Glycogen Synthase Kinase 3 Is a Potential Drug Target for African Trypanosomiasis Therapy


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Development of a safe, effective, and inexpensive therapy for African trypanosomiasis is an urgent priority. In this study, we evaluated the validity of Trypanosoma brucei glycogen synthase kinase 3 (GSK-3) as a potential drug target. Interference with the RNA of either of two GSK-3 homologues in bloodstream-form T. brucei parasites led to growth arrest and altered parasite morphology, demonstrating their requirement for cell survival. Since the growth arrest after RNA interference appeared to be more profound for T. brucei GSK-3 “short” (Tb10.161.3140) than for T. brucei GSK-3 “long” (Tb927.7.2420), we focused on T. brucei GSK-3 short for further studies. T. brucei GSK-3 short with an N-terminal maltose-binding protein fusion was cloned, expressed, and purified in a functional form. The potency of a GSK-3-focused inhibitor library against the recombinant enzyme of T. brucei GSK-3 short, as well as bloodstream-form parasites, was evaluated with the aim of determining if compounds that inhibit enzyme activity could also block the parasites’ growth and proliferation. Among the compounds active against the cell, there was an excellent correlation between activity inhibiting the T. brucei GSK-3 short enzyme and the inhibition of T. brucei growth. Thus, there is reasonable genetic and chemical validation of GSK-3 short as a drug target for T. brucei. Finally, selective inhibition may be required for therapy targeting the GSK-3 enzyme, and a molecular model of the T. brucei GSK-3 short enzyme suggests that compounds that selectively inhibit T. brucei GSK-3 short over the human GSK-3 enzymes can be found.

The vector-borne parasitic disease African trypanosomiasis, caused by members of the Trypanosoma brucei complex, is a serious health threat. It is estimated that 300,000 to 500,000 humans in sub-Saharan African are infected. If the disease is left inadequately treated, it often has a fatal outcome (9). Once infection is established, safe and effective therapy is critically important, yet it has been difficult to achieve. Despite the critical need, the available therapies are becoming less satisfactory due to the rising level of resistance to the available drugs, the long period of treatment required to achieve a cure, and the unacceptable and sometimes severe adverse effects associated with current therapies (9). An urgent priority is to identify and validate new targets for the development of safe, effective, and inexpensive therapeutic alternatives.

Recent advances in the area of parasite genomics and biochemical investigation of the physiologically important enzymes necessary for the parasite’s survival have identified protein kinases as potential drug targets in treatments for trypanosomatiid diseases (3, 14, 23). Protein kinases play an important role in cell survival by phosphorylating and regulating many activities of the cell, including protein synthesis, gene expression, the subcellular localization of proteins, and the protein degradation machinery. Many kinases have been examined for the physiological relevance of their phosphorylation activities in other organisms, and glycogen synthase kinase 3 (GSK-3) has been found to be essential in many fundamental cellular processes (22, 30).

Far from being simply important in glycogen synthesis, the activity of GSK-3 is now recognized as key in mammalian cell signaling pathways for many cellular and physiological events (26). GSK-3 has been targeted for the treatment of several diseases, such as diabetes mellitus and Alzheimer’s dementia, and this enzyme has been found to be amenable to selective targeting with small-molecule drugs (22). GSK-3 has two isoforms in human cells, GSK-3α and GSK-3β. The GSK-3α and GSK-3β isoforms rarely diverge outside the N- and C-terminal regions. Within the ATP binding site of GSK-3, where most GSK-3 inhibitors bind, there appears to be only a single amino acid difference (Glu196 in GSK-3α, Asp133 in GSK-3β), and most inhibitors target both isoforms. GSK-3 generally requires a substrate that is prephosphorylated by a priming kinase (6, 7, 10, 32) (Fig. 1), leading to a role in signaling cascades. GSK-3 is regulated by autophosphorylation and phosphorylation by other enzymes.

Even though orthologs exhibit a high degree of sequence similarity within their catalytic domains (7, 22), there exists evolutionary differences between human and parasite homologues that might be sufficient to allow the design of parasite-specific inhibitors. Compounds that inhibit T. brucei GSK-3 activity and not host GSK-3 might be required for therapy for...
FIG. 1. Enzymatic action of GSK-3. With most GSK-3 substrates, another (priming) kinase first places a phosphate (PO4) on a serine or threonine (S/T) residue separated by three amino acids (X) in the carboxy direction to target S/T residues. GSK-3 then phosphorylates target S/T groups. Occasionally, GSK-3 has been shown to phosphorylate nonprimed peptide substrates and has been shown to autophosphorylate GSK-3 on S/T or tyrosine (10, 32).

Table 1: Oligonucleotide primers used

<table>
<thead>
<tr>
<th>Primer use and no.</th>
<th>ORF position</th>
<th>Primer designation</th>
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<td>RNAi</td>
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<td></td>
<td></td>
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<tr>
<td>1</td>
<td>176–578</td>
<td>GSKshort1Fwd</td>
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<td>GSKLongFwd</td>
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<td>7</td>
<td></td>
<td>GSKLongprobe1b</td>
<td>CACCTGACCCGGAACCTTCG</td>
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<td>8</td>
<td></td>
<td>GSKLongprobe2b</td>
<td>CAGCCCAAGGTGTAAAATTC</td>
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<td>GSK-3 protein expression</td>
<td>1–1059</td>
<td>T. brucei GSKLICFwd</td>
<td>CTCACCCACCCACCCACCTATAGTTGCTCAAC</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>T. brucei GSKLICRev</td>
<td>ATTCATCTTACCTTACCTTCTTCAGCATAG</td>
</tr>
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</table>

* Used as probes for Northern blot analysis of T. brucei GSK-3 long.
PCR and ligase-independent cloning. Regions of the *T. brucei* GSK-3 short gene open reading frame (ORF) were amplified from *T. brucei* 427 genomic DNA with the primers *T. brucei* GSKLICFwd, *T. brucei* GSKLICRev (primers 9 and 10, respectively, Table 1). The primer and probe sequences used in this study are listed in Table 1. The PCR product was cloned into the maltose-binding protein (MBP)-AVA0421 fusion vector. MBP-AVA0421 is a ligase-independent cloning vector that expresses proteins as an N-terminal six-His-MBP–3C cleavage site–ORF fusion. MBP-AVA0421 was generated from AVA0421 by ligating the PCR-amplified MBP sequence from eight-His–MBP–AVA0421 fusion vector.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Human GSK-3β</th>
<th><em>T. brucei</em> GSKLIC Rev</th>
<th><em>T. brucei</em> GSK-3 short</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human GSK-3β</td>
<td>67.4 (1e-177)</td>
<td>29.7 (2.2e-66)</td>
<td>35.6 (2.7e-82)</td>
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<tr>
<td>Human GSK-3β</td>
<td>33.1 (1.0e-71)</td>
<td>40.9 (9.1e-89)</td>
<td>30.6 (4.6e-66)</td>
</tr>
</tbody>
</table>

*The smallest sum probability by BLASTP search is shown in parentheses.*

**TABLE 2.** Comparison of amino acid sequence identities of the two human orthologs of GSK-3 and *T. brucei* GSK-3 long and GSK-3 short.

**FIG. 3.** Southern blot of *T. brucei* genomic DNA with the *T. brucei* GSK-3 short-specific probe. Genomic *T. brucei* 427 SM DNA was digested, blotted, and probed with the RNAi region for GSK-3 short 1 (S1) to verify that only a single band corresponding to the *T. brucei* GSK-3 short gene hybridizes in the *T. brucei* genome. The predicted restriction digest sizes are as follows: BamHI-PstI, 1,702 bp; BamHI-Aval, 2,214 bp; NcoI-PstI, 2,655 bp; NcoI-Aval, 3,167 bp.

**FIG. 2.** RNAi of *T. brucei* GSK-3 short homologs. (Top) Growth of *T. brucei* BSF as the number of days after tetracycline addition (day 0). *T. brucei* strain 427 SM cells were transfected with plasmids, which, after transfection, resulted in RNAi inhibition of target gene expression. Both homologs of GSK-3, *T. brucei* GSK-3 long and *T. brucei* GSK-3 short (two different constructs of *T. brucei* GSK-3 short), were separately targeted, SM cells served as no-plasmid controls. With the addition of tetracycline (+), dramatic growth inhibition compared with that for the no-tetracycline control (−) occurred when either the long or the short *T. brucei* GSK-3 homolog was targeted. (Bottom) Northern blots showing the reduction in mRNA levels (Gene) for the *T. brucei* GSK-3 short (S1 and S2) and *T. brucei* GSK-3 long (L) transcripts with tetracycline induction (lanes +) and no tetracycline induction (lanes −). The result for a tubulin mRNA control (Tub) differed little after RNAi induction with tetracycline.
of 10% trifluoroacetic acid in 37.5 mM EDTA and 750 μM unlabeled ATP. The content of each reaction mixture was spotted on P-81 filters, and the filters were washed three times in 2.5% phosphoric acid. The SPA was used to screen for inhibitors and determine the 50% inhibitory concentrations (IC₅₀) of 303 inhibitors against the T. brucei GSK-3 short version at the Kₘ determined for ATP and the peptide. The mixture used for the SPA included 20 ng (46.8 nM) of enzyme, 2.4 μM peptide substrate, 4.5 μM unlabeled ATP, and 4 nM γ-³²P-labeled ATP. The reaction mixture was incubated for 30 min. Quantitative measurement of the levels of phosphate incorporation and binding to BioGSP-2 by the T. brucei GSK-3 short enzyme was done with 100 μl of 20 mg/ml streptavidin-coated SPA beads in 37.5 mM EDTA–75 μM unlabeled ATP. In each assay, human GSK-3β (Upstate Cell Signaling Solutions, Temecula, CA) and assay buffer were used as positive and negative controls, respectively. The emission of light by this SPA bead simulation reaction was measured as counts per minute on a Chameleon 425-104 multilabel plate scintillation counter (Hidex; Oy, Turku, Finland).

**Parasite cultures and in vitro compound screening assays.** BSF T. brucei brucei strain 427 was cultured in HMI-9 medium supplemented with 10% heat-deactivated fetal bovine serum, 1% penicillin, and streptomycin at 37°C in a 5% CO₂ atmosphere, as described previously (15). The in vitro susceptibilities of the parasites to the compounds were tested in 96-well plates with an initial inoculum size of 1 × 10⁵ trypomastigotes per well. Compound stock solutions were prepared in dimethyl sulfoxide at 10 to 20 mM and diluted in HMI-9 medium to 20 μM. They were then added to the plates in serial dilutions for a final volume of 200 μl/well. The growth of triplicate cultures was quantitated after 48 h by the addition of Alamar blue (Alamar Biosciences, Sacramento, CA) (28). Pentamidine isethionate (Aventis, Dagen-

![Figure 4](image)

**FIG. 4.** Purity of recombinant T. brucei GSK-3 short produced in E. coli. The enzyme was purified by metal affinity chromatography, followed by size-exclusion chromatography. Three lanes representing 25 μg of protein from fractions from-size exclusion chromatography (fractions 1 to 3, respectively) that were pooled to make the working batch of enzyme for further study are shown. The purity was judged to be greater than 98%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC₅₀ (nM) for T. brucei GSK enzyme</th>
<th>EC₅₀ b (nM)</th>
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<tr>
<td>GW8510</td>
<td><img src="image" alt="Structure" /></td>
<td>1</td>
<td>119</td>
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<td>CDK-1 and -2 inhibitor III</td>
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<td>20</td>
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<tr>
<td>2-Cyanoethyl alsterpaullone</td>
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<td>SB-415286</td>
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<td>1,000</td>
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</table>

a The inhibitors were selected from among a panel of 48 kinase inhibitors.

b The EC₅₀ were determined by the T. brucei BSF growth assay.

**TABLE 3. Commercial kinase inhibitors with activity against the GSK enzyme and T. brucei BSFs**
ham, United Kingdom) was included in each assay as a positive control. The effective concentration which caused 50% growth inhibition (EC50) was calculated by nonlinear regression analysis with Prism software (Graphpad Software, Inc. La Jolla, CA).

Modeling of T. brucei GSK-3 homology and inhibitor docking. The Protein Data Bank contains 14 structures of human GSK, each with variations in the motion of the hinge domain between the N- and C-terminal lobes of the enzyme. For modeling of the three-dimensional structure of the T. brucei GSK-3 short version (Protein Data Bank accession no. 1R0E), human GSK-3 complexed with a 3-indolyl-4-arylmaleimide inhibitor was ultimately chosen as the template because the resulting model led to the best quantitative structure-activity relationships with the six Merck-Serono inhibitors with a 2(Z)-2-[4-(tert-butythiazol-2[3H]-ylidene)-2-(2-aminoxyrimidin-4-yl) acetonitrile core structure. Creation of the protein model with the Homology module of the INSIGHTII program (Accelrys Software, Inc., San Diego CA) was straightforward, since no insertions or deletions occurred in the binding site. Molecular docking of inhibitors was performed by Metropolis Monte Carlo procedures with the FLO/QXP program (19). Inhibitors were treated as fully flexible, as were the side chains of amino acid residues in the binding site.

RESULTS

Sequence analysis and RNAi. Two GSK-3-encoding genes were found in the T. brucei genome by BLAST homology searches: a long version (Tb927.7.2420, which encoded a predicted 501-amino-acid protein) and a short version (Tb10.61.3140, which encoded a predicted 352-amino-acid protein). Homology comparisons did not reveal which homolog might be equivalent to the GSK-3α or the GSK-3β mammalian form (Table 2), and both T. brucei GSK-3 long and T. brucei GSK-3 short are more homologous to human GSK-3β than to human GSK-3α. The knockdown of T. brucei GSK-3 short or long mRNA was accomplished by overexpressing double-stranded RNA in mammalian BSFs of T. brucei. Two RNAi constructs were separately tested to knock down the expression of the T. brucei GSK-3 short gene. At 48 h, Northern analysis demonstrated a 65% mRNA reduction for T. brucei GSK-3 short construct 1 and a 76% mRNA reduction for T. brucei GSK-3 short construct 2 (Fig. 2). Along with the reduction of T. brucei GSK-3 short mRNA, the induction of double-stranded RNA led to the striking inhibition of cell proliferation (Fig. 2). Induction of the T. brucei GSK-3 long RNAi construct yielded an 82% reduction in the mRNA for that gene (Fig. 2), but the inhibition of growth was not as complete as that produced by the T. brucei GSK-3 short constructs (Fig. 2). Because of the more pronounced growth inhibition by RNAi directed against T. brucei GSK-3 short, we chose to focus further work on the T. brucei GSK-3 short enzyme.

Southern blot analysis. Hybridization of a Southern blot membrane with a radiolabeled GSK-3 short-specific probe (the same sequence used for RNAi) revealed a single band that hybridized with four double restriction enzyme digests to rule out a possible off-target effect for the RNAi sequence. In each case, the molecular weight of the hybridizing band was predicted from the T. brucei GSK-3 short genomic sequence (Fig. 3). The presence of a single band for each digest at the molecular weight predicted for T. brucei GSK-3 short indicates that there is only one sequence complementary to the RNAi segment used in the genome of T. brucei, and it is the gene for T. brucei GSK-3 short.

Expression of highly active recombinant T. brucei GSK-3 short enzyme. The recombinant T. brucei GSK-3 short construct was produced in Escherichia coli as a C-terminal fusion with MBP (Fig. 4), because the non-MBP-fused T. brucei GSK-3 short enzyme was insoluble in E. coli. The Km of the enzyme substrate for ATP was determined to be 4.5 μM, while the Km for the phosphorylated peptide (GSP-2) was 2.4 μM. The specific activity of the purified T. brucei GSK-3 short enzyme was found to be 1,000 U/mg (where 1 U was defined as the incorporation of 1 nM phosphate into 1.2 μM GSP-2 per min at 30°C with a final ATP concentration of 1.2 μM), which is comparable to the value of 922 U/mg cited for commercially available human GSK-3β (lot 28407U; Upstate Cell Signaling Solutions).

Activities of kinase inhibitors against T. brucei GSK-3 short construct and T. brucei BSF parasites. Screening of 48 commercially available protein kinase inhibitors revealed that GW8510 (Table 3) has activities at 1 nM (IC50) against the T. brucei GSK-3 short enzyme and about 100 nM (EC50) against BSFs of T. brucei. We have also identified other compounds (Table 3) with activities against the enzyme, but they may be inhibiting additional targets in the T. brucei cells, as suggested by the comparatively low EC50 in comparison to their IC50.

In a screen against 255 compounds with known activity against human GSK-3β (Merck-Serono), we observed a correlation between enzyme and cellular activity, which supports the chemical validation of T. brucei GSK-3α as a drug target (examples are shown in Table 4). An excellent correlation between enzyme inhibition and cell growth inhibition was demonstrated for the compounds of this series that inhibited the T. brucei GSK-3 enzyme and T. brucei BSF growth. Further research is required to establish if the “hit” compounds identified kill T. brucei cells by specifically inhibiting GSK-3 short, but the chemical data support validation of GSK-3 short as a drug target for T. brucei.

Model of T. brucei GSK-3 with Merck-Serono inhibitors. A homology model of T. brucei GSK-3 short was constructed on the basis of a crystal structure of the human GSK-3β enzyme in

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>IC50 (nM) for T. brucei GSK enzyme</th>
<th>ED50 (nM)</th>
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<tr>
<td>1</td>
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<tr>
<td>6</td>
<td>168</td>
<td>705</td>
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</tbody>
</table>

* The inhibitors were selected from a panel of 255 GSK-3β inhibitors.
* The EC50 were determined by the T. brucei BSF growth assay.
complex with an inhibitor. Subsequently, the six Merck-Serono inhibitors listed in Table 4 were docked to validate the model by determination of the structure-activity relationship. A linear regression between the predicted binding energies and experimental energies derived from the IC50s showed an excellent correlation ($R^2 = 0.85$; $n = 6$; $F = 23.3$, which indicates a significance level of 99.0%) (Fig. 5). The predicted binding mode of 30 nM Merck-Serono inhibitor 2 is shown in Fig. 6. It features a double hydrogen bond recognition pattern that is typical for many kinase inhibitors and that involves the 2-amino-pyrimidinyl group of the inhibitor and a consecutive hydrogen bond donor and acceptor from the protein backbone. Several hydrophobic contacts are present between the thiazolyl and Cys-170, as well as the Phe-31 originating from the glycine-rich loop characteristic for kinases; the nitrile and the “gatekeeper” residue Met-101; and the pyrimidinyl and Leu-159. The N-methyl-piperidinyl makes a hydrogen bond to the carbonyl of Pro-105 and is further enveloped by Phe-103 and Arg-110, which is in a salt bridge with Glu-106. By comparison of the human enzyme structure and the model of the T. brucei enzyme structure, it is predicted that the specificities of the GSK-3 kinase inhibitors can be found because of differences in seven residues in the binding site (Table 5).

**DISCUSSION**

In this paper, we report genetic and chemical validation data that support the hypothesis that T. brucei GSK-3 short is a drug target for T. brucei. First, RNAi experiments targeting T. brucei GSK-3 short demonstrated dramatic inhibition of the growth of T. brucei BSFs. It is likely that the RNAi construct inhibits only the target T. brucei GSK-3 short gene because probing with the same DNA fragment used for the RNAi Southern analysis showed only a single hybridizing band (in four separate digests) with the molecular weight expected for the T. brucei GSK-3 short gene. Thus, there was insufficient nucleotide identity for cross hybridization by Southern analysis. Furthermore, the nucleotide sequence of the T. brucei GSK-3 short RNAi construct had only 47% identity to the corresponding region of T. brucei GSK-3 long, with no stretches of nucleotide identity longer than 11 bases, indicating that it is unlikely that the T. brucei GSK-3 short RNAi would affect T. brucei GSK-3 long expression. Therefore, it is very likely that RNAi causes the specific effect only on T. brucei GSK-3 short gene expression. This also suggests that the growth-inhibitory effect of T. brucei GSK-3 short RNAi occurs independently of T. brucei GSK-3 long inhibition and that T. brucei GSK-3 short and long have nonoverlapping functions that cannot be complemented by one another.

Second, the anti-BSF cellular activities and the activities against the T. brucei GSK-3 short enzyme of a series of kinase inhibitors directed against GSK-3 correlated well. We cannot exclude the possibility that these compounds inhibit targets in the cell other than GSK-3 and thus could exert their effects by additive actions. For instance, it is known that many GSK-3 inhibitors also inhibit CDK-1 and -2 (11, 31), which have active sites very similar to the active site of GSK-3 (27). The inhibition of CDKs has been shown to arrest the growth of T. brucei (12, 13, 31). Recombinant CDK homolog enzymes are not currently available from T. brucei due to difficulty with the heterologous expression of these proteins; thus, we have been unable to test for the cross-activities of the compounds to the CDKs. It is likely that many kinase inhibitors act on targets other than T. brucei GSK-3 short to inhibit BSF cell growth, as some protein kinase inhibitors effectively impaired cell growth but had little activity against T. brucei GSK-3 short. However, there is a strong correlation between T. brucei GSK-3 short enzyme activity and BSF cell proliferation inhibition. Thus, the chemical and genetic evidence favors T. brucei GSK-3 short as a good target for drugs, which would thus have activities against T. brucei.

The ability to selectively target the T. brucei GSK-3 enzymes over the mammalian GSK-3 orthologs may be important to avoid the toxicity caused by effects on cell signaling and cell cycle regulation. Human GSK-3β and T. brucei GSK-3 short are only 41% identical, and thus, it seems possible that selec-
The different pairs are shown in boldface.

Only the backbone is in the binding site.

tive inhibitors of \( T. \) \( \text{brucei} \) GSK-3 short can be found. Indeed, the molecular modeling studies show differences in the active sites that should be able to be translated into the development of selective inhibitors. An example of potential selectivity from this study is that one of the interactions of the Merck-Serono sites that should be able to be translated into the development of the molecular modeling studies show differences in the active sites.

### ACKNOWLEDGMENTS

We acknowledge the key collaborations initiated by Solomon Nwaka of UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, who continues to advise us on the project. We also acknowledge the support of Wim Hol and the entire Medical Structural Genomics of Pathogenic Protozoa group, who initiated the cloning and characterization of \( T. \) \( \text{brucei} \) GSK-3 short.

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### REFERENCES