study the effect of Wortmannin and Src-1-inhibitor on relevant amoebic virulence factors. Results show that both inhibitors affect amoebic motion by a direct effect on the structure of the actin cytoskeleton. The proteolytic activity was diminished by Wortmannin, but not by Src-inhibitor; however, the phagocytic capacity of the parasite was significantly diminished by

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Wortmannin and Src-inhibitor-1; finally, the *in vivo* virulence of *E. histolytica* was markedly reduced only by Wortmannin.

MATERIALS AND METHODS

Cells

Trophozoites of *E. histolytica* HM1-IMSS strain were axenically cultivated in TYI-S-33 medium (Diamond *et al*. 1978), supplemented with 10% (v/v) bovine serum and 3% (v/v) Diamond vitamin Tween 80 solution (JRH Biosciences) for 48 h in glass screw cap tubes at 37 °C. After that cells were incubated on ice for 10 min, collected by centrifugation at 900 ***g*** for 10 min, and washed 3 times in TYI-S-33 medium without serum. These trophozoites were treated or not with Wortmannin (50 nM) or Src-inhibitor-1 (30 μM) for 2 h, then they were washed with TYI-S-33 medium without serum and cell viability was tested using Trypan blue exclusion test (in all experiments cell viability was >90%). Trophozoites treated or not with inhibitors were used to determine movement, proteolytic activity, phagocytic capacity and the ability to develop ALA in the hamster model for amoebiasis (Tsutsumi and Shibayama, 2006).

Type O human erythrocytes (Rh+) were freshly obtained in Alsever's solutions, and washed 3 times in the same solution to remove white blood cells. The erythrocytes were counted and used in a 1:10 (trophozoites:erythrocytes) ratio in erythrophagocytosis assays.

Confocal microscopy and movement analysis

Trophozoites treated or not with the indicated inhibitors were fixed with freshly prepared 4% formaldehyde, permeabilized with 0·2% Triton X-100, and blocked with 10% BSA. Actin was stained with rhodamine-phalloidin (1:25, Molecular Probes; Oregon, USA) for 30 min at 37 °C. Coverslips were mounted with Vectashield (Vector Laboratories; Ontario, Canada) and actin-polymerized structures were analysed by confocal microscopy in an LSM 700 microscope (Carl Zeiss Micromagin GmbH, Carl Zeiss, Germany). Movement of live trophozoites was evaluated by Leica Kasertechnik GMB microscopy using the track object feature of the Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Maryland, USA). Motion dynamics were measured by making frame-to-frame comparisons.

Substrate gel-electrophoresis

Substrate-gel electrophoresis using Heussen's method was followed as previously described (Heussen and Dowdle, 1980). Total extracts of proteins (10 μg) from both, treated or non-treated trophozoites, were resolved onto 12·5% sodium dodecyl sulfate (SDS)-polyacrylamide gels, co-polymerized with 0·4% porcine-skin gelatin (SIGMA-ALDRICH; St Louis, MO, USA) at 4 °C. Then the gels were washed once in 2·5% Triton X-100 and incubated in the same solution for 2 h, washed twice again in double-distilled water, and incubated for 12–16 h in activation buffer (0·1 M Tris-HCl, pH 7·0) at 37 °C. Gels were stained with Coomassie blue.

Fibronectin purification

Fibronectin (FN) was purified by a modification of the gelatin-Sepharose affinity chromatography method (Ruoslahti *et al*. 1982) from fresh human blood collected in VD vacutainer tubes with 10·8 mg EDTA. Protein purity was monitored in 5% SDS discontinuous polyacrylamide gels. Purified FN was dialysed against 0·15 M NaCl, 0·05 M Tris-HCl, pH 7·4, and stored at −70 °C. Purified plasma FN was quantified using an extinction coefficient of 1·28 at 280 nm.

Fibronectin fibre disruption and degradation assay

Glass coverslips were coated with FN (100 μg/ml) and incubated for 12 h at room temperature under UV light to allow FN to adhere and form fibres. Trophozoites treated or not with inhibitors were allowed to attach and interact with FN-coated coverslips for 15 min. The adhered trophozoites were detached by incubation of coverslips on ice for 10 min; the slides were then washed, blocked with 10% BSA and incubated 1 h with an anti-FN antibody (1:100) at 37 °C, and then the slides were stained with anti-rabbit IgG H&L (FITC) secondary antibody (Invitrogen, NY, USA). Coverslips were mounted with Vectashield (Vector Laboratories). Finally, the slides were analysed by confocal microscopy.

Erythrophagocytosis

After treatment with inhibitors, trophozoites were washed in TYI-S-33 without bovine serum to eliminate drug residues. Then erythrocytes were added, and the interaction was carried out for 15 min at 37 °C in TYI-S-33 without bovine serum using a 1:10 amoeba-erythrocyte ratio. In order to stop erythrophagocytosis, cells were fixed with 2·5% glutaraldehyde and analysed by phase-contrast microscopy.

For a quantitative determination, non-fixed trophozoites were washed with Turk's solution, to eliminate non-ingested erythrocytes. Then the trophozoites were lysed with formic acid and the amount of haemoglobin was measured by spectrophotometric analysis at 400 nm.

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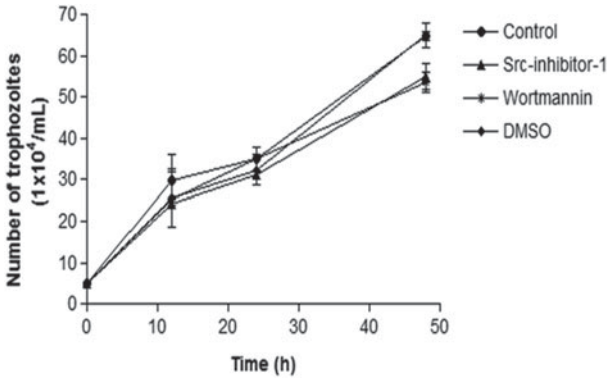

Fig. 1. Effects of Src-inhibitor-1 and Wortmannin on *Entamoeba histolytica* growth. Growth curves for untreated and Src-inhibitor-1 (30 μM) or Wortmannin (50 nM) or DMSO treated *E. histolytica*.

Amoebic liver abscess formation

Male hamsters *(Mesocricetus auratus)* of approximately 80–100 g (3 animals per group) were intrahepatically infected (Tsutsumi *et al*. 1984) with $1{\cdot}5 \times 10^6$ treated or non-treated trophozoites. Seven days p.i., animals were anaesthetized with sodium pentobarbital (94·5 mg/kg of body weight) and killed by exsanguination. Livers were dissected and weighed before and after removing the amoebic abscesses.

Statistical analysis

The significant difference between control and treated cells was statistically analysed by paired Student's *t*-test ($p < 0{\cdot}05$). All statistical analyses were carried out using Stata software version 17.0.

RESULTS

Treatment with Src-inhibitor-1 and Wortmannin did not significantly affect parasite growth but did affect actin cytoskeleton rearrangement and amoebic movement of E. histolytica

In order to elucidate the effects of Src-inhibitor-1 and Wortmannin on parasite viability and growth capacity, non-treated trophozoites or trophozoites pre-treated for 2 h with inhibitors were grown for 12, 24, and 48 h in culture medium. As shown in Fig. 1, there were no statistically significant differences in the growth rate for up to 48 h between trophozoites treated with Src-inhibitor 1 and Wortmannin compared to untreated or DMSO-treated parasites.

Re-organization of the actin cytoskeleton is the primary mechanism of cell motility and PI3 K and Src have been implicated in this process (Platek *et al*. 2004; Li *et al*. 2005). Under normal conditions, *E. histolytica* trophozoites form diverse actin structures such as phagocytic invaginations, adhesion plates, actin dots in association with adhesion plates and stress fibres, pseudopodia, and occasionally the cortical actin belt (Talamás-Rohana and Rios, 2000). Figure 2 shows that in control and DMSO-treated trophozoites, most of these structures are present: a few phagocytic invaginations, adhesion plates, stress fibres and a few actin dots. Whereas in Src-inhibitor-1 treated trophozoites, the absence of several actin structures is evident, some cortical actin filaments, stress fibres, and the actin mesh-like structures remain. Moreover, the presence of spike-like structures can be observed. In the case of Wortmannin-treated trophozoites an accumulation of stress fibres at the zone of the actin ring that forms in dividing cells can be observed, and also the formation of pseudopodia. Other structures such as adhesion plates and phagocytic invaginations are missing (Fig. 2A). After determining affectation of the actin cytoskeleton, we then evaluated the movement with an Image-Pro Plus 5.1 software finding that amoebic movement was significantly reduced by Src-inhibitor and Wortmannin but not by DMSO (Fig. 2B).

Wortmannin but not Src-inhibitor-1 decreases the proteolytic activity of E. histolytica

Another essential virulence factor in *E. histolytica* is the proteolytic activity (Tillack *et al*. 2007). Therefore, we decided to analyse the effect of these drugs on the proteolytic activity. Figure 3A shows that non-treated trophozoites or Src-1-inhibitor treated trophozoites had higher proteolytic activity; however, cells treated with Wortmannin showed a decreased proteolytic activity. To confirm that the inhibition of the proteolytic activity was specific, we tested another unrelated inhibitor, NS398, a specific cyclooxygenase 2 (COX-2) inhibitor dissolved in DMSO, finding that this treatment did not affect the proteolytic activity of the cells (data not shown).

Considering that collagen and FN are two ECM components that are usually degraded by *E. histolytica* trophozoites, we analysed the effect of drug treatment on FN fibre disruption and degradation. As shown in Fig. 3B, regions where cells were attached showed loss of FN fibre staining and black holes, due to degradation of FN by non-treated trophozoites or trophozoites treated with DMSO and Src-inhibitor-1; FN fibre degradation was substantially diminished in the case of Wortmannin-treated parasites. Furthermore, quantification of fluorescence intensity confirmed the degradation of FN, except when the parasites were treated with Wortmannin (data not shown). These results confirm that Wortmannin reduced the capacity of *E. histolytica* to degrade FN.

Src-inhibitor-1 and Wortmannin reduce erythrophagocytosis by E. histolytica

Erythrophagocytosis is considered to be indicative of amoebic virulence (Bhattacharya *et al*. 2002). When

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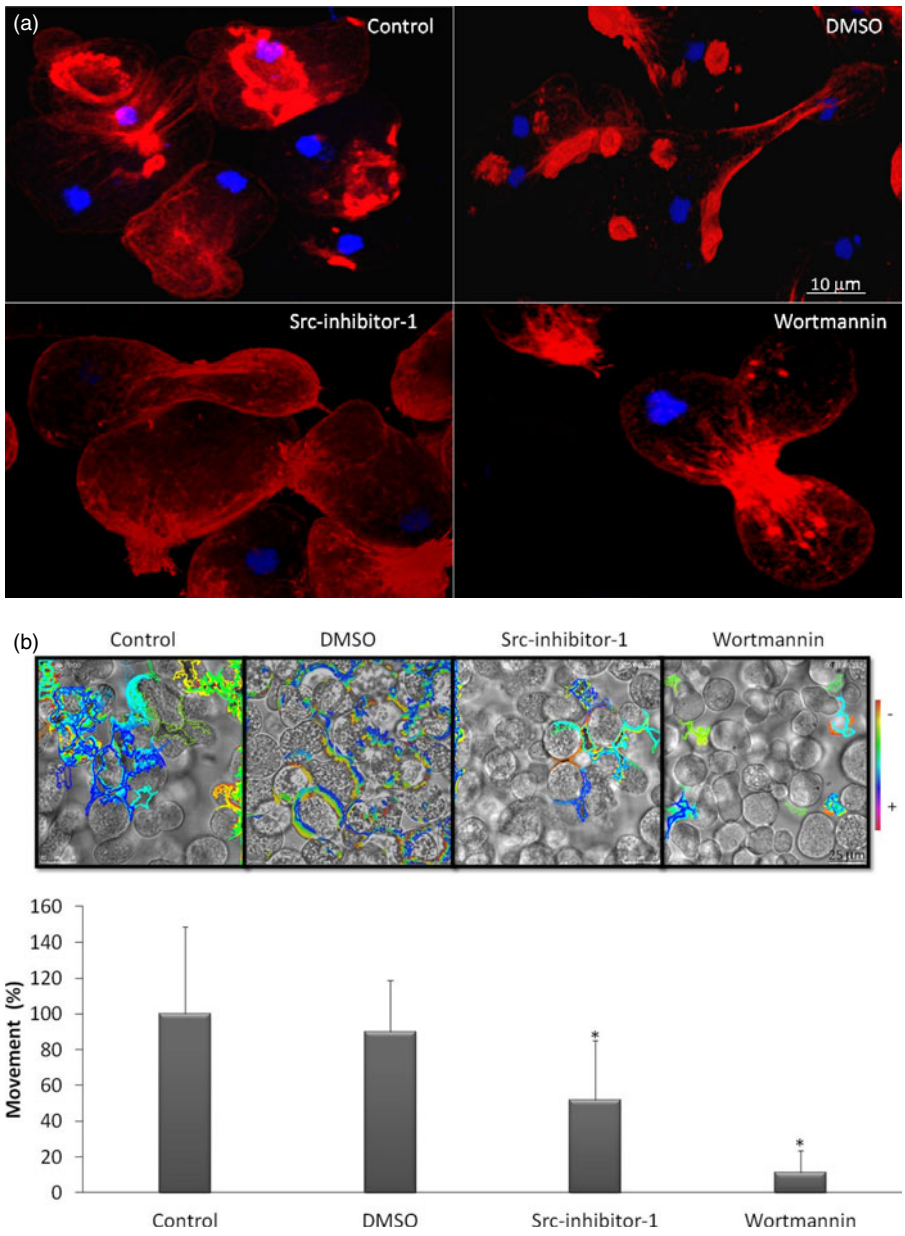

Fig. 2. Src-inhibitor-1 and Wortmannin affect the actin cytoskeleton re-arrangement and amoebic movement of *Entamoeba histolytica*. (A) Trophozoites treated or not with Src-inhibitor-1 (30 μM) or Wortmannin (50 nM) were fixed and stained with rhodamine-phalloidin and DAPI. (B) The movement of live trophozoites treated or not with inhibitors was evaluated as mentioned in the Materials and Methods section; colours for tracks were generated randomly, so every track has a different colour. Results are representative of 3 different experiments and the analysis represents the comparison of 500 frames.

the effect of these inhibitors on the erythrophagocytic capacity was analysed, spectrophotometric analysis of ingested haemoglobin showed that both Src and PI3 K inhibitors reduced the erythrophagocytosis by *E. histolytica* (Fig. 4B). As in the case of proteolytic activity, to confirm that erythrophagocytosis reduction was specific, we treated parasites with NS-398 and showed that this treatment did not affect the level of ingested haemoglobin. Similarly, by phase-contrast microscopy, we demonstrated that human erythrocytes could not be ingested by *E. histolytica* when trophozoites were treated with Src or PI3 K inhibitors, but not with NS-398, instead, erythrocytes accumulated on the surface membrane of Wortmannin or Src-inhibitor-1 treated parasites (Fig. 4A). These results also showed that Wortmannin had a more pronounced effect than Src-inhibitor-1.

Wortmannin but not Src-inhibitor-1 inhibits ALA development in hamsters

The most relevant marker of *E. histolytica* virulence is its capacity to produce liver abscesses in hamsters. Thereby we decided to evaluate the effectiveness of

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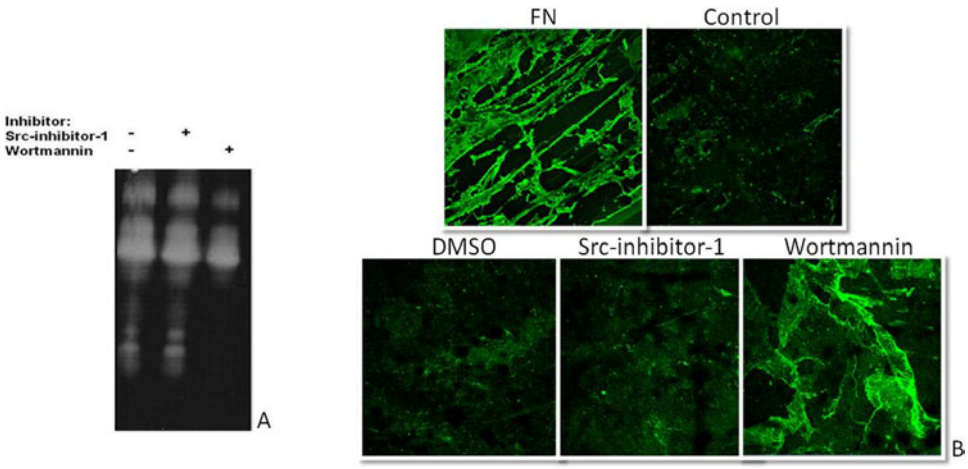

Fig. 3. Wortmannin decreases the proteolytic activity of *Entamoeba histolytica*. (A) Total extract of trophozoites treated (+) or not (-) with Src-inhibitor-1 (30 μM) or Wortmannin (50 nM) were resolved in a 12·5% substrate gel co-polymerized with gelatin. After incubation at 37 °C for 16 h to activate proteases, gels were stained with Coomassie blue. This result is representative of 3 independent experiments. (B) Visualization of degraded FN after 15-min incubation of FN-coated slides with live amoebae, and staining of FN with a polyclonal anti-FN antibody (1:100) and a second FITC-labelled antibody (1:1000).

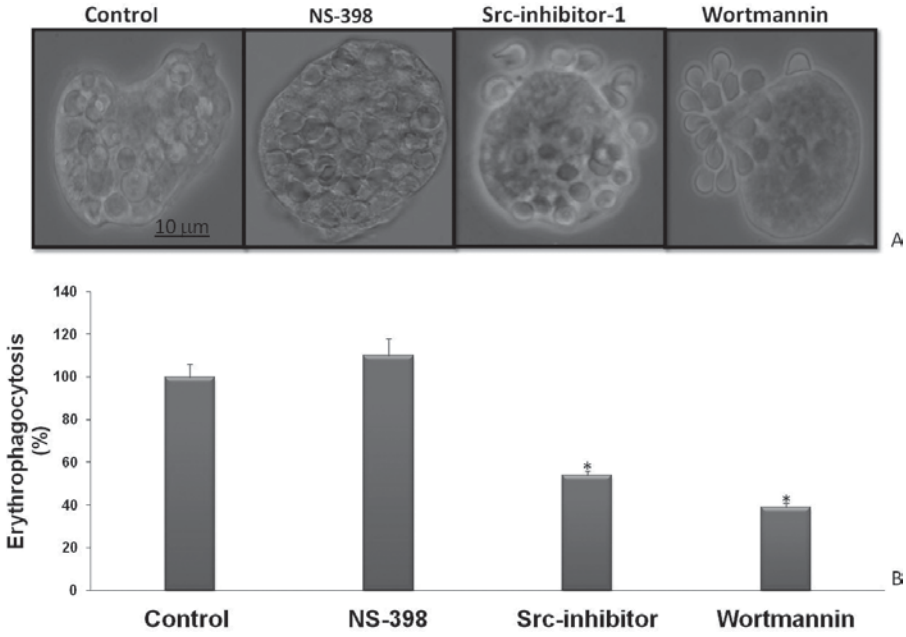

Fig. 4. Src-inhibitor-1 and Wortmannin reduce erythrophagocytosis by *Entamoeba histolytica*. Phase-contrast microscopy revealed that Src-inhibitor-1 and Wortmannin reduce erythrophagocytosis in live trophozoites of *E. histolytica*. (B) Quantitative analysis of ingested haemoglobin by *E. histolytica* was evaluated as mentioned in the Material and Methods section. The graphic shows data of 3 different experiments and represents the average ± S.D. **P* < 0·05.

these kinase inhibitors on amoebic liver abscess development. Results showed that Wortmannin inhibited the amoebic liver abscess formation, whereas, Src-inhibitor-1 only reduced the damage compared with untreated or NS-398 treated trophozoites (Fig. 5). Liver abscesses produced with Src-1-inhibitor treated trophozoites were not as typical as those produced by non-treated amoebae; in the former case, lesions showed a more granulomatous appearance.

DISCUSSION

In this work, we evaluated the effectiveness of Wortmannin and Src-1-inhibitor on amoebic virulence factors. PI3 K is a lipid kinase that produces the intracellular second messengers phosphatidylinositol $(3,4,5)P_3$ and phosphatidylinositol$(3,4)P_2$, which are critical regulators of a wide variety of cellular processes, including cell migration (Vanhaesebroeck *et al*. 2005; Hawkins and Stephens, 2007; Barberis

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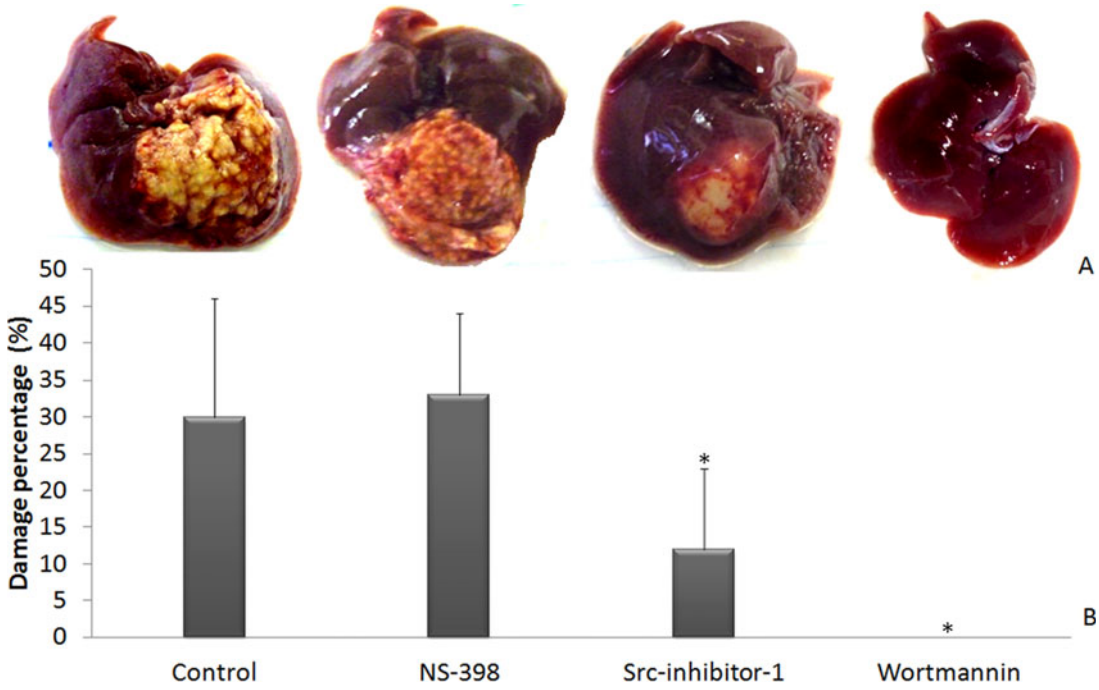

Fig. 5. Wortmannin inhibits ALA development in hamsters. (A) Liver abscess development after 7 days post-infection with live trophozoites of *Entamoeba histolytica* treated or not with Src-inhibitor-1 (30 mM) or Wortmannin (50 nM) or 50 μm NS-398. (B) The graphic shows data of 3 different experiments and represent the average ± S.D. ($n = 9$). *$P < 0{\cdot}05$.

and Hirsch, 2008). Accordingly, we detected that activity was reduced by Wortmannin in live trophozoites; however, when the actin cytoskeleton was visualized by confocal microscopy after phalloidin stain, it seemed that Wortmannin did not affect actin polymerization. These observations are in agreement with previous studies carried out in *E. histolytica*, where Wortmannin-treated parasites were able to produce lamelipodia and to form pseudopods (Batista and De Souza, 2004), but a reduction of amoebic movement was detected, such as occurs in the pancreatic line cell SW 1990 where Wortmannin downregulates cell motility (Teranishi *et al*. 2009).

Src is a tyrosine kinase involved in a variety of biological processes that are associated with cytoskeletal re-organization (Haskell *et al*. 2001; Frame *et al*. 2002). In this work, it has been demonstrated that in *E. histolytica,* Src could be playing a role in amoebic activity and phagocytosis, because their inhibition disturbs re-arrangement of the actin cytoskeleton. This result agrees with another study carried out in rat-1 fibroblasts, where Src caused a profound remodelling of the cortical actin cytoskeleton (Platek *et al*. 2004). Furthermore, Src-knockout fibroblasts exhibit impaired motility and spreading on plastic, which can be restored by transfection with an expression vector for normal, but non kinase-defective Src (Kaplan *et al*. 1995).

Entamoeba histolytica possesses a large number of genes coding for proteolytic enzymes (Tillack *et al*. 2007) that function during tissue invasion by degrading mucus and ECM. Here, we find that the proteolytic activity is reduced by Wortmannin but not by Src-inhibitor-1, this is in accordance with other reports which have demonstrated the role of PI3 K on the regulation of proteases (Ralston and Petri, 2011). FN is one of the major components of the ECM, and *E. histolytica* can degrade this component (Talamás-Rohana and Meza, 1998) during the invasion process. For this reason, we investigated whether Wortmannin could reduce the FN degradation, and our results demonstrated that Wortmannin inhibited FN degradation; this is in agreement with a previous report where Wortmannin blocked FN degradation in human melanotic melanoma cells (Nakahara *et al*. 2003). Proteases have been suggested as attractive potential targets for treatment of amoebiasis; they are necessary for pathogenesis and this work strengthens this feature by the reduction of the *in vivo* virulence through the reduction of proteolytic activity.

The ability of *E. histolytica* to phagocytose red blood cells is in some way related to its pathogenic activity (Bhattacharya *et al*. 2002). It has been demonstrated that PI3 K participates in the erythrophagocytosis process by *E. histolytica* (Ghosh and Samuelson, 1997; Batista and De Souza, 2004), and our results support this idea. In relation to Src, this kinase is found in phagocytic cells and Src-deficient cells are less effective than the wild-type cells in mediating phagocytosis (Hunter *et al*. 1993). This agrees with our results; we found a remarkable reduction of phagocytic capacity when trophozoites

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were treated with Src-inhibitor-1, confirming a role for Src during phagocytosis by *E. histolytica*.

Finally, the virulence of *E. histolytica* is defined by their ability to generate ALA in animal models (Santi-Roca *et al*. 2009). The main animal model used for the study of hepatic human amoebiasis is the hamster because it is a susceptible model and mimics the human disease (Tsutsumi *et al*. 2006). In this study, we evaluated the effect of Src and PI3 K inhibitor treatment of parasites on their capacity to produce amoebic liver abscess. According to our results, Wortmannin inhibits this capacity, and this could be explained by the reduction of motility, and phagocytic and proteolytic capacities. However, Src-1-inhibitor did not prevent *in vivo* virulence. This result could be explained by the fact that Src-1-inhibitor did not diminish the proteolytic activity such as Wortmannin did. Eventually the motility and phagocytic capacities were reduced by Src-1-inhibitor. This could also explain the formation of a non-typical amoebic liver abscess, different from that formed with non-treated trophozoites.

In summary, we have demonstrated that both Src and PI3 K are essential components of critical amoebic functions such as cell movement, erythrophagocytosis and proteolytic activities, and the trophozoites' ability to develop amoebic liver abscesses. Moreover, we have shown that PI3 K is a non-dispensable element in the virulence of *E. histolytica*. Finally, these results provide the basis for the development of new therapies for amoebiasis, based on the inhibition of specific kinases of *E. histolytica*.

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