Published in final edited form as:

Mol Biochem Parasitol. 2011 April ; 176(2): 98–108. doi:10.1016/j.molbiopara.2010.12.009.

Structure Determination of Glycogen Synthase Kinase-3 from *Leishmania major* and Comparative Inhibitor Structure-Activity Relationships with *Trypanosoma brucei* GSK-3

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Abstract

Glycogen synthase kinase-3 (GSK-3) is a drug target under intense investigation in pharmaceutical companies and constitutes an attractive piggyback target for eukaryotic pathogens. Two different GSKs are found in trypanosomatids, one about 150 residues shorter than the other. GSK-3 short (GeneDB: Tb927.10.13780) has previously been validated genetically as a drug target in *Trypanosoma brucei* by RNAi induced growth retardation; and chemically by correlation between enzyme and *in vitro* growth inhibition. Here, we report investigation of the equivalent GSK-3 short enzymes of *L. major* (LmjF18.0270) and *L. infantum* (LinJ18_V3.0270, identical in amino acid sequences to *Ldon*GSK-3 short) and a crystal structure of *Lmaj*GSK-3 short at 2 Å resolution. The inhibitor structure-activity relationships (SARs) of *L. major* and *L. infantum* are virtually identical, suggesting that inhibitors could be useful for both cutaneous and visceral leishmaniasis. *Leishmania* spp. GSK-3 short has different inhibitor SARs than *Tbru*GSK-3 short, which can be explained mostly by two variant residues in the ATP-binding pocket. Indeed, mutating these residues in the ATP-binding site of *Lmaj*GSK-3 short to the *Tbru*GSK-3 short equivalents results in a mutant *Lmaj*GSK-3 short enzyme with SAR more similar to that of *Tbru*GSK-3 short. The differences between human GSK-3β (*Hs*GSK-3β) and *Lmaj*GSK-3 short SAR suggest that compounds which selectively inhibit *Lmaj*GSK-3 short may be found.

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Keywords

Glycogen synthase kinase-3; *Leishmania* spp.; *Trypanosoma brucei*; African sleeping sickness; drug development; protein kinase

1. Introduction

Pathogenic protozoa kill or permanently injure millions of people living in underdeveloped regions of the world [1,2]. Parasitic diseases such as leishmaniasis not only afflict humans, but impair local economies and sicken livestock and pets [3]. *Leishmania* spp. are responsible for a disabling skin disease and visceral involvement that is often fatal if untreated. Due to the immune evasion mechanisms of *Leishmania* spp., vaccine development remains difficult and challenging [4]. Available therapies are toxic, difficult to administer, and are becoming increasingly ineffective [3,5]. Development of new anti-leishmanial therapeutics that are oral, inexpensive, and non-toxic remains an urgent priority if the debilitating effect of leishmaniasis is to be reduced. Recent advances have increased the prospect of discovering druggable enzyme targets based on biochemical and physiological differences between pathogens and host [1].

We have recently demonstrated that *Trypanosoma brucei* glycogen synthase kinase-3 (*Tbru*GSK-3) short (GeneDB: Tb927.10.13780) is a promising drug target for *T. brucei* based on: 1) RNA interference (RNAi) data suggesting essentiality of *Tbru*GSK-3 short for *T. brucei* blood-stream-form (BSF), and 2) excellent correlations between BSF cell-activity and enzyme inhibition by compounds from a focused small molecule inhibitor library [6]. As previously reported [7], there are two GSK-3 orthologs in the *Leishmania* spp. genomes: a short form and a long form. These are equivalent to the two orthologs found in *T. brucei* [6]. Humans also have two orthologs, an alpha (α) and a beta (β) form. The trypanosomatid short GSK-3 orthologs are the closest in sequence to *Hs*GSK-3 β (see Supplementary Fig. 1 for sequence alignment).

GSK-3 is a serine-threonine protein kinase under investigation for a variety of therapeutic uses in humans [8]. Glycogen synthase kinase 3 was initially defined as a regulator of glycogen metabolism [9]. It phosphorylates residues on glycogen synthase, the rate-limiting enzyme involved in glycogen synthesis [10]. GSK-3 generally acts as a secondary protein kinase, in that it phosphorylates a serine or threonine of a protein substrate that was previously phosphorylated several residues upstream. However, “glycogen synthase kinase” is somewhat of a misnomer because GSK-3 has numerous other functions. GSK-3 phosphorylates many key regulatory proteins and is itself involved in processes such as transcription and cell cycle regulation. This family of protein kinases is highly conserved and homologs have been found in mammals, yeast, mold, plants and protozoans, some of which may or may not possess glycogen or glycogen synthase but nonetheless require GSK-3 homologs activities in signaling pathways for many cellular and physiological events [7–9,11]. GSK-3 inhibition has been shown to be useful in animal models of several diseases, including diabetes mellitus [12,13] and Alzheimer’s dementia [14]. The GSK-3 enzyme has also been found to be amenable to selective and safe targeting with small molecule drugs [8]. Lithium has long been used to treat mania [14] and is now known to be an inhibitor of GSK-3 activity. Long-term lithium administration at therapeutic levels for mania has not been associated with toxicity and lithium use has been associated with lower rates of cancer [15]. Several classes of GSK-3 specific small molecule inhibitors do not lead to any evident toxicity in animal models [13,16–19]. Thus, there are obvious reasons to believe that GSK-3 inhibitors could be administered to humans for treatment of parasitic diseases with minimal toxicity [16–19].

Validated drug targets for *T. brucei* can sometimes be extended to *Leishmania* species since these parasites share a conserved core proteome in large syntenic polycistronic gene clusters [20] and also share distinctive core biochemical processes. *Tbru*GSK-3 short and *L. major* GSK-3 short (*Lmaj*GSK-3) short have 65 % amino acid sequence identity (Table 1, Supplementary Fig. 1). Biological evidence that GSK-3 short enzyme could be a potential target for anti-leishmanial drug development was recently described for *L. donovani* [7]. However, optimization of lead candidates for development of potent therapeutics remains a challenge. Detailed understanding of inhibitor SAR within the context of X-ray crystal structures and computational modeling can effectively guide optimization of known inhibitors by predicting functional groups needed to improve potency, selectivity, and alterations allowed to improve pharmacokinetic properties.

In this report, we describe biochemical, structural, and inhibitor structure-activity relationships of *Leishmania* spp. GSK-3 enzymes. We expressed and purified recombinant GSK-3 short from *L. major* (a cause of “Old World” cutaneous leishmaniasis) and *L. infantum* (a cause of Mediterranean visceral leishmaniasis identical in amino acid sequence to *L. donovani* GSK-3 short [*Ldon*GSK-3 short] [7]). We then solved the crystal structure of *Lmaj*GSK-3 short to a resolution of 2.0 Å. It was found that a variety of protein kinase inhibitors inhibit *Lmaj*GSK-3 short and their SAR was observed to be recapitulated with *Linj*GSK-3 short. Despite the high similarity in *Tbru*GSK-3 short and *Lmaj*GSK-3 short, these enzymes have distinct structure-activity relationships (SAR) for some GSK-3 inhibitors. A structural model of *Tbru*GSK-3 short was created based on the structure of *Lmaj*GSK-3 short which explains the disparate SAR. Specifically, the structural comparison shows that there are two key amino acids in the active site that are different from those of *Lmaj*GSK-3 short. These residues are predicted to reduce compound-protein interactions within the ATP-binding cavity in *Lmaj*GSK-3 short compared to *Tbru*GSK-3 short. Mutation of these two amino acids in *Lmaj*GSK-3 short to the equivalent residues found in *Tbru*GSK-3 short abolished the difference in SAR. Thus, our studies define the structural constraints for the further development of potent GSK-3 inhibitors that target *T. brucei* as well as for all *Leishmania* species.

2. Materials and Methods

2.1. Bioinformatics

The *Hs*GSK-3 β (UniProt P49841) orthologs *Lmj*F18.0270 and *Lin*J18_V3.0270, from the genomes of *L. major* and *L. infantum* respectively, were identified by BLASTP [21]. A comparison of amino acid identities (%) of human GSK-3 β vs. *L. major*, *L. infantum* and *T. brucei* GSK-3 short is shown in Table 1 and alignments of their predicted amino acid sequences are shown in Supplementary Fig. 1.

2.2. Compound Library

Protein kinase inhibitors purchased from Calbiochem (San Diego, CA) included GSK-3 β Inhibitor VIII (Catalog no: 361557), TrkA Inhibitor (Catalog no: 648450), Angiogenesis inhibitor (Catalog no: 175580), RO-31-7549 (Catalog no: 557508), Indirubin-3'-monoxime-5-sulphonic Acid (Catalog no: 402088), JAK3 Inhibitor VI (Catalog no: 420126), Alster-paullone, 2-cyanoethyl (Catalog no: 126871), Cdk1/2 Inhibitor III (Catalog no: 217714), Hymenialdisine, *Stylissa damicornis* (Catalog no: 400085). The remaining inhibitors, GW8510 (Catalog no: G7791) and SU9516 (Catalog no: S1693) were obtained from Sigma Chemical Co. (St. Louis, MO). They were dissolved and stored at -20° C in 100% DMSO at a final concentration of 20 mM. Final compound concentration per well in screening assays was 10 μ M, while further serial dilutions to determine IC₅₀ values when IC₅₀ values were <10 μ M were done after the initial screening assays.

2.3. Molecular Cloning, Expression and Characterization of Parasite GSK-3 short enzymes

The complete coding region of GSK-3 short genes of *L. infantum* strain MHOM/BR/82/BA-2 and *L. major* Friedlin strain genomic DNA [21] were PCR amplified using the primers *Lmaj*GSKFwd (5'- GGGTCCTGGTTCGATGTCGCTCAACGCTGCCGCT -3'), *Lmaj*GSKRev (5'- CTTGTTCGTGCTGTTTATTACTTGCGAACTAGCTTGGCCTTC -3'). A previously described set of primers, *Tbru*GSKLICFwd and *Tbru*GSKLICRev, were used to amplify the ORF of *Tbru*GSK-3 short from the *Tbru*GSK-3(MBP)-AVA0421- expression vector [6]. These were cloned into the ligation independent cloning (LIC) site of expression vector AVA0421 [23,24]. This vector was derived from pET14b and encodes an N-terminal His(6)-Tag followed by an optimized 3C protease cleavage recognition site. The inserts were all sequenced and confirmed to correspond to GenBank entries for *Tbru*GSK-3 short (XM_822768), *Lmaj*GSK-3 short (XM_00168238) and *Lin*fGSK-3 short (XM_001464807). Site-directed mutagenesis of residues within the ATP-binding cavity of *Lmaj*GSK-3 short was performed on the expression plasmid using the QuikChange protocol (Stratagene, Santa Clara, CA).

Recombinant GSK-3 short proteins were expressed in *E. coli* BL21(DE3)* (Invitrogen, Carlsbad, CA) using Studier auto-induction protocols at 20°C [25]. Soluble *Tbru*GSK-3, *Lmaj*GSK-3 and *Lin*fGSK-3 short enzymes were purified by immobilized metal-affinity chromatography (IMAC) followed by size-exclusion chromatography (SEC). Soluble recombinant GSK-3 proteins were applied to a Ni²⁺-NTA (Qiagen, Valencia, CA) column in binding buffer composed of 20 mM HEPES pH 7.25, 500 mM NaCl, 5% glycerol, 30 mM imidazole, 0.5% CHAPS, and 1 mM TCEP. The Ni²⁺ column was washed with binding buffer without CHAPS, and the proteins were eluted with the same buffer supplemented with 250 mM imidazole. The eluted GSK-3 proteins were concentrated by centrifugal ultrafiltration and loaded into a 26/60 Superdex 75 SEC column. Fractions corresponding to GSK-3 were pooled and concentrated (e.g. Fig. 1A). The GSK-3 homologs were used for enzyme assays after IMAC and SEC purification.

Analysis of the Ni²⁺ SEC purified *Lmaj*GSK-3 short by SDS-PAGE showed the presence of two distinct but closely spaced major bands with molecular weights (MW) that closely matched that predicted for *Lmaj*GSK-3 short (Fig 1A). The SEC fractions with *Lmaj*GSK-3 short were pooled and dialyzed against 20 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol and 1 mM TCEP in the presence of 3C protease at a ratio of 1:50 overnight at 4°C. The protein sample was recovered from dialysis and the salt concentration of the buffer was adjusted to 500 mM NaCl and 30 mM imidazole. The 3C-treated sample was further purified by passage through a second gravity flow 3 mL Ni²⁺ chromatography column to remove the His-Tag 3C protease and any uncleaved *Lmaj*GSK-3 short. The recombinant protein was further resolved by cation exchange chromatography to separate the phosphorylated and unphosphorylated versions of the protein. The Ni²⁺ SEC/3C cleaved protein sample was desalted into 50 mM MES pH 7.0 with 5% glycerol and 1 mM TCEP, loaded into a 6 mL Resource S column and eluted using a linear gradient to 500 mM NaCl (Buffer B) in 40 column volumes. The cation exchange column step yielded 2 major peaks (Fig. 1B). Pure samples of Ni²⁺ SEC purified *Lmaj*GSK-3 short after the cation exchange step were analyzed by MALDI/TOF as previously described [26]. The first peak was composed of phosphorylated-GSK and the second peak was composed of non-phosphorylated GSK (Fig. 1C–D). LC/MS/MS was used to further characterize the phosphorylation by loading protein sample into an SDS-PAGE, excising the band corresponding to *Lmaj*GSK-3 short from the gel, and digesting it with trypsin. The resulting trypsin-cleaved peptides were analyzed by LC/MS/MS.

2.4. Activity assays

Protein kinase activity of recombinant GSK-3 homologs was assayed by Scintillation Proximity Assay (SPA) [6] using streptavidin-coated beads, [γ - ^{33}P] ATP (Amersham Biosciences, Piscataway, NJ), and biotinylated peptide substrate (BioGSP-2. Peptide sequence: Biotin-C6-Tyr-Arg-Arg-Ala-Ala-Val-Pro-Ser-Pro-Ser-Leu-Ser-Arg-His-Ser-Ser-Pro-His-Gln-Ser(PO_3H_2)-Glu-Asp-Glu-Glu-Glu) (American Peptide Company, Inc. Sunnyvale, CA) [6,27]. Further measurement of activity and inhibition of protein kinase phosphorylation properties by small molecule inhibitors in wild type and mutant enzymes was done by a non-radioactive Kinase-Glo luciferase assay reagent (Promega, Madison, WI). This reagent evaluates protein kinase activity by measuring changes in initial ATP concentration after GSK-3 phosphorylation reaction via luminescence [28]. A filtration assay, measuring direct incorporation of [γ - ^{33}P] onto BioGSP-2 peptide substrate and its subsequent binding to a P-81 filter (Whatman, Florham Park, NJ), was used to confirm inhibition of protein kinase phosphorylation properties, K_m of ATP and K_m of BioGSP-2 peptide substrate of GSK-3 enzymes. Enzyme activity assays in the presence of 3.2 μM BioGSP-2; 8.2 nM *Lmaj*GSK-3, *Lmaj*EGSK-3, *Lmaj*FGSK-3 or *Lmaj*FEGSK-3; or 2.8 nM *Linf*GSK-3; 2.7 nM *Tbru*GSK-3 with or without inhibitor(s) were performed in a buffered solution containing 25 mM Tris-HCl (pH 7.5), 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mM DTT (dithiothreitol), 0.1 mg/mL BSA plus 2 U/mL heparin. The reaction was initiated with addition of 2 μM ATP and incubated for 90 minutes at 30°C. The K_m for ATP and peptide substrate (BioGSP-2) was measured in a filtration assay based on the incorporation of [γ - ^{33}P] into the peptide after 90 minutes at 30°C and its subsequent binding to a P-81 filter (Whatman, Florham Park, NJ) [6]. Human GSK-3 β , enzyme assay buffer, and control wells with no peptide substrate were included in each assay plate as internal positive and negative controls. Kinase-Glo luminescence and SPA reaction results were read as counts per second and counts per minute, respectively, on a Chameleon 425-104 multi-label plate scintillation counter (Hidex, Oy, Turku Finland).

2.5. Protein Crystallization

Purified *Lmaj*GSK-3 short protein was screened at the high-throughput crystallography facility at Hauptman-Woodward Institute [29] as previously described [30]. After 4 weeks, there was a crystal. The initial conditions were subsequently optimized for crystal growth using vapor diffusion from sitting drops. Crystals for diffraction measurement were grown by adding 1 μL protein at 15 mg/mL to 1 μL reservoir solution consisting of 70% v/v TacsimateTM (Hampton Research, Aliso Viejo, CA), 0.1 M bis-Tris, pH 7.0. TacsimateTM contains 1.8305 M malonic acid, 0.25 M ammonium citrate tribasic, 0.12 M succinic acid, 0.3 M DL-malic acid, 0.4 M sodium acetate trihydrate, 0.5 M sodium formate, and 0.16 M ammonium tartrate dibasic.

2.6. X-ray diffraction and structure determination

Crystals of *Lmaj*GSK-3 short were frozen directly in liquid nitrogen. Data were collected to 2.0 Å resolution from a single crystal on a MarMosaic 300 mM CCD detector on beamline 23 ID-D at the Advanced Photon Source, Argonne IL, at a wavelength of 0.9184 Å. Data were processed and scaled using HKL2000 [31]. Data collection statistics are given in Table 2. The structure was solved by molecular replacement using the *Hs*GSK-3 β structure (PDB 1GNG) [32] after removal of loops and ligands. Molrep [32,34] placed two copies of the model into the asymmetric unit yielding a correlation coefficient 0.48. Iterative manual model building using Coot [35] and refinement in REFMAC continued until R and Rfree reached 0.226 and 0.224, respectively. Model quality was validated using Coot and MolProbity [35,36]. The structure has been deposited in the Protein Data Bank with access code 3E3P.

2.7. Modeling of glycine-rich loop

In order to be able to perform docking studies with inhibitors, Modeller (v8.2) [37] was used to generate coordinates for the glycine-rich loop and the hinge regions of *Lmaj*GSK-3 short, which are disordered in the crystal structure. The template for modeling was *Hs*GSK-3 β (PDB_ID: 1PYX) [38].

2.8. Modeling of inhibitors

The inhibitors used in the assays were docked into the *Lmaj*GSK-GH model using the GOLD software (Genetic Optimization of Ligand Docking, GOLD 4.0.1, CCDC, UK) [39]. The ligand-binding site in *Lmaj*GSK-3 was defined as all residues within a 10 Å radius of the main chain carbonyl oxygen atom of Glu 101. The number of poses for each inhibitor was limited to 10, and they were clustered so that conformations within 1.5 Å RMSD (root mean square difference) of each other were considered identical. The individual binding poses of each ligand were ranked according to the GOLD score. The top ranked conformation of each ligand was selected and visualized using Hermes 1.0.1 (Hermes 1.0.1, CCDC, UK) [39] to examine the mode of protein-inhibitor binding, then examined individually.

Prior to docking inhibitors into the *Lmaj*GSK-3 short structure, we verified that the GOLD software was able to reproduce the correct pose of the substrate analog phosphoaminophosphonic acid-adenylate ester ANP. The binding mode of ANP is known when bound to human GSK (PDB 1PYX) [38]. The RMSD between the docked pose of ANP in the *Lmaj*GSK-3 short structure and its bound conformation in the crystal structure 1PYX [38] was 0.56 Å when the protein structures were superimposed. Thus, it seems likely that the docking results with *Lmaj*GSK-3 short are reasonable.

3. Results

3.1. Leishmania spp. GSK-3 short: Expression, Protein kinase Activity, and Structure-Activity Relationships of Inhibitors

The short GSK-3 orthologs of *T. brucei*, *L. major* and *L. infantum* were analyzed in a protein kinase assay using a phosphopeptide commonly used to assay GSK-3 enzymes [6] and found to be active in transferring phosphate group from ATP to the peptide substrate. The K_m values of the two substrates, ATP and BioGSP2 (Table 3), were determined by varying the concentrations of both substrates at constant enzyme concentration for each GSK-3 homolog.

Eleven commercially available protein kinase inhibitors were assayed for activity against *Lmaj*GSK-3 short and *Linf*GSK-3 short and compared for activity against *Tbru*GSK-3 short (Table 4) using an ATP consumption assay in which changes in initial ATP concentration were monitored by luciferase and light production (Kinase-Glo). The inhibitor results demonstrate that the SARs for the *Leishmania* spp. GSK-3 short are nearly identical. Surprisingly, the SAR of *Tbru*GSK-3 short was distinct from the *Leishmania* spp. GSK-3 short for some of these inhibitors. The most obvious example is the GW8510 inhibitor, a potential candidate for further chemical and structural optimization. GW8510 was earlier identified as a versatile scaffold for the development of an effective neuroprotective compound that could have therapeutic value in the treatment of human neurodegenerative diseases [40]. GW8510's IC₅₀ value for *Tbru*GSK-3 short was 21 nM compared to 840 nM for *Lmaj*GSK-3 short and 628 nM for *Linf*GSK-3 short (Table 4, Fig. 2). The two most potent inhibitors of *Lmaj*GSK-3, GW8510 and CDK 1/2 inhibitor III showed competition for the ATP binding site. This was demonstrated by a reduction in velocity when increasing concentrations of the inhibitors were added to the reaction (Fig. 3).

3.2. Phosphorylation and Mass spectrometry analysis of *Lmaj*GSK-3 short

Analysis of *Lmaj*GSK-3 short, prepared in *E. coli*, showed the presence of two protein forms with MW suggesting a phosphorylated form and an unphosphorylated form (Fig. 1A). The recombinant protein was further resolved by cation exchange chromatography, yielding 2 major peaks (Fig. 1B). Analysis by SDS-PAGE showed that both peaks were composed of a 41-kDa protein band, which matched the predicted MW of 3C-cleaved *Lmaj*GSK-3 short (Fig. 1C). MALDI/TOF analysis of the resulting peaks showed that the first peak was composed of phosphorylated-GSK and the second peak was composed of non-phosphorylated GSK (Fig. 1D). LC/MS/MS analysis showed the presence of 4 peptides with phosphorylated amino acids corresponding to Ser2 ($\text{MS}^{\text{P}}\text{LNAAAAADER}$), Ser232 ($\text{GDNS}^{\text{P}}\text{AGQLHEIVR}$), Tyr264 ($\text{LNPSHTDVL}^{\text{P}}\text{YNSK}$) and Tyr305 ($\text{MKPY}^{\text{P}}\text{EALCHPYDELHDPATK}$). Both phosphorylated and non-phosphorylated *Lmaj*GSK-3 short were highly active in the kinase activity assay. There was no detectable doubly phosphorylated *Lmaj*GSK-3 short and none of the detected phosphorylation sites were within the activation loop. This does not however rule out the possibility of physiological phosphorylation within the activation loop since activation phosphorylation may occur in *Leishmania* spp. by other kinases.

3.3. Overall structure description

The structure of the non-phosphorylated form of *Lmaj*GSK-3 short was solved to 2.0 Å resolution by molecular replacement (Table 2). Residues 7–23 and 34–353 are well defined by electron density for monomer A and 7–23 and 33–353 for monomer B. The protein (Fig. 4) has a typical two-domain kinase fold [41] in which each domain is connected via a linker or hinge region (residues 102–106). The β -strand domain at the N-terminal end consists of a β sheet of five antiparallel strands in the order of 1–2–3–5–4 (residues 20–22, 35–39, 45–52, 94–101, and 79–86, respectively). The β strands 1–3 lie between two helices α 1 (10–18) and α 2 (59–72). The α -helical domain at the C-terminal end consists of 17 helices (Fig. 5). The ATP-binding site and the activation loop (residues 169–187) lie between the two domains near the hinge. The missing residues of 24–33 make up the glycine-rich loop, which forms the top of the active site (Fig. 4 and 5).

3.4. Active site comparison

The ATP-binding site that can be occupied by inhibitors consists of five areas (Table 5): 1) the adenine site next to the hinge region of the enzyme, 2) a hydrophobic pocket close to the N6 of the adenine ring whose size is controlled by the so-called gatekeeper residue, 3) a pocket next to N3 of the adenine ring, 4) a fairly open region where the ribose binds, and 5) the binding site for triphosphate plus Mg (II) [42]. Most inhibitors only occupy some of these areas [42].

The 21 residues lining each of these five areas in *Lmaj*GSK-3 short and their counterparts in the related *Tbru*GSK-3 and *Hs*GSK-3 β are listed in Table 5. Only 3 residues differ between *Lmaj*GSK-3 short and *Hs*GSK-3 β . They line the adenine, gatekeeper, and the N3 pockets. Ala 26 of *Lmaj* and *Tbru*, which originates from the so-called glycine-rich loop, sits above the adenine plane and is replaced by an isoleucine in the human enzyme. In principle, the presence of a smaller residue in the parasite enzymes provides more space for larger, bulkier inhibitors. However, most protein kinase inhibitors cannot exploit this difference because they mimic the adenine by a planar ring system that forms hydrogen bonds to the hinge region analogous to those formed by the N1 and N6 of adenine, thereby presenting their planar ring perpendicular to Ala 26, a direction which is unreachable by substituents attached to such scaffolds.

Another residue in the adenosine pocket, Tyr 102 in *Lmaj*GSK-3 short, which originates from the hinge region of the bilobal enzyme, might offer opportunities for selectivity in *T. brucei*, which has a phenylalanine in that position. Unfortunately the homologous residue in *Hs*GSK-3 β is also Tyr 134 (Table 5), thus selectivity cannot be obtained between human and *Lmaj*GSK-3 short. In the gatekeeper position of *Lmaj*GSK-3 and *Tbru*GSK-3 there is a methionine (Met 100 or 101, respectively), whereas *Hs*GSK-3 β gatekeeper is a Leucine (Leu 132). Both Met and Leu residues have approximately the same van der Waals volume, but maybe the difference between an unbranched and a branched hydrophobic residue can be exploited. Finally, in a pocket near N3 of the adenine ring, *Lmaj*GSK-3 but not *Tbru*GSK-3, possesses Asp 105 instead of the Glu 137 as seen in the human enzyme. Comparison of the crystal structures reveals a difference at this position. Glu 137 of the human enzyme makes a salt bridge with Arg 141, but the shorter Asp 105 of *Lmaj*GSK-3 is apparently unable to partner with Arg 109 and instead forms a hydrogen bond with the backbone carbonyl of Pro 104, thereby partially filling the pocket. Thus, *Lmaj*GSK-3 short is expected to be more restrictive to inhibitor size in this region compared to the human and the *T. brucei* enzymes.

3.5. The SAR of inhibition of *Lmaj*GSK-3 short by compounds can be explained by molecular modeling

Despite repeated attempts, we were unable to obtain *Lmaj*GSK-3 short structures co-crystallized with inhibitors. Since the glycine-rich loop was missing in the structure, yet is known to interact with inhibitors, we created a model of the GSK-3 short (Fig. 5). The inhibitors listed in Table 4 were docked with GOLD into the *Lmaj*GSK-3 short model. Their predicted poses agree with those observed experimentally in various crystal structures of these inhibitors in complex with *Hs*GSK-3 β . For example, in the case of CDK1/2 inhibitor III the diamino-substituted, triazole is predicted to bind to the adenine pocket and form hydrogen bonds with the backbone carbonyl of Glu 101 and the backbone nitrogen and carbonyl of Val 103 (Fig. 4 and 6A). The attached phenylsulfonamide is predicted to sit in the pocket near adenine N3. The difluorophenyl is predicted to occupy the PPP pocket and form hydrogen bonds to the amidino group of Arg 109 and the backbone of Ala 26. This CDK1/2 inhibitor III has been observed in several protein kinase structures in the same binding pose: with casein kinase 1 gamma 3 [PDB: 2CHL], ste20-like kinase [PDB: 2J51; 2JFL] [43], and CAM4K [PDB: 2W4O; unpublished SGC structure].

In the case of GW8510, the thiazolo-indolinone ring of the inhibitor is predicted to sit in the adenine pocket and form hydrogen bonds with the backbone carbonyl of Glu 101 and the backbone nitrogen of Val 103 (Fig. 6B). The attached p-aminophenyl is predicted to make a hydrogen bond to the backbone carbonyl of Val 103. The terminal pyridine group is predicted to sit against Ala 26 and to form additional hydrogen bonds to Met 25 compared to CDK1/2 inhibitor III. These additional interactions may account for GW8510's enhanced potency as an inhibitor, compared with CDK 1/2 inhibitor III. The GW8510 inhibitor was observed in the same pose as in human CDK2 [PDB: 1FVV] [44].

3.6. Differences in the SAR between *Leishmania* spp. GSK-3 and *T. brucei* can be explained by 2 residues that line the ATP-binding pocket

As noted above, there were two amino acid variations that caused differences in the ATP-binding pocket of *Leishmania* spp. GSK-3 and *T. brucei* GSK-3 short, namely Tyr102Phe [Y102F] and Asp105Glu [D105E] (1st AA is from *Lmaj*GSK-3 short, # based on *Lmaj*GSK-3 short, 2nd AA is the homologous AA in *Tbru*GSK-3 short). We hypothesized that these residues in the *Lmaj*GSK-3 short might inhibit the binding of inhibitors, such as GW8510 and CDK1/2 Inhibitor III, thus explaining the more potent inhibition of GW8510 and CDK1/2 Inhibitor III against *Tbru*GSK-3 short vs. *Lmaj*GSK-3 short. To test this

hypothesis, we made recombinant *Lmaj*GSK-3 short mutants with single substitutions Y102F or D105E and with both substitutions. All of these mutants were found to be enzymatically active. The mutation Y102F of *Lmaj*GSK-3 short had a partial effect, conferring more than 10-fold sensitivity to GW8510 inhibition, while mutation D105E had nearly 2 fold effect (Table 4, Fig. 2). However, the double mutant (Y102F and D105E) was remarkably sensitive to GW8510 and CDK1/2 Inhibitor III, with a value roughly equal to that of the IC₅₀ of *Tbru*GSK-3 short (Table 4, Fig. 2). The double *Lmaj*GSK-3 short mutant had a decreased IC₅₀, compared to that of wild type *Lmaj*GSK-3 short, for GW8510 and CDK1/2 Inhibitor III (Table 4, Fig. 1). GW8510 and CDK1/2 inhibitor III were predicted to have limited contact with Tyr 102 (Y102) and Asp105 (D105) amino acids in *Lmaj*GSK-3 short wild type enzyme and clearly these interactions increase with mutant Phe 102 (F102) and Glu 105 (E105) residues. The greatest single mutation effect was observed when the mutants were tested against GW8510. The *Lmaj*GSK-3 short IC₅₀ value of 840 nM for GW8510 was reduced to 93 nM for the *Lmaj*GSK-3 short Y102F mutant. These observations support our inhibitor-binding mode model that suggests a better interaction in the adenine binding pocket between the aromatic rings of GW8510 with the hydrophobic phenylalanine residue relative to the less hydrophobic and more acidic tyrosine residue occupying the same position in WT *Lmaj*GSK-3. However, it is notable that *Hs*GSK-3 β has the same tyrosine residue as *Lmaj*GSK-3 short at the homologous residue, but it is rotated 180 degrees away from the inhibitor-binding site. Thus other dynamic processes must explain the increased affinity for GW8510 for binding *Hs*GSK-3 β vs. wild type *Lmaj*GSK-3 short. The observations also suggest that compounds that fill more space and hydrogen bonds where they contact Asp 105 will be more successful inhibitors of *Lmaj*GSK-3 short. Therefore, potency of selected compounds may be improved by making analogues with a bulkier functional group having tighter contact with Asp 105 of *Lmaj*GSK-3 short.

4. Discussion and Conclusion

Increasing interest in GSK-3 enzymes [6,7,11] for anti-parasitic drug development warrants detailed understanding of their structure and SAR. This study presents the first solved structure of a parasitic protozoan GSK-3 enzyme, i.e. *Lmaj*GSK-3 short. There are 2 orthologs of GSK-3 enzyme in *Leishmania* spp. and *T. brucei*, GSK-3 short and GSK-3 long. We focused on the short orthologs of *Leishmania* spp. (*Lmaj*GSK-3 short (GeneDB LmjF18.0270) and *Lin*fGSK-3 short (GeneDB LinJ18.0270)) since the orthologous *Ldon*GSK-3 short (98% sequence identity with *Lmaj*GSK-3 short and 100% identity with *Lin*fGSK-3 short) and the *Tbru*GSK-3 short were recently shown to be potential drug targets [6,7]. Similarly, there was no obvious evidence of overlap in functional characteristics that can suggest compensation of roles when *Tbru*GSK-3 short or *Tbru*GSK-3 long gene products were separately ablated by RNAi [6]. Furthermore, similarities in the ATP binding domains of GSK-3 short and GSK-3 long may be sufficient enough for an inhibitor to hit both targets at the same time. By extrapolating from the *Tbru*GSK-3 genes, it would be anticipated that inhibitors of *Leishmania* spp GSK-3 short would be sufficient to stop the growth and proliferation of these parasites. Moreover, Xingi *et al* [7] recently identified *Ldon*GSK-3 short as the predominant intracellular target of 6-Br-5methylinindirubin-3'-oxime (5-Me-6-Bio) in *L. donovani* and provided genetic evidence for a potential role in cell-cycle progression and apoptosis-like death (note that our results from *Lin*fGSK-3 short inhibitors will be the same for that of *Ldon*GSK-3 short as the amino acid sequences of these enzymes are identical). Thus, there is reasonable evidence that GSK-3 short is a promising drug target for *Leishmania* spp. and their GSK-3 long activities may not provide sufficient compensation to reverse potent inhibitors' effects

All 11 protein kinase inhibitors are predicted to bind within the enzyme active site at the ATP-binding pocket of *Lmaj*GSK-3 short. Direct evidence of inhibitor competition for ATP

binding site was demonstrated by a decrease in velocity when different concentrations of the most active compounds CDK1/2 Inhibitor III and GW8510 were added to the reaction across increasing concentrations of ATP (Fig. 3). *Lmaj*GSK-3 short has a hydrophobic cavity in its active site, and it is expected that these inhibitors are capable of interacting with the hydrophobic pocket and more sub-sites near this pocket; thus, further optimization could lead to more potent inhibitors and possibly leishmania-specific inhibitors. Such an expectation is consistent with our bioassay results, as exemplified by species inhibitory differences among all inhibitors (Table 4).

The compounds CDK1/2 Inhibitor III and GW8510 have lowest IC₅₀ values against *Lmaj*GSK-3 short. All attempts to co-crystallize these and other inhibitors with *Lmaj*GSK-3 short failed. However the structure and molecular modeling suggests that CDK1/2 Inhibitor III and GW8510 occupy almost the whole active pocket of GSK-3 and interact with conserved residues in the hinge region via hydrogen bonding, thus explaining their potency (Fig. 6A and B).

It is important to note that many of the compounds in this study are known to inhibit multiple human protein kinases; hence, off-target effects are a potential drawback of these compounds. However, with our knowledge and understanding of compound interaction with residues within the ATP-binding cavity described above, there is a greater possibility of designing inhibitors that will be relatively specific for *Leishmania* spp GSK-3. A screen for new scaffolds inhibiting *Lmaj*GSK-3 short, using high-throughput screening of focused protein kinase inhibitor libraries, might yield new leads that have selective activity against *Lmaj*GSK-3 short.

Alternatively, non-selective compounds that effectively target both *Leishmania* GSK-3 and *Hs*GSK-3 β may be suitable for anti-leishmanial chemotherapy, as GSK-3 inhibitors have not been found to be toxic when administered to rodents [13,16–19]. Indeed, GSK-3 inhibitors are being actively studied by pharmaceutical companies for the therapy of Alzheimer's, diabetes, and mania [13–19].

Recent studies have shown that *Hs*GSK3 β inhibition is associated with the inhibition of inflammation by attenuating activation of the pro-inflammatory transcription factor NF κ B. *Hs*GSK3 β inhibition has also been shown to induce secretion of the anti-inflammatory cytokine IL-10 [45–47]. Thus, anti-inflammatory effects of *Hs*GSK3 β inhibitors may reduce the inflammatory pathology of leishmaniasis. Both anti-parasitic and anti-inflammatory properties of GSK3 inhibitors would aid in the successful treatment of leishmaniasis.

In conclusion, the GSK-3 short enzymes of *L. major* and *L. infantum* have been recombinantly expressed and the crystal structure of *Lmaj*GSK-3 short was solved. Our structural and docking studies combined with mutational studies demonstrate a solid understanding of the binding mode of various inhibitors and delineate ways to predict inhibitors that might be useful for the therapy of leishmaniasis. The inhibitor SAR of *Lmaj*GSK-3 short and *Linj*GSK-3 short (and by inference, *L. donovani*) are virtually identical, suggesting that compounds inhibiting GSK-3 short could be useful for the therapy of both cutaneous and visceral leishmaniasis.

Summary

This study presents the first solved structure of a parasitic protozoan GSK-3 enzyme and concluded that *Lmaj*GSK-3 short structure reported here will aid in the design and development of potent GSK-3 inhibitors for all *Leishmania* species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (awards GM64655, AI067921, AI080625) and UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). K.K.I. was supported by a US National Institutes of Health grant from the Fogarty International Center 2D43 TW000924. We thank Molly C. Reid, Isolde LeTrong and Eric Larson for assistance with data collection. GM/CA CAT has been funded in whole or in part with Federal funds from the National Cancer Institute (Y1-CO-1020) and the National Institute of General Medical Science (Y1-GM-1104). Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under contract No. DE-AC02-06CH11357. K.K. Inampudi thanks the Cambridge Crystallographic Data Centre for providing the GOLD software.

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Fig. 1.

A. SDS-PAGE coomassie blue stained gel of recombinant *Lmaj*GSK-3 short protein fractions, eluted during size exclusion chromatography (fractions 4–13 shown), demonstrating the presence of two distinct but closely spaced major bands with MW that closely match the predicted MW of *Lmaj*GSK-3 short. The M lane is molecular weight standards with the 40 and 50 kDa standards marked to the left. B. UV 260nm absorbance tracing showing peak 1 and 2 eluted from the cationic exchange column. The steady upsloping line shows increasing buffer B during elution of the cation exchange. C. SDS-PAGE coomassie blue stained protein fractions (peak 1 and peak 2) eluted from the cationic exchange. The M lane is molecular weight standards with the 40 kDa standard marked to the left. D. LC-MS tracings demonstrates the band in peak 1 correlated with mono-phosphorylated *Lmaj*GSK-3 short (41092 Da) while the major peak 2 band correlated with non-phosphorylated *Lmaj*GSK-3 short (41010 Da).

Fig. 2.

Titration of GW8510 and the varying forms of *LmajGSK-3* short (and mutant forms), *LinfGSK-3* short, and *TbruGSK-3* short. *LmajE* and *LmajF* represent mutants of *LmajGSK-3* short with substitutions in the Y102F (*LmajF*) and D105E (*LmajE*) sites (Table 4) to represent residues similar to that of *TbruGSK-3* short. *LmajFE1* represents preparations of double mutants with both Y102F and D105E substitutions.

Fig. 3. Untransformed plots for the effects of different concentrations of ATP competitive inhibitors GW8510 and Cdk1/2 inh. III on the velocity of *LinfGSK-3* short (A) and *LmajGSK-3* short (B).

Fig. 4.

Crystal structure of *Lmaj*GSK-3 short modeled with the CDK1/2 Inhibitor III. The α -helical and β -strand domains are colored cyan and blue, respectively. The hinge and activation loop of the active site are highlighted in yellow. The glycine-rich loop that is disordered in the crystal structure is depicted as magenta dots. The CDK1/2 Inhibitor III is absent from the crystal structure, but drawn here to depict the location of the inhibitor-binding site.

Fig. 5. Overlay of the *LmajGSK-3 short* crystal structure (blue) and model (grey). The modeled glycine-rich loop is not visible in the crystal structure and is colored in red. The right panel is rotated 30 degrees compared to the left.

Fig. 6.

A) Model of *Lmaj*GSK-3 short with CDK 1/2 inhibitor III showing sidechain interactions to Arg 109 and backbone interactions to Glu 101, Val 103, and Ala 26. B) Model of *Lmaj*GSK-3 short with GW8510 showing similar interactions as in CDK 1/2 inhibitor III, but with additional backbone interactions to Met 25.

Table 1

Comparison of amino acid identities (%) of human GSK-3 β vs. *T. brucei* GSK-3 short and *L. maj* GSK-3 short.

	<i>Hs</i> GSK-3 β	<i>Lmaj</i> GSK-3 short	<i>Tbru</i> GSK-3 short
<i>Lin</i> fGSK-3 short	40.9	98.0	63.4
<i>Hs</i> GSK-3 β		40.9	43.5
<i>Lmaj</i> GSK-3 short			63.9

Table 2

Data-collection and refinement statistics for L. major GSK-3 short

Wavelength (Å)	0.9184
Resolution (Å)	50 – 2.0 (2.00 – 2.07)
Space group	C222 ₁
Unit cell (Å)	a = 70.2, b = 155.7, c = 158.8.
Total number of observations	358269
Unique reflections	54336 (3879)
R _{merge}	0.087 (0.691)
Completeness (%)	92.3 (66.6)
Mean I/σ(I)	20.1 (1.7)
Redundancy	6.6 (5.0)
Wilson B factor (Å ²)	32.1
Refinement	
Resolution (Å)	40 – 2.0
R/R _{free}	0.226/0.271
Rmsd bonds (Å)	0.022
Rmsd angles (°)	1.968
Protein atoms	5415
Water molecules	196
Mean B protein (Å ²)	46.1
Mean B other atoms (Å ²)	39.8
Residues in most favored regions (%)	96.1
Residues in allowed regions (%)	100.0
Residues in disallowed regions (%)	0

Table 3

Recombinant Glycogen Synthase Kinase-3 short kinetic characteristics

	<i>Tbru</i> GSK-3	<i>Lmaj</i> GSK-3	HsGSK-3b	<i>Linf</i> GSK-3
$K_m(\text{ATP})$ (μM)	9.42 ± 2	8.66 ± 3	9.25 ± 0.84	6.86 ± 1.7
$K_m(\text{BioGSP-2})$ (μM)	6.56 ± 3.6	6.61 ± 2.42	6.83 ± 0.86	5.41 ± 0.65

Inhibitors: Mean IC ₅₀ in μM (SD)						
	Indirubin-3'-monoxime-5-sulphonic Acid	JAK3 Inhibitor VI	Alster-paullone, 2-cyanoethyl	SU9516	Cdk1/2 Inhibitor III	Hymeni-aldisine, <i>Stylissa dami-cornis</i>

	2.22 (0.07)	4.04 (2.49)	1.04 (0.40)	1.87 (0.09)	0.26 (0.09)	18.25 (3.83)
	2.84 (1.01)	6.79 (0.07)	1.00 (0.22)	3.04 (0.24)	0.32 (0.09)	13.13 (4.51)
	8.63 (0.88)	5.82 (0.92)	4.76 (1.97)	3.15 (0.29)	0.46 (0.15)	10.42 (0.07)
	0.93 (0.15)	2.43 (0.05)	0.31 (0.04)	1.40 (0.35)	0.15 (0.05)	16.07 (2.77)
	2.29 (0.37)	6.22 (2.62)	0.87 (0.29)	1.29 (0.27)	0.18 (0.06)	9.25 (0.55)
	0.48 (0.02)	1.70 (0.48)	1.05 (0.29)	0.59 (0.09)	0.12 (0.04)	3.95 (0.47)
	0.06 (0.01)	1.16 (0.37)	0.02 (0.01)	0.41 (0.14)	0.21 (0.05)	0.10 (0.02)

Table 5

ATP binding site analysis

	L. major	L. infantum	T. brucei	Human
Adenine pocket	A26	A26	A26	I62
	V34	V34	V34	V70
	A47	A47	A47	A83
	E101(bb)	E101(bb)	E102 (bb)	D133 (bb [*])
	Y102	Y102	F103	Y134
	V103(bb)	V103(bb)	V104(bb)	V135(bb)
	L158	L158	L159	L188
Gatekeeper pocket	V77	V77	V77	V110
	M100	M100	M101	L132
“Near adenine N3” pocket	P104	P104	P105	P136
	D105	D105	E106	E137
	T106	T106	T107	T138
	R109	R109	R110	R141
Ribose region	G27	G27	G27	G63
	H155(bb)	H155(bb)	H156(bb)	Q185(bb)
PPP+Mg(II) pocket	Q28(bb)	Q28(bb)	Q28(bb)	N64(bb)
	G29	G29	G29	G65
	K49	K49	K49	K85
	K153	K153	K154	K183
	N156	N156	N157	N186
	D170	D170	D171	D200

* bb = the residue contributes to the binding site only through the backbone atoms.