Pharmacological Assessment Defines Leishmania donovani Casein Kinase 1 as a Drug Target and Reveals Important Functions in Parasite Viability and Intracellular Infection

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Protein kinase inhibitors have emerged as new drugs in various therapeutic areas, including leishmaniasis, an important parasitic disease. Members of the Leishmania casein kinase 1 (CK1) family represent promising therapeutic targets. Leishmania casein kinase 1 isoform 2 (CK1.2) has been identified as an exokinase capable of phosphorylating host proteins, thus exerting a potential immune-suppressive action on infected host cells. Moreover, its inhibition reduces promastigote growth. Despite these important properties, its requirement for intracellular infection and its chemical validation as a therapeutic target in the disease-relevant amastigote stage remain to be established. In this study, we used a multidisciplinary approach combining bioinformatics, biochemical, and pharmacological analyses with a macrophage infection assay to characterize and define Leishmania CK1.2 as a valid drug target. We show that recombinant and transgenic Leishmania CK1.2 (i) can phosphorylate CK1-specific substrates, (ii) is sensitive to temperature, and (iii) is susceptible to CK1-specific inhibitors. CK1.2 is constitutively expressed at both the promastigote insect stage and the vertebrate amastigote stage. We further demonstrated that reduction of CK1 activity by specific inhibitors, such as D4476, blocks promastigote growth, strongly compromises axenic amastigote viability, and decreases the number of intracellular Leishmania donovani and L. amazonensis amastigotes in infected macrophages. These results underline the potential role of CK1 kinases in intracellular survival. The identification of differences in structure and inhibition profiles compared to those of mammalian CK1 kinases opens new opportunities for Leishmania CK1.2 antileishmanial drug development. Our report provides the first chemical validation of Leishmania CK1 protein kinases, required for amastigote intracellular survival, as therapeutic targets.

Leishmaniasis is an important human disease caused by protozoan parasites of the genus Leishmania. This parasite has two developmental stages, an extracellular promastigote form present in the insect vector and an intracellular amastigote form predominantly located inside phagolysosomes of macrophages in mammalian hosts. There are several types of the disease, cutaneous, mucocutaneous, and visceral leishmaniasis, with the last type being the most severe form, as it can affect vital organs (1). Several treatment options are available, and these either show severe side effects (pentavalent antimonials, miltefosine, amphotericin B [AMB]), are unaffordable (liposomal amphotericin B), or lead to toxic effects (pentavalent antimonials, miltefosine, amphotericin B) in the mammalian hosts. There are several types of the disease, cutaneous, mucocutaneous, and visceral leishmaniasis, with the last type being the most severe form, as it can affect vital organs (1). Several treatment options are available, and these either show severe side effects (pentavalent antimonials, miltefosine, amphotericin B [AMB]), are unaffordable (liposomal amphotericin B), or lead to toxic effects (pentavalent antimonials, miltefosine, amphotericin B) in the mammalian hosts.

Protein kinases represent one of the most important groups of drug targets currently in development and are the targets of several Food and Drug Administration (FDA)-approved drugs against cancer (3). Targeting Leishmania through its essential protein kinases is an attractive strategy, which can be broadly applied to diseases caused by unicellular parasites (4). Phosphorylation is indeed essential for almost all cellular processes, and as a result, inhibition of protein kinases should lead to interference with the regulation of key processes in metabolism, proliferation, or differentiation. In Leishmania, different protein kinases have already been identified as potential drug targets, such as mitogen-activated protein kinases (MAPKs) or cdc2-related kinase 3 (CRK3) (5–7). Members of another family of kinases have emerged as potential drug targets, casein kinase 1 (CK1), a family of multi-functional Ser/Thr protein kinases that are characterized by a highly conserved kinase domain and a specific C-terminal domain implicated in its regulation and localization (8). Although CK1 homologs in various protozoa share properties that are similar to those of higher eukaryotes, their functions have not yet been identified (9–14). There are six CK1 isoforms in Leishmania major, LmjF35.1000 (UniProtKB/Swiss-Prot accession number Q9NHE2; LmaCK1.1), LmjF35.1010 (UniProtKB/Swiss-Prot accession number Q9NHE1; LmaCK1.2), LmjF04.1210 (UniProtKB/Swiss-Prot accession number Q96999), LmjF25.1580 (UniProtKB/
Swiss-Prot accession number Q4Q9T3), LmjF27.1780 (GenBank accession number E9ADF5), and LmjF30.3470 (UniProtKB/Swiss-Prot accession number Q4QSU1), for which the biochemical properties and the physiological functions are largely unknown.

According to recent proteomic analyses, the most studied CK1 in Leishmania, CK1.2, is also the most abundant isoform (13, 15–17). This kinase can be shed in the extracellular environment, where it can directly phosphorylate extracellular substrates, such as the C3a polypeptide of the human complement system, or can be exported into infected host cells by exosomal release from intracellular parasites, with potential immunosuppressive effects through the phosphorylation of cellular host proteins, including, for example, human IFNAR1, a receptor essential for alpha/beta interferon (IFN-α/β) signaling (15, 17–19). These data suggest that Leishmania CK1.2 might be a suitable target to identify efficient antiparasitic inhibitors. This kinase displays an unusual ATP binding pocket substantially different from that of mammalian CK1, despite their very similar kinase domains (20), and its inhibition by trisubstituted pyrrole blocks promastigote growth (13). However, many aspects of this protein remain unknown, such as its implication in intracellular amastigote survival, as the only available data on the impact of CK1 inhibition on parasite survival were obtained on cultured promastigotes. Yet, proteomics and phosphoproteomics studies revealed important differences in stage-specific protein expression and protein phosphorylation between promastigotes and amastigotes (21–23). As a consequence, pharmacological data obtained for CK1.2 in promastigotes cannot be directly extrapolated to axenic amastigotes or intracellular parasites.

Here we used D4476 {4-[4-(2,3-dihydro-1,4-benzodioxin-6-yl)-5-(2-pyridyl)-1H-imidazol-2-yl]benzamide}, an established CK1 inhibitor, to investigate the effects of casein kinase 1 inhibition on axenic amastigotes or intracellular parasites.

Two independent experiments were performed in triplicate, and 300 macrophages were analyzed per coverslip. Results were expressed as mean values ± standard deviations.

Ethics statement. All animals were housed in an A3 animal facility in compliance with the guidelines of the A3 animal facilities at the Institut Pasteur, approved by the Comité d’Ethique pour l’Expérimentation Animale (CETEA; approval number 89), as referenced by the Ministry of Research of France. Animal housing conditions and the protocols used in the work described herein were approved by the Direction des Transports et de la Protection du Public, Sous-Directoire de la Protection Sanitaire et de l’Environnement, Police Sanitaire des Animaux, under approval numbers B75-15-27 and B75-15-28, in accordance with the Ethics Charter of Animal Experimentation, which includes appropriate procedures to minimize pain and animal suffering. G.F.S. and E.P. are authorized to perform experiments on vertebrate animals (licenses B75-1159 and 75-1265, respectively, issued by the Direction Départementale de la Protection des Populations de Paris) and are responsible for all the experiments conducted personally or under their supervision, as governed by the laws and regulations relating to the protection of animals.

Bioinformatics. Multiple-sequence alignments (MSA) were computed using the PSI-Coffee mode of the T-Coffee program (29), which uses homology extension against Non Redundant (version 8.91). The re-
sulting alignments were visualized using the Jalview Java alignment editor (30, 31). To determine the conservation of *Leishmania* protein kinases across *Leishmania* species, homologs of all Pfam *Leishmania* major protein kinases were identified by BLAST analysis against a subset containing only eukaryotic proteins, as released by UniProt in September 2011 (32, 33). Only hits aligned to the *L. major* proteins with an E value lower than 10^{-10} were used for the analysis. *L. braziliensis*, *L. infantum*, and *L. mexicana* were included to evaluate the average similarity inside the genus.

**Cell death analysis.** Parasites were diluted in phosphate-buffered saline (PBS) and incubated with 4 μg/μl propidium iodide (Sigma-Aldrich). Cells were analyzed with a FACSCalibur flow cytometer (excitation λ, 488 nm; emission λ, 617 nm; Beckman Coulter). Analysis was performed using the Watson pragmatic model of FlowJo (version 7.6) software (Tree Star, Inc., San Carlos, CA).

**Protein extraction.** Parasites were harvested from logarithmic-phase cultures by centrifugation at 1,500 × g for 10 min. Cells were washed in PBS without CaCl2 and MgCl2, and resuspended in lysis buffer containing 50 mM Tris HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 100 μM MgCl2, 100 U/ml Benzonase, and protease inhibitor cocktail (Complete EDTA-free tablets; Roche Applied Science). Samples were incubated on ice for 30 min and vortexed every 10 min, followed by 5 min sonication (10 x on/20 s off). The lysates were centrifuged for 15 min at 12,000 × g to eliminate cell debris. Supernatants were either used immediately or stored at −80°C.

**Expression and purification of recombinant LmaCK1.2-His6.** The open reading frame of the *Leishmania major* CK1.2 homolog LmjF35.1010 (termed LmaCK1.2) was synthesized by GeneCust and cloned into pBAD-thio-Topo (Invitrogen). pBAD-thio-LmaCK1.2 was also subjected to site-directed mutagenesis to generate a pBAD-thio-LmaCK1.2-K40A mutant using a QuikChange kit (Stratagene) and primers 5′-AGTTCGCTATTTACGCTGAAACAG-3′ and 5′-CTCTGTTTCCAGTGC AATAGCAACT-3′, where the lowercase nucleotides represent the mutated nucleotides. After induction, cells were harvested and resuspended in lysis buffer (60 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM dithiothreitol, 150 mM sodium chloride, 10 mM imidazole) supplemented with protease inhibitor cocktail (Complete EDTA-free tablets; Roche Applied Science). After sonication, Triton X-100 (final concentration, 0.1%) was added and the sample was incubated for 30 min at 4°C (shaking) and centrifuged for 30 min at 4°C. The proteins from the supernatant were purified on a NiNTA-agarose column (Pierce) and eluted in a PBS elution buffer at pH 7.5 (60 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM dithiothreitol) containing 300 mM imidazole. The eluate was supplemented with 15% glycerol and stored at −80°C.

**Protein kinase assay.** Both recombinant LmaCK1.2 and parasite lysates were assayed in buffer C at pH 7.0 (60 mM β-glycerophosphate, 30 mM p-nitrophenyl phosphate, 25 mM MOPS [morpholinepropanesulfonic acid]), 5 mM EGTA, 15 mM MgCl2, 1 mM dithiothreitol, 0.1 mM sodium vanadate) with 15 μM [γ-33P]ATP or [γ-32P]ATP in a 30-μl final volume. We used the following substrates: 27 μM CK-specific peptide substrate CK-S (RRKHAACGSKYHT: synthesized by Proteogenix [Oberhausen, Germany]), 36 μM dephosphorylated casein, 16 μM histone H1, or 6 μg myelin basic protein (MBP). After 30 min of incubation at 30°C, the reaction was stopped by adding 25 μl of the reaction mixture onto Whatman P81 phosphocellulose paper. The filters were washed in 1% phosphoric acid and then assayed in a scintillation counter. Alternatively, for protein substrates, the reaction was stopped by adding an equal volume of 2X electrophoresis loading buffer to the 20-μl reaction mix. Incorporated 32P or 32P was monitored by SDS-PAGE and autoradiography. Native mammalian CK1 isoforms (essentially a mixture of CK1α and CK1ε) were extracted from porcine brain and purified by affinity chromatography on an immobilized axin fragment (34). The kinase activities were expressed as a percentage of the maximal activity, i.e., the activity in the absence of inhibitors. Controls were performed with appropriate dilutions of DMSO.

**Results**

LmjF.35.1010 is a highly conserved casein kinase I homolog. LmjF35.1010 (LmaCK1.2) is 353 amino acids long and has a predicted molecular mass of 39,75 kDa. We found LmaCK1.2 to be more closely related to trypansomatid CK1 (80% identity with Trypanosoma cruzi Tc00.1047053580541.240 and up to 81% identity with T. brucei CK1 Tb927.5.800; data not shown) than to the other *Leishmania* CK1 isoforms (32 to 67% identity; Table 1). LmaCK1.2 is also characterized by its high degree of conservation among *Leishmania* species (L. major, L. infantum, L. donovani, L. mexicana, and L. braziliensis), showing more than 99% identity (Fig. 1A), which is particularly high, given that, on average, orthologous *Leishmania* kinases display only 88% identity (data not shown). LmjF35.1010 lacks only one amino acid compared to the L. infantum (LinJ35.1030) or L. donovani (LdbPKP-351030.1) ortholog and shows three and four amino acid changes compared to the L. mexicana (LmxM34.1010) and L. braziliensis (LbrM34.1000) orthologs, respectively. All these differences are restricted to the last 30 C-terminal amino acids (Fig. 1A). This level of conservation among *Leishmania* species does not apply to all CK1 isoforms but is unique to LmaCK1.2, which, in fact, is the most conserved kinase in *Leishmania* (Fig. 1B, horizontal axis). This genomic stability of LmaCK1.2 is not due to its chromosomal location, as the adjacent CK1 homolog (LmaCK1.1, which is 76% identical to LmaCK1.2) does not show the same degree of sequence preservation (data not shown). This finding indicates that...
TABLE 1 Percent identity between the six Leishmania CK1 family members and six human CK1s

<table>
<thead>
<tr>
<th>Kinase (accession no.)</th>
<th>Catalytic domain % identity to LmaCK1.2</th>
<th>Whole protein % identity to LmaCK1.2</th>
</tr>
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<tr>
<td>LmjF35.1000 (Q9NH2E)</td>
<td>72</td>
<td>67</td>
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<tr>
<td>LmjF30.3470 (Q4Q6E1U)</td>
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<tr>
<td>Lmg10.1y (Q9Y6M4)</td>
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<td>52</td>
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</table>

*The percentages were obtained from multiple-sequence alignment of the catalytic domain or the whole protein and were computed using the PSI-Coffee mode (version 8.91).*

There is a very strong purifying selection against mutations within LmaCK1.2, probably pointing to very important physiological functions of this CK1 family member. This high level of conservation is common to other protozoan parasites. For example, *Plasmodium falciparum* CK1 (PfCK1) is more than 99% homologous to the CK1 orthologs of other *Plasmodium* species, such as *P. berghei* CK1 (PbCK1), *P. knowlesi* CK1 (PkCK1), *P. chabaudi* CK1 (PcCK1), *P. reichenowi* CK1 (PrCK1), and *P. yoelii* (PyCK1), with no more than two amino acid changes (data not shown). The conservation was also high when considering human CK1. LmaCK1.2 was 59% identical to CK1β and CK1ε and 58% identical to CK1α. The percent identity increased up to 71% and 66%, respectively, when restricting the comparison to the catalytic domain. These data are significantly higher than the average percent identity between human and *Leishmania* kinases (37%; Fig. 1B). When comparing the *Leishmania* kinome with the human kinase, LmaCK1.2 is the kinase with the highest similarity to its human ortholog (Fig. 1B, vertical axis). Even though *Leishmania* CK1.2 is highly conserved among eukaryote CK1 (Fig. 1C), its sequence displays some characteristics specific to trypanosomatids, such as a GGA insertion between domains III and IV in the kinase domain (Fig. 1C, bar) (11) or the presence of six amino acid changes identical in all trypanosomatids (Fig. 1C, dots). The biological significance of these insertions or amino acid changes is unknown, though they could offer possibilities for the design of trypanosomatid-specific CK1 inhibitors.

**rLmaCK1.2 is intrinsically active and phosphorylates the CK1-specific substrate CK-S.** To biochemically characterize *Leishmania* CK1.2, a tagged recombinant protein was generated, using the sequence from *L. major*, the only sequence available at the time that this study was initiated. However, because the whole-protein sequence of CK1.2 from *L. major* is more than 99% conserved in CK1.2 from *L. donovani* and 100% conserved if we consider only the kinase domain, we regarded LmaCK1.2 to be equivalent to *L. donovani* CK1.2 (LdCK1.2) for all subsequent experiments. A codon-optimized gene encoding LmaCK1.2 was synthesized in order to improve bacterial expression and cloned into the pBAD-thio-Topo vector. Additionally, a recombinant LmA CK1.2 kinase-dead mutant was generated. Because in earlier reports CK1ε-R38A was described to be kinase dead, we mutated the corresponding lysine 40 in LmaCK1.2 into an alanine (LmaCK1.2-K40A) by site-directed mutagenesis (35). The resulting proteins, tagged at the C terminus with both V5 and His6, were purified from *Escherichia coli* on cobalt resin (Fig. 2Aa and Ab).

We first characterized the purified recombinant LmaCK1.2 (rLmaCK1.2) by an *in vitro* kinase assay using a known CK1 peptide substrate, CK-S (34). Whereas rLmaCK1.2 could phosphorylate CK-S very efficiently, no phosphorylation was detected with rLmaCK1.2-K40A (Fig. 2B). This indicates that CK-S phosphorylation is specifically due to rLmaCK1.2 and not to a bacterial copurified kinase. We next assessed the specificity of rLmaCK1.2 using casein, histone H1, and MBP as the substrates. As shown in Fig. 2C, all three substrates were phosphorylated by rLmaCK1.2 but not by rLmaCK1.2-K40A; casein, as expected, was the best substrate (Fig. 2C). Altogether, these data showed that rLmaCK1.2 is an intrinsically active casein kinase 1.

*Leishmania* faces dramatic environmental changes during the infectious cycle: promastigotes are adapted to 26°C and a neutral pH inside the sand fly vector, while amastigotes reside inside host cell phagolysosomes at 37°C and pH 5.5 (36). As LmaCK1.2 has been previously identified as an exokinase secreted into the host environment, we investigated the sensitivity of rLmaCK1.2 activity to pH and temperature. We tested the phosphorylation of CK-S by rLmaCK1.2 at pH 5.5, 6.5, 7.5, and 8.0. rLmaCK1.2 is inactive at pH 5.5; its activity is first detected at pH 6.5 and steadily increases to attain the maximum of 5.1 pmol of phosphate incorporated/min at pH 8.0 (Fig. 2D). The pH thus has a strong impact on rLmaCK1.2 kinase activity, suggesting that although LmaCK1.2 could be released by the parasite directly into the phagolysosome, it is unlikely that it would phosphorylate substrates inside this compartment. Likewise, rLmaCK1.2 kinase activity is decreased by about 60% at the temperature encountered inside the mammalian host (Fig. 2E). The increase in temperature has an influence on rLmaCK1.2 activity but does not completely inactivate the kinase, suggesting either that in the amastigote the activity of LmaCK1.2 is reduced or that LmaCK1.2 might require the presence of chaperones to maintain its normal activity.

**Recombinant Leishmania CK1.2 is sensitive to known CK1 inhibitors.** Mammalian CK1 isoforms are sensitive to different pharmacological inhibitors, such as D4476 (37), IC261 (38), and CK1-7 (39), whose structures are presented in Fig. S2 in the supplemental material. CK1-7 was the first described CK1 inhibitor and has a 50% inhibitory concentration (IC50) greater than 10 μM against most human CK1 isoforms (39). D4476 is the most specific and potent CK1 inhibitor known to date and has an IC50 in the submicromolar range (37, 40), whereas IC261 specifically inhibits CK1δ and CK1ε with an IC50 of 1 μM (38). We tested the effect of 10 μM of these inhibitors on rLmaCK1.2, using the phosphorylation of peptide substrate CK-S as the readout. At this concentration, CK-S phosphorylation by rLmaCK1.2 was reduced by 97% and 50% using D4476 and CK1-7, respectively, while IC261 had no effect on kinase activity (Fig. 3A). When we tested the effect of these inhibitors on LmaCK1.2 using casein as a substrate, we found that D4476 remained very efficient, while the other inhibitors did not have any effect (Fig. 3B). We noticed that CK1-7 partially inhibited CK1.2 activity toward peptide substrates but not toward protein substrates. Together, these data demonstrate that, like human CK1s, LmaCK1.2 is inhibited by the specific CK1 inhibitor D4476 and is therefore a druggable *Leishmania* kinase.

We next asked whether, despite the high degree of homology of...
their catalytic domains, rLmaCK1.2 and mammalian CK1s could be differentially inhibited. We thus compared the IC50s of selected inhibitors against rLmaCK1.2 and porcine brain CK1 (Sus scrofa CK1 [SsCK1] [34]). We performed kinase assays in the presence of increasing concentrations of inhibitors. For both kinases, we used the amount of protein that corresponded to an activity of 10,000 cpm and we measured the decrease in activity as stated by Reinhart et al. (34). D4476 inhibited rLmaCK1.2 more potently than it inhibited SsCK1, with IC50s of 0.09 ± 0.1 µM and 0.3 ± 0.1 µM, respectively (Fig. 3Ca). CKI-7 (Fig. 3Cb) and IC261 (Fig. 3Cc) were more potent toward SsCK1 (0.47 ± 0.1 µM and 4.8 ± 1.5 µM, respectively) than rLmaCK1.2 (>10 µM for both). The IC50 of IC261 for rLmaCK1.2 was closer to that obtained for Schizosaccharomyces pombe (SpCK1; 1.6 µM) than that obtained for Spcki and Spckiε (1 µM), suggesting that although its protein sequence is more closely related to that of CK1δ or CK1ε, the ATP binding pocket of LmaCK1.2 might be structurally more closely related to that of CK1α [38].

Transgenic CK1.2-V5-His6 is sensitive to D4476. To gain access to the endogenous CK1.2 and determine whether it is expressed in promastigotes as well as in amastigotes, we raised a polyclonal antibody designated SY3535 that recognizes the kinase...
Homo sapiens anti-protein extract was analyzed by Western blotting with SY3535 or control. As shown in Fig. 4A, SY3535 recognized rLmaCK1.2 (a band (Fig. 4B, top). As judged by growth and cell cycle analysis, the extracts from showing the specificity of the SY3535 antibody. With total protein above CK1.2-V5-His6, that was absent in the mock-transfected controls and a higher band between 40 and 55 kDa, corresponding to endogenous CK1.2 present in all the samples. Western blotting with SY3535 antibody were unsuccessful, we cloned CK1.2-V5-His6 into transgenic L. donovani (Fig. 4A, lanes 1, 2, 3). These findings demonstrate that LdCK1.2 is also expressed in axenic amastigotes. Similar results were obtained using anti-HsCK1α (Fig. 4A, lane 3), a band corresponding to the endogenous LdCK1.2 (39 kDa) was detected. Similar results were obtained using anti-HsCK1α (Fig. 4A, lanes 1’ to 3’). These findings demonstrate that LdCK1.2 is also expressed in axenic amastigotes and not only in promastigotes (13, 18).

As our attempts to purify endogenous LdCK1.2 using SY3535 antibody were unsuccessful, we cloned CK1.2-V5-His6 into the leishmanial expression vector pLEXY to overexpress LmaCK1.2-V5-His6 in parasites. Proteins were extracted from transgenic L. donovani promastigotes and axenic amastigotes containing either the pLEXSY or the pLEXSY-CK1.2-V5-His6 vector. Twenty micrograms of total protein extracts was analyzed by Western blotting with SY3535 antibody. We detected one band corresponding to endogenous CK1.2 present in all the samples and a higher band between 40 and 55 kDa, corresponding to CK1.2-V5-His6 that was absent in the mock-transfected controls (Fig. 4B, top). As judged by growth and cell cycle analysis, the overexpression of CK1.2-V5-His6 did not affect parasite proliferation in vitro (data not shown).

To test for activity, CK1.2-V5-His6 was immunoprecipitated from promastigote and amastigote total extracts containing either pLEXSY or pLEXSY-CK1.2-V5-His6 using the anti-V5 antibody. Equal amounts of total extract and purified CK1.2-V5-His6-bound beads were analyzed by Western blotting with the anti-V5 antibody. As presented in Fig. 4C, CK1.2-V5-His6 was detected in both promastigote and axenic amastigote extracts. In the promastigote extract, we could observe an additional faint band at about 40 kDa that was recognized by the anti-V5 antibody but that was absent in transgenic axenic amastigotes or in mock-transfected parasites (Fig. 4C). This protein was also enriched by immunoprecipitation and could correspond to an N-terminal degradation product of CK1.2-V5-His6.

To determine the relative activity of CK1.2-V5-His6, in promastigotes and in axenic amastigotes, we incubated equal amounts of purified beads, corresponding to equal amounts of protein (Fig. 4C) from transgenic or mock-transfected promastigotes and axenic amastigotes at 30 or 37°C for 30 min in the presence of [γ-32P]ATP, 27 μM CK-S. From the mock-transfected controls, we detected a weak kinase activity—higher in promastigotes than in amastigotes—that could use CK-S as a substrate and be inhibited by D4476, suggesting that the agarose beads could directly or indirectly bind endogenous CK1 (Fig. 4D). We detected a higher phosphatransferase activity in promastigotes (2.5 pmol of phosphate incorporated/min) than in axenic amastigotes (1.7 pmol of phosphate incorporated/min). This activity decreased dramatically when the samples were treated with D4476. Similar to the recombinant CK1.2, the transgenic CK1.2 was inhibited by D4476. We also confirmed the influence of temperature on trans-
genic CK1.2 and showed a decrease of activity of transgenic CK1.2 upon a temperature shift from 30°C to 37°C, which could explain the observed drop in activity between promastigotes and amastigotes (see Fig. S1 in the supplemental material).

Endogenous CK1 activity is sensitive to D4476. We took advantage of the CK1-specific peptide substrate and the CK1-specific inhibitor D4476 to assess CK1 activity in total protein extracts; this unique combination allowed us to gain access to endogenous CK1 activity. We first tested whether CK1 inhibitors could prevent the phosphorylation of CK-S by endogenous CK1 activities. Twenty micrograms of total protein extracts was incubated in the presence of 1% DMSO alone or D4476, IC261, and CKI-7 at 10 μM in 1% DMSO. Only the most potent inhibitor of rLmaCK1.2, D4476, prevented CK-S phosphorylation (Fig. 5A). The CK1 activity detected in Leishmania protein extracts showed a sensitivity to D4476, IC261, and CKI-7 comparable to that of the recombinant LmaCK1.2. Because CK1.2 seems to be the main casein kinase 1 expressed in Leishmania (13, 15–17), we could estimate the contribution of CK1.2 to the total kinase activity. We thus performed a kinase assay with total protein extracts as a source of kinases but no substrate other than endogenous substrates. The reactions were performed with or without 10 μM D4476, IC261, or CKI-7. We observed a decrease in the phosphorylation of numerous substrates upon addition of D4476 (Fig. 5B) but not upon addition of IC261 or CKI-7. The level of inhibition of D4476 on endogenous protein phosphorylation was subsequently estimated by quantitative imaging to be about 46% of the total endogenous kinase activity in promastigotes and 58% in amastigotes (Fig. 5B). Neither IC261 nor CKI-7 showed a significant effect. Altogether, these results suggest that CK1 might be one of the major protein kinase families expressed and active in Leishmania at both stages.

FIG 3 LmaCK1.2 is sensitive to known CK1 inhibitors. (A) Kinase assay using 27 μM CK-S and 1 μg recombinant LmaCK1.2 in the presence of either 1% DMSO alone or D4476, IC261, and CKI-7 at 10 μM in 1% DMSO. (B) Kinase assay using 36 μg dephosphorylated casein and 1 μg of recombinant LmCK1.2 in the presence of either DMSO or inhibitors at 10 μM (as described for panel A). (a) Autoradiography; (b) Coomassie staining. (C) Determination of the IC50 of D4476 (a), CKI-7 (b), and IC261 (c) for mammalian CK1 from porcine brain (SsCK1) and recombinant LmaCK1.2. We performed kinase assays using SsCK1 or LmaCK1.2 in the presence of increasing concentrations of the respective CK1 inhibitors.
Inhibition of CK1 is cytostatic in promastigotes and cytotoxic in axenic amastigotes. We have shown thus far that D4476 inhibits recombinant, transgenic, and endogenous CK1.2. To validate this kinase as a therapeutic target, we investigated the effect of its inhibition on parasite viability. Previously, Allocco et al. showed that the LmaCK1.2 inhibitors pyrrole and imidazopyridine strongly reduced promastigote growth (13), but they did not investigate their effect on the viability of amastigotes. We thus assessed the effect of our three CK1 inhibitors on L. donovani promastigotes as well as on axenic amastigotes by measuring the percentage of metabolically active cells using the alamarBlue method (26). D4476, the most efficient CK1 inhibitor, was also the most efficient compound against parasites, with an EC₅₀ of 30 μM on promastigotes and an EC₅₀ of 40 μM on axenic amastigotes (Table 2; see Fig. S3A in the supplemental material). The EC₅₀ of D4476 for promastigotes of L. amazonensis, L. braziliensis, and L. major, determined in triplicate in three independent experiments, were 25 ± 2, 20 ± 3, and 47 ± 6 μM, respectively, suggesting that D4476 has an impact on the causative agents of cutaneous, mucocutaneous, and visceral leishmaniasis. IC261 had a weak effect on promastigotes, with an EC₅₀ of 70 μM, and no effect on axenic amastigotes (EC₅₀ > 100 μM; Table 2; see Fig. S3B in the supplemental material). CK1-7 did not reduce the percentage of metabolically active cells in promastigotes or axenic amastigotes, which is consistent with the absence of inhibition toward the recombinant kinase (EC₅₀ > 100 μM; Table 2; see Fig. S3C in the supplemental material). These results show that targeting CK1 with D4476 has an impact on promastigote and, more importantly, axenic amastigote biosynthetic activity. Each compound showed stage-specific potency, demonstrating the necessity to test com-

FIG 4 Transgenic CK1.2 is sensitive to D4476. (A) One microgram of recombinant LmaCK1.2 (lanes 1 and 1’), 50 μg of promastigote total protein extract (lanes 2 and 2’), and 50 μg of axenic amastigote total protein extract (lanes 3 and 3’) were analyzed by Western blotting using SY3535 (lanes 1 to 3) or an anti-HsCK1 antibody (lanes 1’ to 3’). (B) Twenty micrograms of extracts obtained from transgenic promastigotes or axenic amastigotes expressing either pLEXSY (mock transfection) or pLEXSY-CK1.2 was analyzed by Western blotting using SY3535 (top) and stained by colloidal Coomassie to evaluate the loading (bottom). (C) As described for panel B, total extracts or immunopurified extracts were analyzed by Western blotting using an anti-V5 antibody (top and middle) and stained by colloidal Coomassie to evaluate the loading (bottom). Numbers to the left of the gels in panels B and C are molecular masses (in kilodaltons). (D) Kinase assay using 27 μM CK-S and 10 μl of V5-coated beads purified from transgenic promastigotes or axenic amastigotes expressing either pLEXSY (mock transfection) or pLEXSY-CK1.2 with or without 10 μM D4476 in 1% DMSO. The activities in the reaction mixtures were measured using a scintillation counter.
pounds on amastigotes and not only on promastigotes. To determine whether the effect of D4476 is cytostatic or cytotoxic, we followed the effect of a 1 × EC_{50} dose (40 μM) of D4476 on the equivalent volume of DMSO at different time points during axenic amastigote differentiation. We treated the cells at 0 h, when they were first exposed to environmental changes; at 24 h, when morphological changes occurred; and at 48 h, when axenic amastigotes began to mature and multiply. The untreated cells proliferated for 72 h (Fig. 6Ba, closed circles) and then entered into stationary phase, which correlated with an increase in the percentage of dead cells (Fig. 6Ba, open circles). For all the treated samples, we observed an increase in the percentage of dead cells about 24 h after the treatment was administered. When samples were treated at 0 h, we did not observe an increase in cell density (Fig. 6Bb, closed circles) but observed an elevated percentage of dead cells, which reached up to 80% (Fig. 6Bb, open circles). A similar phenomenon was observed when samples were treated after 24 h or 48 h into the differentiation process (Fig. 6Bc and Bd, respectively, closed circles), but with a percentage of dead cells of only 40% (Fig. 6Bc and Bd, respectively, open circles). This difference suggests that CK1.2 activity could be crucial in the early differentiation of axenic amastigotes but remains essential throughout this process. Altogether, we demonstrated that the effect of the EC_{50} dose is only cytostatic in promastigotes, whereas it is cytotoxic in amastigotes. This confirms that CK1.2 is a promising drug target for antileishmanial treatment.

The effect of D4476 on parasites is mediated by inhibition of the CK1.2 isoform. *Leishmania* CK1.2 is the only isoform that has been identified experimentally, by proteomics, suggesting that it may be the most abundant casein kinase 1 (13, 15–18, 41, 42). Consequently, the effect of D4476 on cells could be mediated mainly through inhibition of CK1.2. To investigate this hypothesis, we used two complementary approaches: a drug competition assay and a biological assay. First, we investigated whether D4476 was sufficient to prevent the binding of CK1.2 to an ATP binding column, as described in the Materials and Methods. In the untreated sample, endogenous CK1.2 was mainly found in the elution fraction, and only a small amount was found in the flowthrough (Fig. 7A, bottom). In contrast, in the competition sample, endogenous CK1.2 was exclusively found in the flowthrough and not in the elution fraction (Fig. 7A, bottom). These data indicate that upon competition with D4476, endogenous CK1.2 no longer binds to the ATP-beads, suggesting that CK1.2 has a high affinity for this inhibitor that cannot be easily displaced by ATP. As judged by the elution profile revealed by SYPRO Ruby staining, we did not observe a difference between the proteins eluted from the

### TABLE 2 EC_{50}s of known CK1 inhibitors on *L. donovani* promastigotes and axenic amastigotes as well as macrophages (when required)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Promastigotes (μM)</th>
<th>Amastigotes (μM)</th>
<th>Macrophages</th>
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<tbody>
<tr>
<td>D4476</td>
<td>30 ± 3</td>
<td>42 ± 3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>IC261</td>
<td>78 ± 7</td>
<td>&gt;100</td>
<td>NA</td>
</tr>
<tr>
<td>CKI-7</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NA</td>
</tr>
</tbody>
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*EC_{50}s were determined in triplicate in three independent experiments, unless indicated otherwise. NA, not applicable.

*EC_{50}s were determined in triplicate in two independent experiments.*
treated or the untreated samples, despite the excess of D4476, suggesting that it has no effect on the most abundant ATP binding proteins (Fig. 7A, top).

We next asked whether even a modest increase in CK1.2 protein levels could lead to an increase in the EC₅₀. We assessed the effect of D4476 on *L. donovani* axenic amastigotes overexpressing or not overexpressing CK1.2-V5-His₆ by measuring the percentage of metabolically active cells using the alamarBlue method (as done for the assay whose results are presented in Table 1) (26). We found EC₅₀ of 31 ± 2 μM for the mock-transfected control and 44 ± 2 μM for transgenic parasites (Fig. 7B). Thus, the overexpression of CK1.2-V5-His₆ led to a modest, but significant, increase in the EC₅₀, which is consistent with the fact that CK1.2-V5-His₆ is weakly expressed compared to the level of expression of endogenous CK1.2 (Fig. 4B). While we cannot completely exclude the possibility that D4476 targets other kinases, these results strengthen the idea that CK1.2 might be the main target.

**D4476 reduces the parasite burden in macrophages.** Compounds that are efficient against cultured parasites are not necessarily efficient against intracellular parasites, as previously reported (43, 44). Moreover, the contribution of CK1 kinases to parasite survival within macrophages is largely unknown. We therefore evaluated the effect of D4476, the most efficient CK1 inhibitor, on intracellular amastigote survival. We did not determine the effect of IC261 and CKI-7 on intracellular parasites, as they were inefficient against axenic amastigotes (Table 2). First, we determined the activity of D4476 on *L. donovani*-infected PEMs. Preliminary experiments were performed to assess compound toxicity on macrophages using the alamarBlue method, and we measured an EC₅₀ close to 100 μM (Table 2). Thus, we chose to treat the infected macrophages at 50 μM D4476, corresponding to half of the macrophage EC₅₀, and asked whether this treatment could efficiently trigger intracellular parasite death. As a positive control, cells were treated with 1 μM amphotericin B (AMB), a reference antileishmanial drug. The treatment was performed for 24 h and 48 h, and the number of infected macrophages was determined microscopically, counting 300 cells in two independent triplicate experiments. As expected, AMB was extremely efficient, as less than 1% of macrophages remained infected after 24 h of treatment. Compared to the results for the vehicle (1% DMSO)-treated control, there was a significant decrease in the number of infected macrophages after treatment with 50 μM D4476, with only 45% of the macrophages remaining infected at 24 h post-treatment and 38% remaining infected at 48 h posttreatment (Fig. 8A). This observation suggests that the inhibition of *Leishmania* CK1 kinases has an impact on intracellular *L. donovani* survival. To confirm these results, we used a high-content cell-based assay to determine the activity of D4476 on intramacrophagic *L. amazonensis* amastigotes (27). In this assay, a complete clearance of fluorescent parasitophorous vacuoles (PVs) reflects the presence of a very active antileishmanial molecule. Additionally, the analysis of macrophage nuclei with Hoechst 33342 allows determina-

![FIG 6 D4476 is cytostatic for *L. donovani* promastigotes and cytotoxic for *L. donovani* axenic amastigotes at the EC₅₀ dose. (A) D4476 is cytostatic for *L. donovani* promastigotes. A total of 10⁵ *L. donovani* promastigotes/ml culture were treated either with DMSO or with 30 μM D4476 to follow cell growth and the percentage of cells that died for 8 days. (B) A total of 10⁶ *L. donovani* promastigotes/ml were transferred at pH 5.5 and 37°C, and then the culture was treated either with DMSO (a) or with 40 μM D4476 at different time points during differentiation: 0 h (b), 24 h (c), and 48 h (d). The percentage of cells that died was analyzed using a FACScalibur flow cytometer.](http://aac.asm.org)
D4476 is thus not toxic to host cells at these concentrations. Noticeably, 1% DMSO had no effect on macrophage viability indexes, PV counts, or amastigote loads, as shown by Aulner et al. (27). We next determined the antileishmanial activity of D4476 on infected macrophages by establishing the ratio between the number of PVs and the number of healthy macrophages (Fig. 8Bb). DMSO-treated samples gave an SSMD* close to 0 (no effect), whereas amphotericin B gave an SSMD* of about −15 (maximum effect). With D4476, we observed a modest but significant effect at 1 μM, with an SSMD* of about −2 and an even stronger effect at 10 μM (SSMD*, about −7; Fig. 8Bb). In Fig. 8C, we show representative images of infected macrophages incubated with DMSO, 1 μM D4476, or 10 μM D4476. In the DMSO-treated control, most of the macrophages were infected, as judged by the presence of numerous PVs stained green by LysoTracker dye. In contrast, infected macrophages treated with D4476 showed a decrease in the number of PVs, especially with D4476 at 10 μM. Altogether, these data demonstrate a significant concentration-dependent decrease in parasite load on macrophages treated with D4476. Moreover, these results are in accordance with those obtained with *L. donovani*-infected macrophages and suggest that D4476 is efficient against intracellular parasites down to a concentration of 10 μM. These findings reveal, through a pharmacological approach, the essential biological functions of CK1 kinases at the amastigote stage in two different species and highlight the potential of this protein kinase family as drug targets for antileishmanial therapy.

**DISCUSSION**

In this study, we have characterized LmjF:35.1010 (*LmaCK1.2*) using bioinformatics, biochemical, and pharmacological approaches. Analyzing the effect of CK1-specific inhibitors on (i) recombinant *LmaCK1.2*, (ii) transgenic *CK1.2*, (iii) endogenous CK1 activity detected in parasite extracts, (iv) free parasites, as well as (v) bona fide intracellular *L. donovani* and *L. amazonensis* amastigotes, we provide here the first evidence that CK1 protein kinases are required for intracellular parasite survival and infectivity and thus represent important drug targets in *Leishmania*.

Although we cannot completely exclude the possibility of an off-target effect, the antileishmanial activity of D4476 seems to be mediated mainly through CK1.2 inhibition for the following reasons. First, D4476 is a specific human CK1 inhibitor that targets different CK1 isoforms in the nanomolar range with a similar efficiency (40, 45). It has initially been identified as an inhibitor of activin receptor-like kinase 5 (ALK5), a member of the type I transforming growth factor β receptor family (46). Subsequent assessment of its specificity on a panel of over 70 protein kinases revealed that D4476 is most potent toward CK1. This compound affects only two other protein kinases, PKD1 and p38α, with inhibitory activity 10- and 30-fold lower than that for CK1, respectively (40). Neither ALK5 nor PKD1 has an ortholog in *Leishmania*, limiting any potential off-target effect of D4476 on p38α. Second, four out of the five other *Leishmania* CK1 isoforms are only weakly related to CK1.2, with major differences observed in their amino acid sequences and significant sequence insertions for two isoforms (data not shown). Consequently, the difference in the structures of their ATP binding pockets suggests that their sensitivity to D4476 could be lower than that of CK1.2. Third, CK1.2 is the only isoform for which expression has been detected in *Leishmania*. This protein kinase has been identified in several
FIG 8 Inhibition of CK1 reduces the parasite burden of the infected macrophages. (A) PEMs were incubated with opsonized L. donovani promastigotes, and after 48 h, 1% DMSO (control), 50 μM D4476 in 1% DMSO, or 1 μM AMB in 1% DMSO was added to the culture. Intracellular parasites were detected by nuclear staining with Hoechst 33342 in paraformaldehyde (PFA)-fixed cells after 24 h or 48 h of contact with the drugs. Three hundred macrophages were analyzed per coverslip, and the experiment was performed twice in triplicate. Results are expressed as percentages of the value for the DMSO control. (B) Bone marrow-derived mouse macrophages were infected with L. amazonensis lesion-derived amastigotes and treated for 3 days with DMSO (final concentration, 1%), amphotericin B (0.5 μM), CHX (180 μM), and D4476 (1 and 10 μM). Results of compound activity are expressed as SSMD*s, calculated using DMSO-treated macrophages as a reference group for macrophage viability (a), and antileishmanial indexes (the ratio between the number of PVs and the number of healthy macrophages) (b). About 10,000 macrophages were analyzed per well, and the SSMD*s for replicate wells (4, 32, 16, and 16 replicate wells for D4476-, DMSO-, amphotericin B-, and cycloheximide-treated macrophage cultures, respectively) are shown in a box-and-whisker plot. *, P ≤ 0.001 comparing the DMSO control to D4476. (C) Representative merged images for DMSO- and D4476-treated infected macrophages are shown. A zoomed view of the DMSO control culture is displayed to illustrate more clearly the PVs (green), the macrophage nuclei (blue), and the amastigotes (red). Bars, 50 μm.
proteomic studies, suggesting that CK1.2 could be the most abundant CK1 and could therefore represent the main CK1 activity detected in *Leishmania* (15–18). Moreover, Allocco et al. have shown that CK1.2 is the only isoform responsible for CK1 activity in promastigotes (13). This finding is in agreement with our data, as each inhibitor that we have tested has the same potency toward recombinant LmaCK1.2, endogenous CK1 kinases, or free parasites. Fourth, the binding of CK1.2 to an immobilized ATP matrix could be abolished by preincubation with an excess of D4476, suggesting a strong affinity of this inhibitor for CK1.2 without visible changes in the general profile of the ATP binding proteins. Finally, increasing even slightly the expression of CK1.2 in transgenic axenic amastigotes led to a small but significant increase in the EC₅₀ obtained for D4476.

Our pharmacological approach revealed stage-specific biological functions of CK1.2. In promastigotes, CK1.2 does not seem to be essential. Indeed, treatment at the EC₅₀ dose merely led to a delay in growth, from which cells recovered within 48 h to reach a cell density higher than that for the control treated with DMSO. Only treatment at twice the EC₅₀ dose has a cytotoxic effect on promastigotes, causing annexin V-independent cell death (47). In contrast, treatment of axenic amastigotes at the EC₅₀ dose is sufficient to observe a dramatic effect on cell survival, as within 24 h of treatment the percentage of cells that died strongly increased. Moreover, if cells are treated within the first 24 h after shifting the temperature and pH, the effect on cell survival is further increased. Altogether, our data suggest that CK1.2 plays a critical role within the first 24 h of differentiation but remains essential throughout differentiation. On the basis of the results published by Barak et al., promastigotes start to transform into amastigote-shaped cells in the first hours after exposure to elevated temperatures, a process that occurs synchronously as the parasites undergo cell cycle arrest at G₁ (48). The acidification of the medium allows the release of the cells from this arrest (48). Several reports have shown the involvement of CK1 in cell cycle progression, suggesting that *Leishmania* CK1.2 could be involved in the regulation of cell cycle arrest in G₁ or its release during the first 24 h following exposure of promastigotes to differentiation signals (49). Eukaryotic CK1s have also been shown to be involved in the stress response following DNA damage or viral infection (50), suggesting that the strong effect of CK1.2 inhibition in the first hours of differentiation could also reflect the requirement for CK1.2 for the parasite to resist the stress caused by pH and temperature shock. In addition, it seems that the results obtained with axenic amastigotes could be extrapolated to intracellular amastigotes, as the inhibition of CK1.2 also impaired intracellular parasite survival.

LmaCK1.2, the only *Leishmania* CK1 identified in intracellular parasites, shows a high degree of identity to human CK1s, which attains 71% for the kinase domain (16). Given the evolutionary distance of *Leishmania* and humans, the question of the biological significance of this remarkable similarity arises. For successful host cell infection, *Leishmania* has evolved numerous survival strategies (i) to secure nutrients by exploiting the host cell metabolic pathways for its own benefit, (ii) to defend itself against host cell cytoplastic activities by interfering with, for example, vesicular trafficking or cytokine signaling, and (iii) to subvert host immunity by modulating host cell cytokine production (51). From recent studies, it has emerged that proteins released by parasites into host cells, either free or in vesicles, could regulate these processes (15, 17, 18, 42). Indeed, parasite-derived exosomes have been shown to modulate the immune system by attenuating the cytokine response and IFN-γ treatment, suggesting that parasite proteins released by exocytosis, which include kinases such as MAPKs and CK1.2, could play a major role in the pathogenesis of leishmaniasis (17, 42). Conceivably, the release of *Leishmania* protein kinases into the host cytoplasm could be a potential and potent mechanism by which the parasite modulates the host cell immune response and metabolism for its own needs. A similar phenomenon has been observed in malaria infection. *P. falciparum*-infected red blood cells show an increase in phosphorylated proteins compared to uninfected ones. The majority of these proteins are of parasite origin, but several are of human origin, suggesting that the released parasite kinases phosphorylate host substrates (52). Likewise, exocytosis of *Leishmania* CK1.2 may regulate essential host cell processes through phosphorylation, which would explain the high level of conservation between parasite CK1.2 and human CK1 kinases due to the evolutionary pressure to recognize host cell substrates (15, 17). This hypothesis is supported by several reports showing that *Leishmania* CK1.2 phosphorylates human proteins such as IFNAR1 and the human complement component C3a (18, 19). Hence, inhibition of parasite CK1.2 could have dramatic effects on *Leishmania* intracellular survival and could contribute to rescue an antileishmanial immune response. Moreover, because these vital external CK1.2 functions rely on the capacity of this kinase to recognize and phosphorylate host proteins, the risk for selecting drug-resistant parasites expressing a mutated CK1.2 is extremely low.

For those reasons, *Leishmania* CK1.2 represents a good drug target, but the high degree of conservation between the parasite and human CK1 isoforms may challenge the identification of parasite-specific CK1 inhibitors with limited side effects on host kinases. On the contrary, our data provide important evidence for specific inhibition. First, comparison of the efficacy of different CK1 inhibitors on mammalian CK1 and LmaCK1.2 revealed that the parasite kinase is more sensitive to D4476. Second, although they were inefficient against recombinant CK1.2, IC261 and CK1-7 are quite potent against mammalian CK1, suggesting that *Leishmania* and mammalian CK1 have significant structural differences that can be discriminated by compounds. Finally, we have previously published the finding that purvalanol B has a higher affinity to *Leishmania* CK1.2 than to the human homolog, demonstrating yet again differences in ATP binding pocket conformation (20). This divergence in affinity could be exploited in the future by high-throughput, target-based screening campaigns for the identification of parasite-specific inhibitory compounds.

In conclusion, we have shown for the first time the effects of CK1 kinase inhibition on axenic and intracellular amastigotes, clearly establishing the essential role of *Leishmania* CK1 in host infection and thus validating members of the CK1 kinase family, which have essential biological functions in the survival of the disease-relevant, intracellular amastigote form, as an important target for antileishmanial drug development.

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