TCP1 γ Subunit Is Indispensable for Growth and Infectivity of *Leishmania donovani*

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ABSTRACT T-complex protein-1 (TCP1) is a ubiquitous group II chaperonin and is known to fold various proteins, such as actin and tubulin. In *Leishmania donovani*, the γ subunit of TCP1 (LdTCP1 γ) has been cloned and characterized. It forms a high-molecular-weight homo-oligomeric complex that performs ATP-dependent protein folding. In the present study, we evaluated the essentiality of the LdTCP1 γ gene. Gene replacement studies indicated that LdTCP1 γ is essential for parasite survival. The LdTCP1 γ single-allele-replacement mutants exhibited slowed growth and decreased infectivity in mouse macrophages compared to the growth and infectivity of the wild-type parasites. Modulation of LdTCP1 γ expression in promastigotes also modulated cell cycle progression. Suramin, an antitrypanosomal drug, not only inhibited the luciferase refolding activity of the recombinant LdTCP1 γ (rLdTCP1 γ) homo-oligomeric complex but also exhibited potential antileishmanial efficacy both *in vitro* and *in vivo*. The interaction of suramin and LdTCP1 γ was further validated by isothermal titration calorimetry. The study suggests LdTCP1 γ as a potential drug target and also provides a framework for the development of a new class of drugs.

KEYWORDS *Leishmania donovani*, chaperonin, T-complex protein 1 gamma subunit, visceral leishmaniasis, TCP1 gamma subunit, drug targets, essential for survival, suramin

Leishmaniasis, a neglected tropical disease, is caused by protozoan *Leishmania* parasites and is transmitted to humans by the bite of sand flies, mainly *Phlebotomus* and *Lutzomyia* species (1). *Leishmania donovani* is the causative organism of visceral leishmaniasis (VL) (also known as kala-azar), the most severe form of leishmaniasis, and if left untreated, can lead to death (<https://www.nddi.org/diseases-projects/leishmaniasis/>). It is estimated that between 50,000 and 90,000 people are newly infected by VL every year (2), mostly in 7 countries, viz. Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan. Furthermore, kala-azar is reemerging in India in a particularly aggressive form (post-kala-azar dermal leishmaniasis [PKDL]) (3).

Leishmania spp. lead a digenetic life cycle, alternating between two hosts, as extracellular promastigotes in the midgut of sand flies and as intracellular amastigotes in macrophages of mammals (4). *Leishmania* infection involves engulfment of promastigotes by macrophages, resulting in the formation of phagolysosomes, where shifts in temperature and pH and other microenvironmental changes cause its differentiation into amastigotes (5).

Antimonials (sodium stibogluconate) were in use to treat VL since 1920 but have now been replaced by other drugs in the Indian subcontinent due to the emergence of resistance (6). Amphotericin B (AMB) is highly efficacious but has restricted use because of its toxicity (7). Liposomal AMB is much less toxic but is prohibitively costly (8). Though miltefosine is the only available oral drug for the treatment of leishmaniasis,

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it has its own problems, such as excessive cost, teratogenicity, and a long half-life leading to relapse (9). Hence, there is an urgent requirement to identify new chemotherapeutic targets and to develop new chemotherapeutic agents against leishmaniasis.

TCP1 (T-complex protein-1), or TRiC (TCP1 ring complex) or CCT (chaperonin-containing TCP1), is a group II chaperonin that typically forms high-molecular-weight hetero-oligomeric protein complexes of two identical rings (10, 11). Each ring consists of eight or nine different (though very closely related) subunits that mediate the ATP-dependent cytosolic folding and assembly of a variety of proteins (12–15). The components of TCP1 were primarily characterized in archaea (thermosome) and the eukaryotic cytosol (CCT) (16–18). Because of its complexity, the chaperonin is able to interact with a large range of diverse substrates, including actin and tubulin (19). Until now, very few reports have been available related to the structure and function of TCP1 complexes of protozoan parasites. In *Plasmodium falciparum*, eight subunits have been identified in the parasite's genome (20, 21) and a high-molecular-weight oligomeric complex has been demonstrated in the parasite's cytosol (22). Knockdown of one subunit (theta) of this complex causes a serious growth defect in the parasites, suggesting its essentiality for the parasite's biology (22).

In *Leishmania* spp., the presence of five subunits (β , γ , δ , η , and θ subunits) of the CCT complex have been reported by proteome and transcriptome analysis (23–25). Among them, only one, the gamma subunit of TCP1 of *L. donovani* (LdTCP1 γ), has been cloned and characterized (26). Functional characterization revealed that recombinant LdTCP1 γ formed high-molecular-weight homo-oligomeric complexes and organized into two back-to-back rings of seven subunits each (27). The recombinant LdTCP1 γ also exhibited ATP-dependent refolding of the denatured luciferase enzyme.

In the present study, we report that LdTCP1 γ is essential for *L. donovani* parasite survival. Single-allele-replacement mutants exhibited significantly reduced growth due to delay in cell cycle progression. These mutants also showed reduced infectivity in murine macrophages. On the other hand, overexpression of LdTCP1 γ in parasites caused an acceleration in cell cycle progression and also increased parasite infectivity in macrophages. Suramin, an FDA-approved trypanocidal drug, inhibited the chaperonin activity of LdTCP1 γ and also exhibited antileishmanial efficacy both *in vitro* and *in vivo*. The data show that LdTCP1 γ is indispensable for parasite survival and provide a pathway to explore it as a potential target for future drug development.

RESULTS

Southern blot analysis of LdTCP1 γ . To define the complexity of the LdTCP1 γ gene in the *Leishmania* genome, *L. donovani* genomic DNA was subjected to Southern blot analysis at high stringency, with the LdTCP1 γ probe resulting in a clear single-copy pattern (Fig. 1). Restriction enzymes EcoRI, XbaI, NdeI, and NheI, which do not cut within the gene, resulted in single bands with sizes of ~11 kb, 13.4 kb, 25.6 kb, and 13 kb, respectively (Fig. 1, lanes 1 to 4). NcoI, which cuts once within the gene sequence, gave two bands of ~5.2 kb and 17.5 kb, respectively (Fig. 1, lane 5). The data clearly suggest that LdTCP1 γ is present as a single-copy gene in the *Leishmania* genome.

Generation of single- and double-allele-replacement mutants. In order to determine the essentiality of the LdTCP1 γ gene in *L. donovani*, we tried to replace both alleles of the LdTCP1 γ gene using classical gene replacement experiments (Fig. S1 in the supplemental material). Since *Leishmania* is considered to be a diploid organism, two successive rounds of gene targeting with two dominant selectable markers were undertaken to inactivate the LdTCP1 γ gene completely from both alleles. This was done by generating replacement cassettes having the hygromycin phosphotransferase (HYG) gene or the neomycin phosphotransferase (NEO) gene as the selection marker in between the 5' and 3' flanking regions of the LdTCP1 γ gene (see Materials and Methods). The replacement cassettes were linearized and transfected into wild-type *L. donovani* promastigotes, leading to the generation of heterozygous parasites (single-allele-replacement mutant parasites, Dd8^{+/–}) in which one copy of the LdTCP1 γ gene

FIG 1 Copy number analysis of the *L. donovani* TCP1γ gene by Southern blotting. Lanes 1 to 5, restriction digests of *L. donovani* genomic DNA with EcoRI, XbaI, NheI, NdeI (noncutters), and NcoI (single cutter), respectively. The blot was probed with the 1,656-bp full-length LdTCP1γ gene probe.

was replaced with the HYG gene. This was followed by the replacement of the second allele with the NEO gene to generate LdTCP1γ homozygous null mutant parasites (Dd8^{-/-}).

The episomal complementation mutant strain (Dd8^{+/-/+}) was prepared by transfecting the pKS-NEO-LdTCP1γ episomal construct into heterozygous mutant parasites (Dd8^{+/-}, HYG). LdTCP1γ gene replacement was confirmed by PCR using genomic DNA from various replacement mutants as templates (Fig. 2). PCR analysis using HYG-specific primers (Table 1) showed an ~1,026-bp band in the case of both single- and double-allele-replacement mutants (Fig. 2, lanes 6 and 7), indicating successful replacement of

FIG 2 Agarose gel stained with ethidium bromide showing PCR products of LdTCP1γ, NEO, and HYG genes using genomic DNA isolated from *L. donovani* cell lines as the template, viz., Dd8^{+/+} (wild-type parasites), Dd8^{+/-} (parasites with LdTCP1γ single-allele replacement with HYG), and Dd8^{-/-} (parasites with TCP1γ double-allele replacement with HYG and NEO). Lane 1, 1-kb-plus DNA ladder; lanes 2 to 4, LdTCP1γ PCR products from Dd8^{+/+}, Dd8^{+/-}, and Dd8^{-/-} cells; lanes 5 to 7, HYG PCR products from Dd8^{+/+}, Dd8^{+/-}, and Dd8^{-/-} cells; lanes 8 to 10, NEO PCR products from Dd8^{+/+}, Dd8^{+/-}, and Dd8^{-/-} cells.

TABLE 1 Sequences of primers used in the development of overexpression/knockout constructs, RT-PCR, and verification of LdTCP1 γ allele replacement

Primer no.	Primer name	Sequence ^a	Restriction enzyme(s)
P1	TCP1 γ -F	5'- ACTAGT ATGAATGGGAGCAACCGGT-3'	SpeI
P2	TCP1 γ -R	5'- ACTAGT CGGCTCTGAGCACCATCGGG-3'	SpeI
P3	5FLK-F	5'-GAGTACACACACCCCTCT-3'	None
P4	5FLK-R	5'-ACGCGCTGGTTA CCACAAT-3'	None
P5	3FLK-F	5'-AGCCGAAACAGCAGGATGAG-3'	None
P6	3FLK-R	5'-TGGACAATACACAGCCAGCAA-3'	None
P7	5FLKS-F	5'- ACTAGT GATGGACAGCGTGTCAGC-3'	SpeI
P8	5FLKX-R	5'- CCCGGGT GTTGATTGCTTGCCCTTC-3'	XmaI
P9	3FLKN-F	5'- CCCGGG AAATTAAAG CTAGC GACGGAATACCTGGGAAG-3'	XmaI and NheI
P10	3FLKX-R	5'- TCTAGAT GCAGCTGTTGTTGTTGC-3'	XbaI
P11	HYGX-F	5'-TAAAT CCCGGG ATGAAAAAGCCTGAATCACC-3'	XmaI
P12	HYGN-R	5'- GCTAGC TATTCTTTGCCCTCGGA-3'	NheI
P13	NEOX-F	5'- CCCGGG ATGGGATCGGCCATTGAA-3'	XmaI
P14	NEON-R	5'- GCTAGC TAGAAGAACTCGTCAAGAAGG-3'	NheI
P15	TCP1 γ RT-F	5'-AATGAGATGGAGCGCAACCTGCATG-3'	None
P16	TCP1 γ RT-R	5'-GAGATCCGTGACCACGAATCACG-3'	None
P17	GAPDH-F	5'-GAAGTACACGGTGGAGGCTG-3'	None
P18	GAPDH-R	5'-CGCTGATCACGACCTTCTC-3'	None

^aRestriction sites are in boldface.

one allele with the HYG gene. Similarly, with NEO-specific primers (Table 1), an amplicon of ~800 bp was obtained in the null mutants only (Fig. 2, lane 10), and no amplification was obtained in the case of the heterozygous mutant parasites (Fig. 2, lane 9). An ~1,626-bp band was observed when LdTCP1 γ -specific primers (Table 1) were used in the case of the wild-type parasites and the heterozygous mutant parasites (Dd8^{+/+}, HYG) (Fig. 2, lanes 2 and 3). No amplification of LdTCP1 γ was obtained in the null mutant parasites (Fig. 2B, lane 4), which further confirmed the replacement of both alleles of LdTCP1 γ with HYG and NEO, respectively.

Differential expression of LdTCP1 γ , due to either replacement of a single allele or episomal complementation of the LdTCP1 γ gene, was compared both at the transcriptional (Fig. 3A) and the translational level (Fig. 3B) in various genetically manipulated mutants, viz., the vector control transfectants (Dd8Vc), LdTCP1 γ -overexpressing transfectants (Dd8^{+/+/+}), single-allele-replacement mutants (Dd8^{+/+}), and episomal gene complementation mutants (Dd8^{+/+/+}). As expected, LdTCP1 γ -overexpressing transfectants (Dd8^{+/+/+}) exhibited upregulated expression (2.22-fold) of LdTCP1 γ gene transcripts compared to the gene's expression in wild-type (Dd8^{+/+}) cells (Fig. 3A). Dd8Vc (vector control) parasites showed no significant difference in LdTCP1 γ expression compared to that of wild-type parasites. The gene expression was 2.63-fold downregulated in the case of single-allele-replacement mutants (Dd8^{+/+}). Complementation mutants (Dd8^{+/+/+}) displayed expression of LdTCP1 γ comparable to that of the wild-type parasites (Dd8^{+/+}), thus confirming the regaining of LdTCP1 γ mRNA expression. Similarly, at the protein level (Fig. 3B and C), LdTCP1 γ overexpression transfectants (Dd8^{+/+/+}) exhibited a 1.86-fold increase and the single-allele-replacement mutants showed a 1.6-fold decrease in LdTCP1 γ protein expression compared to its expression in wild-type parasites. However, the vector control (Dd8Vc) and complementation mutant (Dd8^{+/+/+}) parasites showed LdTCP1 γ protein expression comparable to that of wild-type parasites. This further confirmed that LdTCP1 γ expression both at the mRNA and protein level was decreased in single-allele-replacement mutants and increased in overexpression transfectants. The double-allele-replacement mutants (Dd8^{-/-}) failed to survive beyond 20 days of selection under *in vitro* culture conditions even after several attempts of transfection. The observation confirmed the essentiality of the gene for parasite survival. As the Dd8^{-/-} parasites failed to survive, further experiments were carried out with the single-allele-replacement mutants (Dd8^{+/+}).

Effect of differential expression of LdTCP1 γ on promastigote multiplication.

The growth rates of genetically manipulated parasites were compared with that of the wild-type parasites (Fig. 4). LdTCP1 γ overexpression did not have any effect on pro-

FIG 3 RT-PCR and Western blot analysis. (A) Differences in LdTCP1 γ mRNA expression in wild-type (Dd8 $^{+/+}$), vector control (Dd8Vc), overexpression transfectant (Dd8 $^{+/+/+}$), single-knockout (Dd8 $^{+/-}$), and complementation mutant (Dd8 $^{+/-/+}$) promastigotes. (B) Changes in LdTCP1 γ protein expression in wild-type (Dd8 $^{+/+}$), vector control (Dd8Vc), overexpression transfectant (Dd8 $^{+/+/+}$), single-knockout (Dd8 $^{+/-}$), and complementation mutant (Dd8 $^{+/-/+}$) parasites following anti-LdTCP1 γ antibody treatment. GAPDH and α -tubulin were used as controls for normalization in RT-PCR and Western blot analysis, respectively. (C) Quantitative estimation of LdTCP1 γ protein expression in cells by measuring the relative density of each band. The data are representative of the results of three independent experiments. Comparisons were made by one-way ANOVA and *post hoc* Tukey test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Error bars represent standard deviations (SD). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

mastigote multiplication, while single-allele replacement resulted in a great reduction in the multiplication rate of heterozygous mutant parasites (Dd8 $^{+/-}$) compared to that of wild-type Dd8 $^{+/+}$ promastigotes (Fig. 4). The complementation mutants (Dd8 $^{+/-/+}$) regained normal growth.

FIG 4 Effects of modulation of expression of LdTCP1 γ on growth of Dd8 $^{+/+}$, Dd8Vc, Dd8 $^{+/+/+}$, Dd8 $^{+/-}$, and Dd8 $^{+/-/+}$ promastigotes. The data are mean values $\pm$ SD from three independent experiments.

FIG 5 Effects of modulation of expression of LdTCP1 γ on infectivity of parasites (number of infected macrophages per 100 cell nuclei). The data are the mean values $\pm$ SD from three independent experiments. Comparisons were made by one-way ANOVA and *post hoc* Tukey test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

Change in expression of LdTCP1 γ modulates promastigote infectivity and amastigote multiplication. Next, we assessed whether genetic deficiency of LdTCP1 γ or its episomal complementation in *L. donovani* had any impact on the parasite's ability to infect murine macrophage cells (Fig. 5). Interestingly, after 24 h postinfection, overexpressing parasites (Dd8^{+/+/+}) were able to infect 60.203% $\pm$ 2.34% (mean $\pm$ SD) ($P < 0.001$) of mammalian macrophages, while vector control parasites (Dd8Vc) exhibited 40.06% $\pm$ 1.67% infected macrophages (Fig. 5). On the other hand, single-allele-replacement mutants could infect only 20.08% $\pm$ 4.8% ($P < 0.001$) of macrophages, compared to 41.42% $\pm$ 1.1% infection caused by wild-type Dd8^{+/+} parasites (Fig. 5). Thus, single-allele-replacement mutant parasites exhibited significantly reduced infectivity. The complementation mutant parasite (Dd8^{+/-/+}) showed infectivity comparable to that of the wild type.

Parasite loads (number of amastigotes per macrophage cell nucleus) were also evaluated at 6 h, 24 h, 48 h, and 72 h postinfection (Fig. 6). At 24 h, 48 h, and 72 h postinfection, amastigote multiplication of single-allele-replacement mutant parasites (Dd8^{+/-}) was reduced 3- to 4.5-fold compared to the multiplication of wild-type Dd8^{+/+} amastigotes (Fig. 6), while overexpression mutants exhibited 1.5- to 1.84-fold increased proliferation compared to that of wild-type amastigotes (Fig. 6). However, no significant difference was obtained at 6 h postinfection (Fig. 6). In the complementation mutant (Dd8^{+/-/+}), the amastigote numbers were restored to levels comparable to that of the wild-type (Fig. 6). Thus, these observations suggest that the loss of the LdTCP1 γ gene affects not only the ability of the parasites to infect but also their multiplication within murine macrophages.

FIG 6 Effects of modulation of expression of LdTCP1 γ on amastigote multiplication (number of intracellular amastigotes per cell nucleus after 6 h, 24 h, 48 h, and 72 h of infection). The data are representative of the results of two independent experiments (in triplicate). Comparisons were made by one-way ANOVA and *post hoc* Tukey test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

Cell cycle is affected by modulation of LdTCP1 γ expression. Flow cytometry analysis was performed to study whether the slow growth kinetics of single-allele-replacement mutants was due to a defect in the cell cycle. The data in Fig. 7 show the comparative cell cycle progression of Dd8^{+/+}, Dd8^{+/-}, Dd8^{+/-/+}, and Dd8^{+/+/+} promastigotes after arresting growth at the G₁/S boundary using hydroxyurea-mediated synchronization. Cells were harvested at 0 h, 6 h, and 8 h postsynchronization (removal of hydroxyurea) and analyzed for their DNA content using flow cytometry. The experiment was done thrice, and histograms from one representative experiment are shown in Fig. 7A. At 0 h postsynchronization, most of the Dd8^{+/+}, Dd8^{+/-}, Dd8^{+/-/+}, and Dd8^{+/+/+} promastigotes were in the G₀/G₁ phase of the cell cycle, indicating an efficient synchronization process (Fig. 7A and B). Single-allele-replacement mutants (Dd8^{+/-}) moved to the S phase very slowly, as detected after 6 h and 8 h of hydroxyurea removal (Fig. 7B). At 6 h, Dd8^{+/-} parasites showed an increased G₀/G₁ population of cells (60.6% $\pm$ 9.1%) compared to the populations of wild-type parasites (40.34% $\pm$ 1.15%) and complementation mutants (Fig. 7B). After 8 h of synchronization, 34.87% $\pm$ 2.67% of wild-type cells (Dd8^{+/+}) entered G₂/M phase, while in the case of single-allele-replacement mutants (Dd8^{+/-}), only 19.1% $\pm$ 3.96% of cells entered S phase and 55.7% $\pm$ 9.8% of cells accumulated in the G₀/G₁ phase (Fig. 7B). Interestingly complementation mutant cells (Dd8^{+/-/+}) exhibited a comparable cell cycle pattern. So, it may be possible that the S-phase block in the single-allele-replacement mutants is directly or indirectly responsible for the slow growth kinetics of these mutants. Interestingly, by this time point, overexpression transfectants (Dd8^{+/+/+}) had already passed through the G₂/M phase (51.6% $\pm$ 10.01% of cells at 6 h) and entered the G₀/G₁ phase again (40.6% $\pm$ 4.1% of cells at 8 h) (Fig. 7B). This suggested that LdTCP1 γ may have an important role in the cell cycle progression of *L. donovani* parasites.

Effect of chaperonin inhibitors on protein-folding activity of recombinant LdTCP1 γ homo-oligomeric complex of *L. donovani*. We tested three known compounds, suramin, mizoribine, and α -humulene (Fig. S2), for their effects on LdTCP1 γ -mediated luciferase refolding and ATPase activities. These compounds were previously shown to be inhibitors of heat shock protein 60 (HSP60) chaperonins (homo-oligomers) of *Trypanosoma brucei* (28), human (29), and *Chlamydia pneumoniae* (30), respectively. Suramin was found to inhibit the refolding of denatured luciferase by LdTCP1 γ (50% inhibitory concentration [IC₅₀] of $\sim$ 591 $\pm$ 1.2 nM) (Table 2 and Fig. S3); however, it was unable to inhibit the ATPase activity of LdTCP1 γ (Table 2). On the other hand, the other two compounds did not inhibit either the luciferase refolding or the ATPase activity of LdTCP1 γ (Table 2). We also determined the activity of these inhibitors against native luciferase folding and found that none of them had an inhibitory effect (IC₅₀ of $>$ 100 μ M) (data not shown).

Computational studies. We next performed computational studies to further understand the interaction of these inhibitors with LdTCP1 γ . The protein sequence of LdTCP1 γ was obtained from GenBank (accession number [JX088118](#)), modeled using modeller software, and validated as described previously (Fig. S4A) (27), and the previously reported model (27) was used for computational studies. The multichannel surface tool of the Sybyl-X 2.1.1 package (Certara, Inc., Princeton, NJ, USA) was used to predict the possible binding sites for docking with each inhibitor. The top three predicted binding sites were used for generating a grid, and the docking study was performed around it. Figure S4B shows the surface views of the best predicted binding sites. The best pose of suramin showed a docking score of 8.9, while mizoribine and α -humulene showed docking scores of 4.0 and 3.2, respectively. Residues Thr190, Gly191, Val192, Lys193, Arg 199, Tyr 200, Arg318, Lys319, and Arg324 formed hydrogen bonds with the suramin molecule, and residues Ala201, Glu204, Lys205, Thr320, Asn323, Arg327, Glu389, and His393 were involved in van der Waals interactions (Fig. S4C and D). Residues Thr180, Lys198, Ala201, Met393, and Met396 were involved in hydrogen bond interactions with mizoribine (Fig. S4E and F), and residues Glu90, Val91, Cys397, Arg400, Asn401, Glu405, Ala501, Val504, and Gln505 (Fig. S4G and H) showed

FIG 7 Cell cycle analysis of Dd8^{+/+}, Dd8^{+/-}, Dd8^{+/-/+}, and Dd8^{+/+ +/+} promastigotes. (A) Log-phase cells of Dd8^{+/+}, Dd8^{+/-}, Dd8^{+/-/+}, and Dd8^{+/+ +/+} parasites were synchronized at the G₁/S border with 200 μ g/ml hydroxyurea (HU) for 16 h. After removal of hydroxyurea, cells were

(Continued on next page)

TABLE 2 Effects of chaperonin inhibitors on rLdTCP1 γ -mediated protein folding and ATPase activities

Inhibitor	Mean IC ₅₀ $\pm$ SD (μ M) ^a	
	Luciferase refolding	ATPase activity
Suramin	0.591 $\pm$ 0.001	>500
Mizoribine	>100	>500
α -Humulene	>100	>500

^aValues are from three independent experiments.

van der Waals interactions with α -humulene. Among all three compounds, the binding of suramin involved residues that belong to the apical and intermediate domains of the protein (Fig. S5). The apical domain of TCP1 γ is thought to be involved in substrate binding in mice (31). However, the residues which interact with mizoribine and α -humulene were outside the apical domain of the protein. This suggests that suramin probably binds to the apical domain and, thus, may inhibit the binding of the substrate protein (denatured luciferase) to LdTCP1 γ .

Thermodynamics of suramin's interaction with LdTCP1 γ . Since suramin was the only compound able to inhibit the refolding activity of recombinant LdTCP1 γ and the only one that exhibited interaction with active-site residues, we next performed isothermal titration calorimetry (ITC) to obtain thermodynamic insight into the biomolecular interactions between suramin and LdTCP1 γ . The thermodynamic parameters for the interaction of suramin with LdTCP1 γ protein were obtained by fitting the ITC experimental data to a single-site binding model (Fig. 8). An equilibrium dissociation constant (K_d) of $10.38 \pm 1.52 \mu$ M was obtained for the binding of suramin and LdTCP1 γ protein, which depicted strong binding between them. Both enthalpy (ΔH) (-22.96 ± 6.2 kcal/mol) and entropy (ΔS) (-0.055 ± 0.02 kcal/mol.K) were found to be in the negative range, which indicates that the binding interaction is entropically unfavorable ($\Delta S < 0$) and enthalpy driven ($\Delta H < 0$). This further implies that either electrostatic interactions or hydrogen bonds contribute to the underlying binding between suramin and LdTCP1 γ . The negative value of Gibbs free energy of binding (ΔG), -6.69 ± 0.108 kcal/mol, showed that the interaction is spontaneous in nature.

Effects of suramin, mizoribine, and α -humulene on multiplication of promastigotes and amastigotes. All three compounds, suramin, mizoribine, and α -humulene, were tested for their antileishmanial efficacy against *L. donovani* promastigotes and amastigotes. Suramin did not exhibit any effect on promastigote multiplication but was found to be significantly active against the amastigote stage of the parasite, with an IC₅₀ of $10.47 \pm 1.5 \mu$ M (Table 3). On the other hand, α -humulene did not show any antileishmanial efficacy against either stage of the parasite, and while mizoribine did exhibit some antileishmanial efficacy against amastigotes, its IC₅₀ was 6 times higher than that of suramin (Table 3). We also performed cytotoxicity assays of these inhibitors against J774A.1 cells, and suramin showed a safety index (SI) of 61.42.

Suramin reduced the parasite burden in a BALB/c mouse model of experimental visceral leishmaniasis. Next, we evaluated the antileishmanial efficacy of suramin *in vivo* using a BALB/c mouse animal model of *L. donovani* infection and compared the results with the results for the standard drug amphotericin B at doses of 5 mg/kg of body weight for suramin and 1 mg/kg for amphotericin B for 5 consecutive days (Fig. S6). Both drugs were well tolerated at the doses given and showed no signs of *in vivo* toxicity. Parasite burdens in spleen tissues were determined on the 7th day after the last treatment and were compared between the treated and the untreated groups. The

FIG 7 Legend (Continued)

allowed to enter the cell cycle as normal. DNA was stained with propidium iodide at 0 h, 6 h, and 8 h after hydroxyurea removal and analyzed with flow cytometry. Histograms show the fractions of cells in each phase of the cell cycle. G₀/G₁, S and G₂/M phases are represented by M₁, M₂ and M₃ gates, respectively, on the histograms. No detectable cell population was found in the sub-G₀/G₁ phase. (B) Bar graph showing the percentages of cells in G₀/G₁, S, and G₂/M phases of the cell cycle for Dd8^{+/+}, Dd8^{+/-}, Dd8^{-/+}, and Dd8^{+/++} parasites at 0 h, 6 h, and 8 h after hydroxyurea removal. The data represent the mean values $\pm$ standard errors of the means of three independent experiments.

FIG 8 Binding study of suramin with LdTCP1 γ using isothermal titration calorimetry (ITC). Suramin (120 μ M) was titrated into the solution of LdTCP1 γ (12 μ M monomer concentration) in the ITC cell at 25°C. Top, representative binding isotherm. Bottom, integrated data are represented by solid squares and curve fitting by a solid line using a single-site binding model. The molar ratio denotes the binding stoichiometry of suramin to monomeric LdTCP1 γ . Data are representative of three independent experiments.

parasite load in suramin-treated mice was found to be decreased significantly (85.32%) compared to that of untreated infected control mice (Fig. 9). The mice treated with amphotericin B showed significant suppression of the splenic amastigote load, by 89.28% compared to that in the untreated infected control mice.

DISCUSSION

The CCT chaperonin complex is essential for protein folding in eukaryotes (10, 11). It has been implicated in multiple important functions, including folding nascent polypeptides (32), forming multimeric protein complexes (33), and preventing protein aggregate formation (34). There are two divisions of chaperonins based on the number and nature of subunits: group I chaperonins are found in bacteria (e.g., GroEL [HSP60] and GroES [HSP10] of *Escherichia coli*) (35) and eukaryotic organelles, such as chloroplasts and mitochondria, and group II chaperonins are found in archaea (thermosomes) and the eukaryotic cytosol (e.g., chaperonin-containing TCP1 [CCT] complex) (11, 16). The eukaryotic group II chaperonin TCP1 ring complex comprises two identical rings having eight or nine distinct CCT subunits each (18, 36). Thus, TCP1 functions as a

TABLE 3 Effects of chaperonin inhibitors on the cell viability of promastigotes and amastigotes and inhibitor cytotoxicities

Inhibitor	Mean value $\pm$ SD (μ M) ^a			
	IC ₅₀ in:		CC ₅₀ in J774A.1 cells	SI ^b
	Promastigotes	Amastigotes		
Suramin	>100	10.47 $\pm$ 1.5	643.1 $\pm$ 1.51	61.42
Mizoribine	>100	62.6 $\pm$ 1.47	>1,000	>15.9
α -Humulene	>100	>100	>1,000	ND

^aData are representative of two independent experiments (in triplicate).

^bSI, safety index; ND, not determined.

FIG 9 *In vivo* antileishmanial efficacy of suramin in *L. donovani*-BALB/c mouse model after intraperitoneal treatment with suramin (5 mg/kg) for 5 consecutive days. Amphotericin B (AMB) (1 mg/kg) was used as the drug standard. The parasite burden in the spleen, expressed in Leishman-Donovan units (LDU), was determined on the 7th day after the last treatment. Comparisons were made by one-way ANOVA and *post hoc* Tukey test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Values are mean results $\pm$ SD from six animals. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

hetero-oligomeric complex by folding its substrate proteins, such as actin and tubulin. In addition to the role of TCP1 as a hetero-oligomeric complex, TCP1 subunits comprising monomers, homo-oligomers, and complexes with two or more subunits may also have independent activities (37, 38). The role of the cytosolic HSP60 chaperonin TCP1 (CCT) has not been studied as extensively in protozoan parasites. Recently, TCP1 was implicated in the export of *P. falciparum* proteins (20) in red blood cells (RBC). Later, another report, by Spillman et al. (22), established that TCP1 of *P. falciparum* was unrelated to protein trafficking in the RBC compartment but one of its subunits, the theta subunit, was essential for asexual development. This shows the essentiality of TCP1 in the malaria parasite life cycle. We have reported previously (26, 27) that a recombinant single-subunit protein (LdTCP1 γ) forms a homo-oligomeric complex and performs chaperonin activity without other partner subunit(s).

In the present study, we report for the first time that LdTCP1 γ is essential for parasite survival and virulence. To understand the role of LdTCP1 γ in parasite biology, single- and double-replacement LdTCP1 γ mutants were developed by following a classical recombinant replacement strategy (Fig. S1). The gene replacement was accompanied by decreased expression of LdTCP1 γ both at the mRNA and the protein level (Fig. 3A to C). Interestingly, LdTCP1 γ seems to be essential for the survival of promastigotes, as null mutants were unable to survive in cell culture and single-allele-replacement mutant parasites exhibited significantly reduced growth (Fig. 4). Furthermore, by adding the LdTCP1 γ gene back into Dd8^{+/-} cells by episomal complementation, the slowed growth was reverted back to levels exhibited by wild-type Dd8^{+/+} cells. Thus, LdTCP1 γ has been genetically validated as crucial for promastigote survival and multiplication.

To evade the host immune response and to survive within host macrophages, *Leishmania* parasites use several virulence factors that may be surface exposed or actively secreted in multiple forms. Silverman et al. showed that several proteins, including TCP1 γ , were found in the proteome of the secretome of *L. donovani* (23), and so, it may also act as an important virulence factor, as most of the proteins in the secretome were shown to be potential virulence factors (39). Thus, to explore this possibility, we assessed single-allele-replacement mutants and overexpression transfectants for their ability to infect J774A.1 murine macrophages. The single-allele-replacement mutants exhibited decreased infectivity (Fig. 5) and significantly decreased multiplication of amastigotes (Fig. 6). Interestingly, LdTCP1 γ overexpression transfectants showed increased infectivity (Fig. 5) and an increased multiplication rate of amastigote-stage parasites (Fig. 6). The data suggest that LdTCP1 γ is essential for parasite virulence and growth.

The cell cycle analysis of the single-allele-replacement mutants revealed a significant delay at the G_0/G_1 phase that eventually resulted in their growth defects (Fig. 7). An earlier report suggested that TCP1 positively regulates Cdc20 and Cdh1 (substrates of TCP1), which are important for the activation of APC/C (anaphase-promoting complex) at the transition from metaphase to anaphase and during G_1 in yeast (40). Furthermore, Grantham et al. demonstrated that reducing the TCP1 level or affecting its activity disrupts cell cycle progression in yeast (41). In addition, CCT gene expression is upregulated during the transition of the G_1/S phase of the cell cycle (42), while its downregulation results in cell proliferation inhibition, reduced cell viability, cell cycle arrest, and cell apoptosis (43). Knocking down the TCP1 γ subunit caused cell cycle arrest at the S phase in hepatocellular carcinoma cells (44). The observed cell cycle delay in *L. donovani* parasites could also be related to improper processing of actin and tubulin, the major substrates of the eukaryotic CCT complex (45), due to insufficient levels of the TCP1 γ subunit (27). The results are in accordance with earlier reports where eukaryotic CCT has been shown to play a key role in cell proliferation by facilitating the folding of cytoskeletal proteins (e.g., actin, tubulin, and contractin) engaged in cell division (11, 46) and of oncoproteins (e.g., cyclin E, Von Hippel-Lindau [VHL] tumor suppressor protein, cyclin B, and p21ras) (47–49). Depleting CCT complex in cells seems to interfere with both microfilament- and microtubule-based cytoskeleton activities without interfering with actin and tubulin polypeptide synthesis (41). Knockdown of CCT by microinjection of an anti-CCT ϵ antibody (which was shown to change the rate of actin and tubulin processing into cultured cells) resulted in a delay in the G_1/S -phase transition (41), suggesting that high levels of CCT activity are required by the cell (19). The enhanced cell cycle progression observed in overexpressing Dd8 $^{+/+/+}$ cells further confirms that LdTCP1 γ plays a key role in cell cycle progression and suggests that it could be implicated in the cell proliferation of parasites.

As gene deletion data showed that LdTCP1 γ is essential for parasite viability, it can be employed as a potential antileishmanial drug target. For chemical validation, three compounds, namely, suramin, mizoribine, and α -humulene, were selected to analyze their effects on folding and ATPase activities of LdTCP1 γ . Among them, only suramin inhibited the refolding activity of the LdTCP1 γ homo-oligomeric complex (Table 2 and Fig. S3), suggesting that suramin may be targeting recombinant homo-oligomeric LdTCP1 γ and affecting the parasite chaperone machinery. Suramin, an FDA-approved drug, is a symmetrical polysulfonated naphthylamine urea derivative (50) initially developed as a treatment for human African sleeping sickness (51) that has also been shown to have several other activities, viz., it has anticancer activity (52), causes reversal of several autism-related features (53), reduces the levels of prion protein in infected cells (54), and has a strong affinity for a variety of proteins and enzymes (55). It inhibits bacterial HSP104 ATPase, disaggregase, unfoldase, and translocase activities (56). Suramin was also found to inhibit the folding activity of *T. brucei* HSP60 (28). Recently, it has also exhibited *in vitro* antileishmanial activity against *Leishmania major* and *L. donovani* strain AG83 (57). Mizoribine (4-carbamoyl-1- β -D-ribofuranosylimidazo-*l*-ium-5-olate monohydrate), an imidazole nucleotide antibiotic isolated from *Eupenicillium brefeldianum*, has been shown to bind directly to mammalian HSP60 (29) and has inhibited the folding cycle of mammalian HSP60/HSP10 (58), ATPase activity, and the dissociation of HSP10 from HSP60. Interestingly, it does not inhibit either the folding or the ATPase activity of LdTCP1 γ , suggesting that leishmanial LdTCP1 γ is significantly different from mammalian HSP60 chaperonins. α -Humulene has previously been shown to be an inhibitor of chlamydial-HSP60-induced proliferation of rat arterial smooth muscle cells (30). Interestingly, it also did not inhibit the chaperonin activity of LdTCP1 γ (Table 2).

A molecular docking study revealed that the suramin-LdTCP1 γ interaction was stabilized by hydrogen bonds and van der Waals interactions. It is known from previous studies that the apical domain of TCP1 γ is responsible for substrate binding in mouse (31). Interestingly, the docking study indicated that all of the residues that interact with suramin belong to the apical and intermediate domains (Fig. S4 and S5). This probably

suggests that suramin binds to the apical domain of LdTCP1 γ and thus inhibits the binding of the substrate protein with LdTCP1 γ . This is in accordance with our chaperonin activity assay observations that suramin exhibited inhibition only of the luciferase refolding activity of LdTCP1 γ (Table 2 and Fig. S3) and not the ATPase activity (Table 2), as the ATP binding site is different from the substrate binding site (26). Mizoribine and α -humulene interact with low docking scores compared to that of suramin, and the interacting residues were not present in the apical region of LdTCP1 γ ; this could be a probable reason for the ineffectiveness of these two compounds against the protein folding activity of LdTCP1 γ .

Isothermal titration calorimetry (Fig. 8) was conducted to assess the interaction of suramin with LdTCP1 γ . We observed that suramin formed stable adducts with LdTCP1 γ with a K_d of $10.38 \pm 1.52 \mu\text{M}$ (Fig. 8). Slowly binding ligands have apparent K_d values that are higher than the true K_d values, as explained by Hang et al. (59). The apparent K_d value ($10.38 \mu\text{M}$) being higher than the IC_{50} value ($\sim 0.6 \mu\text{M}$) also suggests that suramin binding may have slow kinetics. Furthermore, the binding is enthalpy driven, which means that electrostatic interaction and hydrogen bonds are involved. This analysis confirmed that suramin indeed binds with LdTCP1 γ and, thus, may inhibit its activity.

Interestingly, among the three compounds, only suramin exhibited antileishmanial efficacy against amastigotes, with an IC_{50} of 10.57 and a high safety index (SI) of 61.42, while the other two compounds failed to inhibit parasite multiplication. The parasite burden in spleen tissues of suramin-treated mice was also found to be reduced by 6.4-fold compared to that in untreated control mice (Fig. 9). These observations clearly indicate that LdTCP1 γ inhibition is one of the modes of action of suramin. Interestingly, it has been reported that disruption of a single subunit causes downregulation of other subunits, thus disrupting the function of the whole hetero-oligomeric complex in mammalian (41) and cancer (60) cells. Therefore, there is a possibility that suramin may target a hetero-oligomeric complex of the parasite (if any) by inhibiting LdTCP1 γ . Thus, chemical validation further suggests the essentiality of LdTCP1 γ for parasite survival.

In conclusion, we established that LdTCP1 γ is an essential gene for parasite survival, growth, and infectivity, and hence, it can be employed as a new drug target. Furthermore, suramin, which was initially developed as a treatment for human African sleeping sickness and is an inhibitor of the *T. brucei* HSP60 chaperonin system, also inhibited the luciferase refolding activity of a recombinant LdTCP1 γ homo-oligomeric complex and exhibited both *in vitro* and *in vivo* antileishmanial efficacy. The study provides leads to identify new chemical entities using this target. Suramin can furthermore be used in combinatorial therapies for effective treatment of leishmaniasis.

MATERIALS AND METHODS

Parasites and cell culture. *L. donovani* promastigotes (World Health Organization designation MHOM/IN/80/Dd8), originally obtained from the late P. C. C. Garnham (Imperial College London, London, United Kingdom) and maintained at the CSIR-Central Drug Research Institute in golden hamsters, were used in the present study. The promastigotes were grown at 24°C in medium 199 (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% antibiotic, and antimycotic solution (Sigma) (61). The J774A.1 cell line, obtained from the National Center for Cell Sciences (NCCS), Pune, India, was grown in RPMI 1640 medium (Sigma) supplemented with 10% FBS (Gibco), 1% antibiotic, and antimycotic solution (Sigma) at 37°C with 5% CO_2 .

Gene copy number analysis. Genomic DNA of *L. donovani* promastigotes was isolated as previously described (62). Southern blot analysis was performed using the digoxigenin (DIG) high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany), following the manufacturer's instructions. For southern blotting, 10 μg of genomic DNA was digested with EcoRI, XbaI, NdeI, NheI, and NcoI and subjected to electrophoresis on a 0.7% agarose gel. The transfer of digested DNA to the positively charged nylon membrane (Amersham Biosciences) was carried out according to the standard procedure (63). The PCR product of LdTCP1 γ (1,656 bp) from plasmid pCRT-LdTCP1 γ was labeled using DIG-11-dUTP with DIG high prime DNA labeling reagents (Roche, Mannheim, Germany) to synthesize DIG-labeled LdTCP1 γ probe. Hybridization was done at 42°C, and then chemiluminescence detection was carried out in a MyECL Imager (Thermo Fisher Scientific, Waltham, MA, USA) using CSPD substrate (Thermo Fisher Scientific) according to the manufacturer's instructions.

Development of constructs and parasite transfection. (i) **Overexpression of LdTCP1 γ in *L. donovani* promastigotes.** The complete open reading frame (ORF) of LdTCP1 γ was PCR amplified from plasmid DNA (pCRII-TOPO-LdTCP1 γ) using primers P1 and P2 (Table 1) and cloned into the *Leishmania* pKS-Neo shuttle vector (64). Clone pKS-LdTCP1 γ 1 (with the ORF in the correct orientation) was used to

transfect the *L. donovani* promastigotes by electroporation using the Gene Pulser (Bio-Rad) (65). Parasites transfected with pKS-Neo (empty vector) were taken as a control. Transfectants (Dd8^{+/+/+} and Dd8Vc) were selected and maintained in the presence of G418 (40 µg/ml) in M199 supplemented with 10% FBS.

(ii) Development of LdTCP1γ gene replacement mutants by homologous recombination. One kilobase each of the 5'-flanking (5FLK) and 3'-flanking (3FLK) regions of LdTCP1γ was PCR amplified using *L. donovani* genomic DNA as the template and primers P3, P4, P5, and P6 (Table 1), and the amplicons were cloned in the pCR2.1-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced in both direction to confirm the 5FLK and 3FLK regions of *L. donovani* strain Dd8 (Fig. S1). Furthermore, using these sequences, internal primers with restriction sites (P7 and P8 for 5FLK and P9 and P10 for 3FLK) were designed from the sequenced 5FLK and 3FLK regions. 5FLK and 3FLK were further amplified with the internal primers that were designed, and the amplified products were cloned in the pCR2.1-TOPO vector. The hygromycin phosphotransferase (HYG) and neomycin phosphotransferase (NEO) genes were amplified from pCDNA3 and pXG-GFP vectors (a kind gift from S. M. Beverley). The resistance marker genes were amplified using primers P11, P12, P13, and P14, and the respective amplicons were cloned in the pCR2.1-TOPO vector. The 3FLK fragment was directionally subcloned in the 5FLK-TOPO construct using XmaI and NotI sites, resulting in the construct 5/3-FLK-TOPO. HYG and NEO gene cassettes were further inserted directionally in the 5/3-FLK-TOPO construct using XmaI and NheI sites, resulting in the final gene constructs 5FLK/HYG/3FLK-TOPO and 5FLK/NEO/3FLK-TOPO. Both gene constructs, 5FLK/HYG/3FLK and 5FLK/NEO/3FLK, were removed from the pCR2.1-TOPO vector by restriction enzyme digestion with SpeI and XbaI and purified. First, 5FLK/HYG/3FLK was transfected into *L. donovani* promastigotes (Dd8^{+/+}). Transfectants were selected in the presence of 20 µg/ml hygromycin supplemented with 10% FBS to obtain Dd8^{+/-} LdTCP1γ single-allele-replacement mutants. The Dd8^{+/-} mutants were further transfected with the second allele replacement fragment 5FLK/NEO/3FLK and selected under the drug pressure of 20 µg/ml hygromycin and 20 µg/ml neomycin (G418) in M199 medium supplemented with 10% (vol/vol) FBS. The integration of HYG and NEO genes at the correct position in the *L. donovani* genome was verified by PCR amplification of the genes using genomic DNA of Dd8^{+/+}, Dd8^{+/-}, and Dd8^{-/-} parasites as templates. HYG was amplified with primers P11 and P12, NEO with P13 and P14, and LdTCP1γ with P1 and P2. The PCR products were checked on 1% agarose gels and stained with ethidium bromide.

For episomal complementation, the LdTCP1γ gene, cloned in the pKS-Neo shuttle vector (64), was transfected into Dd8^{+/-} parasites (HYG). The complementation mutants (Dd8^{+/-/+}) were selected and maintained under drug pressure from 20 µg/ml neomycin and 20 µg/ml hygromycin in M199 medium supplemented with 10% (vol/vol) FBS.

RNA isolation and RT-PCR analysis. Total RNA was extracted from mid-log-phase promastigotes of the wild-type strain (Dd8^{+/+}) and laboratory-generated genetically manipulated parasite cell lines using TRIzol reagent (Invitrogen). To prevent any genomic DNA contamination, total RNA (100 µg) was treated with RNase-free DNase I and was further purified using the RNeasy purification kit (Qiagen, Venlo, the Netherlands) in accordance with the manufacturer's instructions. cDNA was synthesized from 5 µg of total RNA using SuperScript II reverse transcriptase and a random hexamer primer (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Real-time PCR (RT-PCR) was performed in triplicate in 30-µl volumes using Dynamo SYBR green mix (Invitrogen) in an RT-PCR machine (Bio Rad). Primers P15 and P16 were used to amplify the LdTCP1γ gene and P17 and P18 were used for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Table 1). Analysis of relative gene expression levels was done based on the threshold cycle (C_T) value of the LdTCP1γ gene normalized to that of the reference GAPDH gene using the method of Livak and Schmittgen (66). The expression ratio was calculated using the formulas $\Delta C_{T(\text{strains})} = C_{T(\text{LdTCP1}\gamma)} - C_{T(\text{GAPDH})}$ and $\Delta C_{T(\text{LdDd8})} = C_{T(\text{LdDd8})} - C_{T(\text{GAPDH})}$, and the ΔC_T of the test sample was normalized to the C_T of the calibrator with the formulas $\Delta\Delta C_T = \Delta C_{T(\text{strains})} - \Delta C_{T(\text{LdDd8})}$ and $2^{-\Delta\Delta C_T}$ = normalized expression ratio. A negative control was set up to check for DNA contamination by amplifying total RNA without reverse transcriptase.

SDS-PAGE and Western blotting. To compare LdTCP1γ expression at the protein level, equal numbers of mid-log-phase promastigotes (4×10^6) of various mutant parasites were harvested by centrifugation ($1,000 \times g$ for 10 min), washed in phosphate-buffered saline, and lysed in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and treated with anti-LdTCP1γ antibody for Western blot analysis as described previously (67, 68). Blots were developed using enhanced chemiluminescence (ECL) reagent (GE Healthcare) and visualized on a MyECL Imager (Thermo Fisher Scientific, Waltham, MA, USA). The quantitative assessment was carried out with ImageJ software (National Institutes of Health). Tubulin was taken as the control for normalization.

Growth curves. The parasite multiplication of various mutant cell lines was compared with the multiplication of the vector control (Dd8Vc) and wild-type (Dd8^{+/+}) parasites. Promastigotes were seeded in triplicate at an initial concentration of 0.5×10^6 parasites/ml and were counted daily for 9 days using a hemocytometer.

Macrophage infection and effects of chaperonin inhibitors on intracellular amastigote multiplication. Parasite infectivity and the multiplication of intracellular amastigotes were evaluated as previously described (69). Briefly, 0.5×10^5 macrophages were layered in 16-well chamber slides (Nunc) and allowed to adhere for 24 h at 37°C in a 5% CO₂-95% air mixture in RPMI medium supplemented with 10% FBS. The adhered macrophages were infected at a ratio of 1:10 for 24 h with stationary-phase promastigotes. The noninternalized parasites were washed from the wells, and infected macrophages were incubated further for 6 h, 24 h, 48 h, or 72 h at 37°C in a 5% CO₂-95% air mixture in RPMI medium supplemented with 10% FBS. At various time points, macrophages were fixed with 90% methanol and stained with Giemsa stain, and amastigotes were counted under the microscope. The number of infected macro-

phages per 100 macrophages (% infectivity) was counted at 24 h of infection. The numbers of amastigotes per cell nucleus after 6 h, 24 h, 48 h, or 72 h of infection were counted for intracellular amastigote proliferation.

To see the effects of chaperonin inhibitors on intracellular amastigote multiplication, J774A.1 murine macrophages were infected with stationary-phase promastigotes at a ratio of 1:10 for 24 h. After washing off noninternalized parasites, macrophages were incubated at 37°C in a 5% CO₂–95% air mixture for 48 h in the absence or presence of increasing concentrations of inhibitors (2.5 μ M to 500 μ M). The cells were fixed in methanol after incubation, stained with Giemsa stain (Sigma), and observed under a microscope to determine the mean amastigote number per 100 macrophages.

Effects of chaperonin inhibitors on promastigote multiplication. The *in vitro* leishmanicidal efficacies of various compounds, namely, suramin, mizoribine, and α -humulene (Sigma) (28–30), against the multiplication of *L. donovani* wild-type promastigotes were investigated. Wild-type mid-log-phase promastigotes and various mutants were seeded in 96-well culture plates at a final concentration of 0.5×10^6 cells/ml in complete M199 medium alone or in the presence of increasing concentrations of compounds in triplicate and allowed to grow at 24°C $\pm$ 1°C for 72 h. The numbers of viable cells were determined by microscopic counting (70).

Cytotoxicity assay. The cytotoxicities of chaperonin inhibitors on J774A.1 murine macrophages were determined by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay (71). Briefly, 1×10^5 cells/100 μ l/well were seeded in a 96-well flat-bottom plate and incubated with different drug concentrations at 37°C and 5% CO₂. Dimethyl sulfoxide (DMSO) was used as the vehicle control for inhibitors that were dissolved in DMSO. After 48 h of incubation, 25 μ l of MTT (Sigma-Aldrich, USA) (5 mg/ml) was added to each well, and the plate was further incubated at 37°C for 4 h. After incubation, the supernatant was removed and 150 μ l of DMSO was added to each well. After 15 min of shaking, the absorbance was measured at 544 nm in a microplate reader (SpectraMax 190 from Molecular Devices). The safety index (SI) for each inhibitor was calculated as the ratio between cytotoxicity (50% cytotoxic concentration [CC₅₀]) and leishmanicidal activity (IC₅₀) against *Leishmania* amastigotes.

Cell cycle analysis. Promastigotes (1×10^7) were harvested and seeded in serum-depleted M199 medium containing 200 μ g/ml hydroxyurea at 24°C. After 16 h of cell synchronization, hydroxyurea was removed and cells were transferred to serum-containing M199 medium and allowed to grow for 0 h, 6 h, and 8 h. Cells were harvested, washed, and resuspended at specified time points in 300 μ l phosphate-buffered saline (PBS). An amount of 700 μ l of chilled ethanol was added to the cell suspension, and the mixture left overnight at 4°C. Samples were acquired on a FACSCalibur instrument (BD Biosciences), and fluorescence intensity was measured on the photomultiplier tube (FL2A with a 585/40 nm bandpass filter) after incubation with propidium iodide solution (50 μ g/ml propidium iodide, 0.05% Triton X-100, and 100 μ g/ml RNase A) for 40 min. At least 10,000 events were analyzed for each sample using BD CellQuest Pro software (BD Biosciences).

Purification of the recombinant LdTCP1 γ protein. LdTCP1 γ was expressed from a T7 promoter of the pCR-T7/NT-TOPO plasmid in *E. coli* strain C41 (DE3) cells as described previously (26). When the optical density of the culture at 600 nm reached approximately 0.6, the expression of recombinant LdTCP1 γ was induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (Thermo Fisher Scientific, Waltham, MA, USA) at 16°C overnight. After overnight induction, bacterial cells were pelleted at $6,500 \times g$ at 4°C for 10 min and suspended in lysis buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride, 1 mg/ml lysozyme, and 10 μ g/ml protease inhibitor cocktail [Sigma]). The cells were lysed by sonication, lysate was cleared by centrifugation at $16,000 \times g$ at 4°C for 20 min, and recombinant protein was purified under native conditions using a preequilibrated Ni-nitrilotriacetic acid (NTA) column (Qiagen) in accordance with the manufacturer's instructions. The sample purity was assessed on a 10% SDS-PAGE gel stained by Coomassie brilliant blue R-250 (Sigma) stain (68). The concentrations of proteins were determined by the Bradford method using bovine serum albumin (BSA) as the standard (72).

ATPase activity assay. The ATPase activity assay was performed as described previously (27, 73). A concentration of 500 nM rLdTCP1 γ was preincubated with inhibitors at varying concentrations (50 to 500 μ M) in a buffer containing 50 mM Tris, pH 7.4, 50 mM KCl, 5 mM MgCl₂, and 1 mM EGTA at 30°C for 10 min. After preincubation, 1 mM ATP was added and the mixture incubated for 60 min at 30°C. Amounts of 10 μ l were transferred to a 96-well plate, and Biomol green (100 μ l) (Enzo Life Science, New York, NY, USA) was added. The samples were incubated for 5 min at room temperature, and absorbance was measured at 630 nm with a SpectraMax 190 reader (Molecular Devices).

Luciferase refolding assay. The luciferase refolding assay was carried out as described previously (27, 74). Briefly, 8 μ M recombinant luciferase (Promega, Madison, WI, USA) was denatured for 60 min in denaturing buffer (6.0 M guanidine hydrochloride, 25 mM HEPES, pH 7.4, 50 mM potassium acetate, 5 mM dithiothreitol). Denatured luciferase was diluted 1:40 in denaturing buffer and a further 1:25 in refolding buffer (25 mM HEPES, pH 7.4, 100 mM potassium acetate, 10 mM magnesium acetate, 2 mM dithiothreitol, 2 mM ATP) with 450 nM rLdTCP1 γ in the presence or absence of various concentrations of inhibitors and incubated for 60 min at 25°C. After 60 min, aliquots were mixed with Steady-Glo reagent (Promega, Madison, WI, USA) at a 1:25 dilution, and luminescence was measured using a GloMax-96 microplate luminometer (Promega, Madison, WI, USA).

ITC. Isothermal titration calorimetry (ITC) experiments were performed at 25°C on a MicroCal VP-ITC calorimeter (MicroCal, Northampton, MA, USA). Both LdTCP1 γ and suramin were prepared in buffer containing 25 mM HEPES, pH 7.4, 100 mM KCl, 10 mM MgCl₂ and degassed for 20 min before each experiment. The ITC experiments were performed by adding aliquots of suramin to LdTCP1 γ . The LdTCP1 γ protein at a concentration of 12 μ M was placed in the sample cell of the ITC, and suramin (the ligand) at a concentration of 120 μ M was injected into the cell. Twenty-eight injections with an injection volume of 10 μ l each were made at intervals of 20 s. The reaction mixture was continuously stirred at 416

rpm during the titration. An equivalent control experiment to subtract the heat of dilution of suramin in buffer was performed (keeping all experimental parameters and ligand concentrations the same as described for titration). ITC data were analyzed using Microcal's Origin software, version 7.0. The ΔH , ΔS , K_d , and binding stoichiometries (n) in each titration were obtained, and ΔG was calculated according to the thermodynamic formula $\Delta G = \Delta H - T\Delta S$, where temperature (T) is 298 K. Mean values and standard deviations were calculated from the results of triplicate analyses.

Computational studies. LdTCP1 γ was modeled as described in a previous report from our laboratory (27). Computational studies were carried out on the protein model to gain molecular insights on the suramin-LdTCP1 γ interaction. The monomer subunit was taken for molecular docking studies. Using the protein preparation module of Sybyl-X 2.1.1 (Certara, Inc., Princeton, NJ, USA), hydrogens and Kollman charges were added to the protein. Protomol generation was done using the multichannel surface tool of the Sybyl-X 2.1.1 suite (Certara, Inc., Princeton, NJ, USA). Predicted binding sites were used for molecular docking studies using the Surflex program in screen mode (75). The structure of suramin was obtained from PubChem (76) and drawn using the sketch module, and energy was minimized using the Tripos force field of SybylX 2.1.1 (Certara, Inc., Princeton, NJ, USA). The interactions analyzed after docking were hydrogen bonding, pi-cation interaction, pi-pi interaction, and van der Waals interactions at a cutoff radius of 3.50 Å. UCSF Chimera (77) and DS Visualizer (78) were used for image generation.

In vivo antileishmanial efficacy assay. Six- to 8-week-old male BALB/c mice, injected intravenously with approximately 5×10^6 freshly isolated *L. donovani* Dd8 amastigotes (79) from hamster, were segregated into 3 groups of 6 mice each. At 30 days postinoculation, mice of groups 1 and 2 were treated intraperitoneally with amphotericin B (1 mg/kg in sterile water) and suramin (5 mg/kg in sterile water) (80), respectively, once daily for 5 consecutive days. Group 3 mice were kept as the untreated control. The animals were humanely euthanized on the 7th day after the last treatment. The weight of the spleen was measured and dabbed imprints on glass slides were made, followed by fixation with 100% methanol and Giemsa staining. Infection was monitored microscopically by using Giemsa-stained imprints, in which parasite burdens were measured by counting the numbers of amastigotes per 500 spleen nuclei and multiplying by the spleen weight (mg) (Leishman-Donovan units [LDU]) (81). The results for the treated and untreated groups were compared. Percent efficacy was calculated by the following equation: $[1 - (\text{mean number of amastigotes in treated mice} / \text{mean number of amastigotes in untreated mice})] \times 100$.

Ethics statement. Animal experiments were performed according to the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Drug Research Institute (IAEC/2018/F-37).

Statistical analysis. Data were expressed as mean values $\pm$ standard deviations (SD) unless mentioned otherwise. IC_{50} values of compounds were obtained from log dose-response data calculated in GraphPad Prism (GraphPad Software, San Diego, CA, USA) via nonlinear regression (curve fit) analysis. Comparisons were made by one-way analysis of variance (ANOVA) and *post hoc* Tukey test using GraphPad Prism (GraphPad Software, San Diego, CA, USA), and differences were considered significant at $P < 0.001$ (***), $P < 0.01$ (**), or $P < 0.05$ (*); a P value greater than 0.05 was considered nonsignificant.

Accession number(s). The complete sequences of cloned 5' and 3' flanking regions of LdTCP1 γ are available under accession numbers [MN147848](#) and [MN147849](#), respectively, in GenBank.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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N.G. conceived the study, designed the experiments, analyzed data, and performed overall supervision. S.Y. performed the experiments and analyzed data. J.K. and M.I.S. did bioinformatic studies. N.G., S.Y., J.K., and M.I.S. wrote the manuscript.

We declare no conflicts of interests.

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