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Glycogen Synthase Kinase 3 β Promotes the Endocytosis of Transferrin in the African Trypanosome

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Abstract

Human parasite *Trypanosoma brucei* proliferates in the blood of its host, where it takes up iron via receptor-mediated endocytosis of transferrin (Tf). Mechanisms of Tf endocytosis in the trypanosome are not fully understood. Small molecule lapatinib inhibits Tf endocytosis in *T. brucei* and associates with protein kinase GSK3 β (TbGSK3 β). Therefore, we hypothesized that Tf endocytosis may be regulated by TbGSK3 β , and we used three approaches (both genetic and small molecule) to test this possibility. First, the RNAi knock-down of TbGSK3 β reduced Tf endocytosis selectively, without affecting the uptake of haptoglobin-hemoglobin (Hp-Hb) or bovine serum albumin (BSA). Second, the overexpression of TbGSK3 β increased the Tf uptake. Third, small-molecule inhibitors of TbGSK3 β , TWS119 (IC₅₀ = 600 nM), and GW8510 (IC₅₀ = 8 nM) reduced Tf endocytosis. Furthermore, TWS119, but not GW8510, selectively blocked Tf uptake. Thus, TWS119 phenocopies the selective endocytosis effects of a TbGSK3 β knockdown. Two new inhibitors of TbGSK3 β , LY2784544 (IC₅₀ = 0.6 μ M) and sorafenib (IC₅₀ = 1.7 μ M), were discovered in a focused screen: at low micromolar concentrations, they prevented Tf endocytosis as well as trypanosome proliferation (GI₅₀'s were 1.0 and 3.1 μ M, respectively).

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Supporting Information

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Additional information on inhibitor screening data, inhibitor chemical structures (SMILES format), CAS numbers, the effect of PKI-166 on endocytosis, the proliferation of TbGSK3 β overexpression clones 1 and 2, and recombinant TbGSK3 β purification and activity (PDF)

Author Contributions

Biological experiments were performed by P.J.G. Enzyme assays and compound screens were performed by S.X. Experimental design, data analysis, and manuscript composition were carried out by all authors.

Notes

The authors declare no competing financial interest.

DEDICATION

Dedicated to the memory of Martin John Rogers (1960–2014), who served with passion, extraordinary vision, and dedication as program officer for Tropical Diseases at NIAID (National Institutes of Health). We have lost a warrior and leader. We continue the battle, John.

These studies show that (a) TbGSK3 β regulates Tf endocytosis, (b) TWS119 is a small-molecule tool for investigating the endocytosis of Tf, (c) endocytosis of GPI-anchored TfR and HpHbR are differentially regulated, and (d) the imidazopyridazine aminopyrazole scaffold of LY2784544 is attractive for a hit-to-lead optimization program in antitrypanosome drug discovery.

Graphical abstract

Keywords

glycogen synthase kinase 3 β ; endocytosis; drug discovery; trypanosome; protein kinase

INTRODUCTION

Trypanosoma brucei is a protozoan parasite that causes human African trypanosomiasis (HAT). During the first stage of infection, the parasite proliferates in the blood and lymph of the vertebrate host, acquiring host transferrin (as an essential source of iron) by receptor-mediated endocytosis.¹ *T. brucei* has a transferrin receptor (TfR) that lacks sequence similarity to human TfR (hTfR). TfR is a GPI-anchored heterodimer of ESAG 6 and ESAG 7 gene products that lack transmembrane and cytoplasmic domains,^{2–4} whereas hTfR is a homodimer comprising transmembrane and cytoplasmic domains.⁴ These differences in receptors for Tf reflect major differences in endocytosis pathways between the parasite and host, fueling expectations that Tf endocytosis may be a good target for the discovery of pharmacological leads for antitrypanosome drug discovery.⁵

In human cells, Tf endocytosis is regulated by nonreceptor protein tyrosine kinase Src.⁶ Trypanosomes are predicted to have 190 protein kinases;⁷ however, none are classic protein tyrosine kinases.⁸ Furthermore, no protein kinase has been identified as a regulator of Tf endocytosis, although a pan-inhibitor of Tyr kinases (tyrphostin-A47) blocks Tf endocytosis in the parasite.^{9,10} Protein kinases that control Tf endocytosis could be valuable targets for drug discovery because of the importance of Tf uptake for trypanosome viability.

Small-molecule perturbants of cellular processes can be useful tools for studying biological systems.^{11–13} The approach is most valuable for deeply diverged eukaryotes whose signaling pathways have deviated significantly from those found in well-studied systems (e.g., yeast and humans). Lapatinib, a small-molecule inhibitor of human epidermal growth factor receptor (EGFR),^{14,15} blocks Tf endocytosis in *T. brucei*. (Guyett, P.J., et al., manuscript submitted.) However, *T. brucei* lacks EGFR. Instead, in the trypanosome, lapatinib associates with four proteins including glycogen-synthase kinase 3 β (TbGSK3 β).¹⁶ The other three enzymes were either implicated in cell division (Tb427.04.5180¹⁷ and

Tb427.05.800¹⁸) or had no loss of fitness when knocked down (Tb427.03.1570^{19,20}). Consequently, we hypothesized that Tf endocytosis is regulated by TbGSK3 β .

In this work, we use three methods to establish TbGSK3 β as a novel regulator of Tf endocytosis in *T. brucei*. First, the knockdown of TbGSK3 β by RNAi decreased the Tf uptake. Second, the treatment of trypanosomes with small-molecule inhibitors of TbGSK3 β reduced Tf endocytosis. Third, the overexpression of TbGSK3 β increases Tf uptake. Because Tf endocytosis is essential for trypanosome proliferation²¹ and a knockdown of TbGSK3 β compromises trypanosome viability,²² we screened a library of compounds to identify new chemotypes that inhibit the enzymatic activity of TbGSK3 β . These novel TbGSK3 β inhibitors were then tested for their effects on Tf endocytosis and trypanosome proliferation. From this directed screen, LY2784544 and sorafenib unexpectedly emerged as inhibitors that are suitable for a follow-up hit-to-lead medicinal chemistry effort in antitrypanosome drug discovery.

RESULTS

Genetic Knockdown of TbGSK3 β Reduces Transferrin Endocytosis

To test the hypothesis that TbGSK3 β regulates Tf endocytosis in *T. brucei*, we knocked down TbGSK3 β using a tetracycline (Tet)-inducible RNA interference (RNAi) system.²³ After 48 h of RNAi induction, TbGSK3 β protein was undetectable by Western blotting (Figure 1a), a fact that coincided with reduced trypanosome proliferation (Figure 1b).

We tested the effect of TbGSK3 β knockdown on trypanosome endocytosis of transferrin after 48 h of RNAi induction (Figure 1c,d). Induced (+Tet) and uninduced (–Tet) cells were normalized to control trypanosomes that were not transfected with the RNAi vector. In the uninduced cells, Tf endocytosis was measured at 90% of the amount taken up by control cells. In contrast, TbGSK3 β knockdown trypanosomes endocytosed 50% less transferrin than control cells. Thus, TbGSK3 β is required for normal levels of transferrin endocytosis.

To determine if a knockdown of TbGSK3 β affected the endocytosis of other macromolecules, the uptake of haptoglobin-hemoglobin (which has a GPI-anchored receptor)²⁴ and bovine serum albumin (BSA), a marker for bulk-phase endocytosis,^{25,26} were monitored. Uninduced TbGSK3 β RNAi cells endocytosed equivalent amounts of Hp-Hb and BSA as control trypanosomes (Figure 1d). Intriguingly, TbGSK3 β knockdown cells endocytosed slightly greater amounts of BSA than did control cells, although the difference in uptake was not statistically significant ($p > 0.05$) (Figure 1d). We conclude that the loss of TbGSK3 β does not inhibit Hp-Hb or BSA endocytosis.

Small-Molecule Inhibitors of TbGSK3 β Decrease Tf Endocytosis in *T. brucei*

To chemically validate TbGSK3 β as a regulator of Tf endocytosis in trypanosomes, we tested the effect of two TbGSK3 β inhibitors, GW8510^{22,27} (IC₅₀ = 8 nM, Table 1) and TWS119²⁸ (IC₅₀ = 600 nM, Table 1, Figure 2a,b) on Tf endocytosis.

GW8510 and TWS119 decreased Tf endocytosis in trypanosomes, with EC₅₀ values of 0.8 and 2.0 μ M, respectively (Figure 2c,d). However, the inhibitors had different effects on bulk-

phase BSA uptake. GW8510 decreased both Tf and BSA endocytosis (Figure 2c). TWS119 did not decrease BSA or Hp-Hb endocytosis although it inhibited Tf endocytosis (Figure 2d), phenocopying a genetic knockdown of TbGSK3 β (Figure 1d). TWS119 inhibits Tf endocytosis with a selectivity ratio (i.e., BSA (EC₅₀): Tf (EC₅₀)) greater than 5 (Table 1). GW8510 is a nonselective inhibitor of endocytosis (selectivity ratio = 1.5 (Table 1)); it may have multiple protein targets that include TbGSK3 β .

The chemical basis for the selective inhibition of Tf endocytosis by TWS119 is not known. To explore the possibility that the property is conferred by its pyrrolopyrimidine chemotype, we tested whether another pyrrolopyrimidine, PKI-166²⁹ had similar biological effects. PKI-166 did not inhibit TbGSK3 β in vitro (IC₅₀ > 20 μ M). Furthermore, PKI-166 did not inhibit the endocytosis of either Tf or BSA (Figure S1). Nevertheless, PKI-166 inhibits trypanosome proliferation (concentration of drug that inhibits growth by 50% (GI₅₀) = 5 μ M).³⁰ We conclude that PKI-166 has a different target other than TbGSK3 β and blocks a different physiological pathway than Tf endocytosis. Therefore, the biological effects of TWS119 on TbGSK3 β and Tf endocytosis are not intrinsic to the scaffold; the substituents on the pyrrolopyrimidine play a major role in conferring the biochemical and biological effects of TWS119.

Overexpression of TbGSK3 β Increases Tf Endocytosis

We performed a direct test, as opposed to the RNAi study, of the role of TbGSK3 β in transferrin endocytosis by over-expressing the enzyme and evaluating the effect on Tf uptake. Production of the enzyme was under the control of a tetracycline inducible T7 RNAP promoter in the pDex777 plasmid.³¹ Two clones selected for analysis both overexpressed TbGSK3 β within 3 h of induction (Figure 3a). Continued overexpression (longer than 3 h) negatively impacted trypanosome proliferation; slower growth was observed at 6 h for both clones (Figure S2). Thus, cells induced for 3 h were used for all subsequent experiments. For clone 1, the increase in TbGSK3 β protein abundance correlated with a significant increase (300%) in Tf endocytosis ($p < 0.05$) (Figure 3c). Clone 2 overexpressed less TbGSK3 β protein (Figure 3c); accordingly, there was less enhancement of Tf uptake (40% increase) (Figure 3a).

We used TWS119 as a chemical probe of the activity of overexpressed TbGSK3 β . Our hypothesis was that if the increase in Tf endocytosis (Figure 3c) was due to the overexpressed enzyme, TWS119, an inhibitor of TbGSK3 β (Figure 2), would reduce the stimulatory effect of the enzyme on Tf uptake. A schematic of the experimental setup is presented in Figure 3b. For clone 1, TWS119 (10 μ M) treatment reduced Tf endocytosis driven by TbGSK3 β overexpression by 300% (Figure 3c), bringing it within error of the amount of endocytosis in cells prior to induction of TbGSK3 β overexpression (Figure 3c). Similar trends were observed with clone 2, although the magnitude of differences was less, as compared to clone 1. These data, combined with the RNAi results, indicate that TbGSK3 β enzyme levels modulate Tf endocytosis in the trypanosome.

Two New TbGSK3 β Inhibitor Scaffolds

Since it is involved in a pathway that is essential for trypanosome proliferation in the host, TbGSK3 β is a good target for discovery of new antitrypanosomal hits.^{22,32} Toward the goal of finding new hits, we established an *in vitro* protein kinase assay using purified recombinant full-length TbGSK3 β expressed in *E. coli* with a 6X His tag. The tagged protein was enriched by immobilized metal affinity chromatography and purified further by size exclusion chromatography (SEC) (Figure S3a). Enzyme activity of recombinant TbGSK3 β (rTbGSK3 β) (from SEC fractions 13 through 15) was confirmed using a synthetic peptide substrate (GSM). The K_M of peptide and ATP substrates were determined (Figure S3b), and found to be within the range previously reported for TbGSK3 β .^{22,33}

To identify novel inhibitor scaffolds for TbGSK3 β , we screened a protein tyrosine kinase inhibitor library (Selleck Chemicals) (Table S1) as outlined in Figure 4a. The library of 70 molecules was initially screened with 20 μ M drug for inhibition of TbGSK3 β enzyme activity. Those compounds that inhibited TbGSK3 β > 80% at 20 μ M were then screened at 2 μ M. Dose–response curves were generated for any compound with at least 20% inhibition at 2 μ M. At each step in the screening protocol, the compounds were also tested for inhibition of the coupled enzymes in the ADP Quest (DiscoverRx, Fremont, CA) assay, in order to verify that the observed inhibition was due to interaction with TbGSK3 β instead of the coupled enzymes (Table S1). A compound was considered a “hit” if it had an IC_{50} value below 2 μ M (Table 1) and had an IC_{50} against the coupled enzymes ($IC_{50, \text{coupled enzyme}}$) greater than 10 μ M; these thresholds were chosen so that any hits would have TbGSK3 β selectivity greater than 5-fold. Two new hits against TbGSK3 β were discovered, namely, LY2784544³⁴ and sorafenib³⁵ (Table 1 and Figure 4b).

Tf Endocytosis Inhibition and Antitrypanosomal Activity of LY2784544 and Sorafenib

The hits were tested for inhibition of endocytosis of Tf or BSA. Sorafenib was a slightly better inhibitor of endocytosis (Figure 5a) with EC_{50} values of 2.7 μ M for Tf, and 5.9 μ M for BSA (Table 1), as compared to LY2784544 (Tf EC_{50} = 7.8 μ M and BSA EC_{50} = 6.2 μ M) (Figure 5b). Antitrypanosomal activity of LY2784544 and sorafenib was tested on bloodstream *T. brucei*. LY2784544 blocked trypanosome proliferation with a GI_{50} of 1.0 μ M, and sorafenib had a GI_{50} of 3.1 μ M (Table 1). Therefore, both LY2784544 and sorafenib are hits for antitrypanosome lead discovery.

MATERIALS AND METHODS

Generation of TbGSK3 β RNAi Construct and Stable Transfection

Chromosomal DNA was isolated from CA427 trypanosomes.³⁶ An approximately 400 bp fragment of the TbGSK3 β gene was amplified using high-fidelity Phusion polymerase (New England Biolabs, Ipswich, MA) and a primer pair designed with assistance from RNAi³⁷ that corresponded to the first 400 bp of the TbGSK3 β gene: forward primer sequence, ctcgaggatccCGGAAC TATTACCGTCGCATGACAAATC; reverse primer sequence, ctcgagaagcttGGAAGTGGCTGCTTGAAGACGTTCTC (lowercase letters are engineered restriction enzyme cut sites, and uppercase letters are specific to the TbGSK3 β gene). The

PCR product was digested with *Bam*HI and *Xho*I and cloned into the p2T7^{TAB}Blue trypanosome RNAi vector.²³

The TbGSK3 β :p2T7^{TAB}Blue construct was transfected into bloodstream-form (BSF) trypanosomes by electroporation with slight modifications from a previously published protocol.³⁸ Briefly, single-marker BSF trypanosomes^{23,39} (10^8 cells) were transfected with 10 μ g of plasmid linearized with NotI-HF (NEB, Ipswich, MA) in 200 μ L of cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM KPi (pH 7.6), 25 mM HEPES (pH 7.6), 2 mM K₂EDTA (pH 8.0), 5 mM MgCl₂, 1 mM hypoxanthine, 0.5% glucose, 5 mM ATP, 5 mM glutathione).³⁸ The electroporation was performed in a 4 mm cuvette with one pulse at 1.35 kV, 50 μ F capacitance, and 360 Ω resistance. The electroporated cells (100 μ L) were diluted in 10 mL of fresh HMI-9 medium and cultured for 15 h. Transfectants were then serially diluted ($1:10^2$, $1:10^3$, $1:10^4$) in HMI-9 containing G418 (6.5 μ g/mL) and hygromycin (5 μ g/mL) and plated (1 mL per well of a 24-well plate). After 5 days of culture, stably transfected clones were selected from plates with fewer than six transfectant-positive wells. The TbGSK3 β RNAi cell line was always cultured in HMI-9 containing G418 (6.5 μ g/mL) and hygromycin (5 μ g/mL) to maintain selection. To induce RNAi, 1 μ g/mL tetracycline (1 μ g/mL) was added to the trypanosomes (5×10^3 cells/mL).

Generation of the TbGSK3 β Overexpression Construct

A full-length TbGSK3 β gene was amplified from *T. brucei* CA427 genomic DNA by PCR (forward primer, aagcttATGTCGCTCAACCTTACCGATGC; reverse primer, ggatccTACTTCTTCAGCAGATACTCCCGC). The bluntend PCR product was cloned into pMiniT cloning vector (New England Biolabs). An internal *Hind*III site was mutated out of the TbGSK3 β gene by QuikChange II site-directed PCR mutagenesis (forward primer, GGAGCCAACAACGAAaCTTCCCAGTGGTAATC; reverse primer, GATTACCACTGGGAAGtTTCGTTGTTGGCTCC) following the recommended protocol (Agilent Technologies). The full-length, silent mutant TbGSK3 β gene was subsequently cloned into pDex777³¹ using *Hind*III and *Bam*HI.

Transfection into BSF trypanosomes was similar to published methods.⁴⁰ Briefly, single-marker trypanosomes (4×10^7) were washed with phosphate-buffered saline supplemented with 1% glucose (PBS-G) and resuspended in 100 μ L of Amaxa human T-cell nucleofection solution (Lonza Group, Basel, Switzerland) containing NotI-linearized pDex777:TbGSK3 β (10 μ g) plasmid. Trypanosomes were nucleofected with one pulse using protocol X-001 on a Nucleofector 2b device (Lonza Group). After nucleofection, trypanosomes were cloned out following described methods (above) with G418 (6.5 μ g/mL) and phleomycin (1.75 μ g/mL) selection. Stable transfectants were routinely cultured with G418 and phleomycin (2.5 μ g/mL) selection. TbGSK3 β overexpression was induced by the addition of tetracycline (1 μ g/mL) to trypanosomes (5×10^5 /mL).

Western Blot

TbGSK3 β RNAi transfected single-marker BSF *T. brucei* (2×10^6) induced or uninduced for 48 h or TbGSK3 β :pDex777 transfected single-marker trypanosomes (1×10^6) induced or uninduced for 3 h were pelleted and washed once in PBS-G. Trypanosomes were lysed in

15 μ L of SDS sample buffer and heated to 95 °C for 5 min, and the proteins were separated by SDS-PAGE (15% acrylamide gel) containing stain-free reagent (Bio-Rad, Hercules, CA). The gel was activated (5 min, following the recommended protocol) and imaged using a ChemiDoc MP system (Bio-Rad, Hercules, CA) to determine the amount of protein in each lane. The proteins were then transferred from the gel to the PVDF membrane using a semidry rapid transfer buffer and apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with PBS, 3% bovine serum albumin, and 0.1% Tween-20 (blocking buffer) for 1 h. After blocking, primary antibody, which was rabbit polyclonal anti-TbGSK3 β antibody (raised against the TbGSK3 β -specific peptide EVNPQRKRSREAPPC (Genscript, Piscataway, NJ)), was diluted (1:2000) in blocking buffer and incubated with the membrane for 1 h. Excess primary antibody was washed three times (5 min each) in PBS and 0.1% Tween-20 (PBS-T), and then secondary antibody, which was alkaline phosphatase-conjugated goat antirabbit (Bio-Rad, Hercules, CA), was diluted (1:2000) in blocking buffer and incubated with the membrane for 1 h. Unbound secondary antibody was washed off twice with PBS-T, and the membrane was developed with Immune-Star AP substrate (Bio-Rad, Hercules, CA) for 3 min. The developed membrane was imaged on a Bio-Rad ChemiDoc MP system.

Endocytosis Assay

Bloodstream CA427 *T. brucei* (5×10^5), 48 h of induced or uninduced TbGSK3 β RNAi transfected single-marker cells or 3 h of induced or uninduced TbGSK3 β overexpression cells cultured in HMI-9 medium were resuspended in 1 mL of serum-free HMI-9. For inhibitor treatment, each compound (at various concentrations) was delivered in 1 μ L of DMSO. The cells were incubated for 3–5 min in the presence of inhibitor and then BSA-AlexaFluor647 conjugate (BSA-A647, 50 μ g/mL, Invitrogen, Eugene, OR) and either transferrin-AlexaFluor488 conjugate (Tf-A488, 25 μ g/mL, Invitrogen, Eugene, OR) or Haptoglobin phenotype 1-1 conjugated to AlexaFluor488 (following AlexaFluor protein labeling kit (Life Technologies, Invitrogen, Eugene, OR)) in complex with hemoglobin (38 nM) were added. Trypanosomes were incubated for 15 min at 37 °C, 5% CO₂. A control aliquot of serum-free HMI-9 medium and Tf-A488 and BSA-A647 without cells was also prepared (no cell control). All samples were transferred to ice. The cells were pelleted (2000g, 5 min, 4 °C), and resuspended in 1 mL of cold PBS containing 10 mM glucose and propidium iodide (PI) (3 μ M). Incubation with PI proceeded for 15 min (on ice), after which the samples were analyzed by flow cytometry (CyAn ADP analyzer (Beckman Coulter), Tf-AlexaFluor488 or Hp-Hb-AlexaFluor488: excitation, 488 laser; emission, 530/40 filter. BSA-A647: excitation, 635 laser; emission, 665/20 filter. PI: excitation, 488 laser; emission, 613/20 filter. Using FlowJo version 9 software (Tree Star, Ashland, OR), median fluorescence of each fluorophore was determined for events with similar forward versus sidescatter profiles that were distinguished from the “no cell” control background and were not permeable to propidium iodide.

Expression and Purification of Recombinant TbGSK3 β

Full-length TbGSK3 β was cloned into the pET21d expression vector in-frame with a C-terminal 6 $\times$ His tag. BL21 (DE3) *E. coli* were transformed with the construct and cultured in terrific broth (TB) supplemented with 1% glucose to an OD (at 590 nm) of 1.8. Expression

of TbGSK3 β was induced with IPTG (0.5 mM) for 3 h at 32 °C. Cells were harvested and resuspended in lysis buffer (30 mM HEPES (pH 8.0), 300 mM NaCl, 10% glycerol, 10 mM imidazole, 0.2 μ M PMSF, and 2 mM β -mercaptoethanol). The bacteria were lysed by sonication and freeze/thaw cycles. Insoluble protein were removed by centrifugation (12 000g, 45 min, 4 °C), and the soluble fraction was loaded onto an immobilized metal affinity chromatography (IMAC) column. The column was washed with 30 mM HEPES (pH 8.0), 0.5 M NaCl, 10% glycerol, 20 mM imidazole, 0.02% Triton X-100, 2 mM β -mercaptoethanol. Recombinant TbGSK3 β (rTbGSK3 β) was eluted from the IMAC column with 6 mL of 30 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, 200 mM imidazole, 2 mM β -mercaptoethanol. Eluted rTbGSK3 β was further fractionated by size-exclusion chromatography (SEC) using a Superdex-200, 10/300 GL column pre-equilibrated with SEC running buffer, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl. Fractions were collected, and a portion of each was analyzed by SDS-PAGE to determine which fraction contained rTbGSK3 β (Figure S3). A band corresponding to the molecular weight of rTbGSK3 β eluted in fractions 13–15 and was considered the purified enzyme. Purified rTbGSK3 β was stored at –80 °C in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20% glycerol until used.

In Vitro Kinase Assay

The enzyme activity of TbGSK3 β was measured with an ADP Quest assay (DiscoverRx, Fremont, CA) using a synthetic peptide substrate, GSM (RRRPASVPPSPSLSRHS(pS)HQRR, where (pS) corresponds to phosphorylated serine). Briefly, inhibitors were serially diluted and incubated with 1.25 nM TbGSK3 β for 10 min at room temperature in assay buffer (15 mM HEPES, pH 7.4, 20 mM NaCl, 1 mM EGTA, 0.02% Tween20, and 10 mM MgCl₂). To initiate the kinase reaction, substrate peptide and ATP were added to a final concentration of 10 μ M each in a final reaction volume of 20 μ L. The reaction was further incubated for 15 min at room temperature. The ADP (product) concentration was determined by the addition of 10 μ L of ADP Quest assay reagent A and 20 μ L of ADP Quest assay reagent B and incubated at room temperature for 15 min. The fluorescence signal at 590 nm was measured with a Varioskan fluorescence plate reader. The corresponding IC₅₀ value of the compound was calculated using GraphPad Prism 6 (GraphPad Software, Inc., CA) according to the equation

$$\% \text{ inhibition} = \min + \frac{\min - \max}{1 + 10^{(\log \text{IC}_{50} - x)n}}$$

where min and max are the *y* intercepts of the lower and higher baselines, respectively (with assumed slopes of 0), *x* is the inhibitor concentration, and *n* is the Hill coefficient.

Proliferation Inhibition Assay

Bloodstream form (BSF) CA427 trypanosomes (2×10^3 cells/mL) were cultured in 1 mL of HMI-9 medium. Each TbGSK3 β inhibitor was added (at various concentrations) to 1 μ L of DMSO, and trypanosomes were cultured for 48 h, after which they were counted with a Coulter Counter (Beckman) using a 100 μ m aperture tube monitoring for particle sizes of between 2.5 and 5 μ m. The growth percentage (compared to the DMSO control) was determined according to the equation

$$\% \text{ growth} = \frac{[\text{final}]_{\text{treated}} - [\text{initial}]}{[\text{final}]_{\text{control}} - [\text{initial}]} \times 100$$

where [initial] is the starting cell density, [final]_{control} is the final cell density in untreated trypanosomes after 48 h, and [final]_{treated} is the final cell density of trypanosomes treated with the inhibitor. The calculated % growth was plotted versus the TbGSK3 β inhibitor concentration, and the dose–response curve was fitted to determine the concentration of inhibitor that inhibited trypanosome growth by 50% (GI₅₀).

DISCUSSION

Genetic and Small-Molecule Evidence that TbGSK3 β Regulates Tf Endocytosis in a Trypanosome

We used three approaches to document the role of TbGSK3 β in Tf endocytosis by *T. brucei*. Genetic manipulation of TbGSK3 β protein levels modulated the amount of transferrin taken up: knockdown (Figure 1) decreased Tf endocytosis while overexpression (Figure 3) increased Tf uptake. Chemical inhibition of the enzyme also decreased Tf endocytosis (Figure 2). The two TbGSK3 β inhibitors, TWS119 and GW8510, represent different chemotypes (pyrrolopyrimidine and thiazoloindole, respectively), which reduces the likelihood of inadvertently blocking some other activity that is not linked to TbGSK3 β . Furthermore, Tf endocytosis was not blocked by PKI-166, a pyrrolopyrimidine that did not inhibit TbGSK3 β (Figure S1). Combined, these data provide compelling evidence that TbGSK3 β is important for Tf endocytosis.

Interestingly, the endocytosis of BSA or Hp-Hb was not affected when TbGSK3 β was knocked down (Figure 1d) or after TWS119 treatment of trypanosomes (Figure 2d). These data illustrate for the first time that the endocytic pathway of two GPI-anchored receptors (TfR and HpHbR) might be differentially regulated. Furthermore, the uptake of BSA²⁵ is functionally distinguishable from the receptor-mediated route of Tf. Similarly, in HeLa cells, the phagocytosis of viral particles may be subjected to differential regulation: GSK3 β knockdown decreases clathrin-dependent infection by the vesicular stomatitis virus but increases caveolin/lipid raft-mediated infection by simian virus 40.⁴¹

In human cells, there is no report that GSK3 β regulates Tf endocytosis. With this in mind, we tested the ability of GW8510 and TWS119, which inhibit human GSK3 β ,²⁸ to inhibit Tf endocytosis in HeLa cells. Neither TbGSK3 β inhibitor affected Tf endocytosis in HeLa cells (Figure 6). As a control to validate our experimental method, we used semiselective protein kinase inhibitor H-89, which inhibits Tf endocytosis in HeLa cells.⁴² In contrast to TWS119 and GW8510, H-89 decreased Tf endocytosis in HeLa cells (Figure 6). These data indicate that TbGSK3 β , although 70% identical in protein sequence to human GSK3 β , has unique functions in trypanosomes as compared to its homologue in human cells.

TWS119 Is a Chemical Tool for Studying Tf Endocytosis in a Trypanosome

Chemical biology can provide new reagents to complement or extend genetic studies of biological processes.^{13,43} In this work, we identified two new compounds that inhibit recombinant TbGSK3 β in vitro at low concentrations. Even though a compound inhibits TbGSK3 β in vitro, it could be active against other enzymes in vivo. In this case, screening the activity of each TbGSK3 β inhibitor against the Tf endocytosis phenotype provides a different metric for target selectivity in trypanosomes. TWS119 has comparable IC₅₀ and GI₅₀ values (in the range of 1 μ M, Table 1). The treatment of trypanosomes with TWS119 (2 μ M) decreased the Tf endocytosis (Table 1), but the probe had no effect on BSA or Hp-Hb endocytosis, phenocopying the genetic knockdown of TbGSK3 β (Figure 1). Also, the TWS119 treatment countered the increase in Tf endocytosis after the overexpression of TbGSK3 β (Figure 3c). Therefore, we propose TWS119 as a chemical probe for studying signaling pathways and effector proteins regulated by TbGSK3 β in the trypanosome. (PKI-166 will be an important control molecule for these studies because it is a close structural analog of TWS119, but it does not inhibit TbGSK3 β in biochemical assays or Tf endocytosis in trypanosomes (Figure S1)).

There are several advantages to using a chemical probe to study trypanosome biology to complement the genetic knockdown of TbGSK3 β . First, the chemical tool shifts the kinetics of experiments to minutes or hours after exposure to the probe, instead of days after a genetic knockdown, to observe the desired phenotype. Second, enzyme inhibition by a chemical tool allows any physiology that is enzyme-activity-dependent to be studied. Finally, the titration of the chemical probe dose and exposure time allows an investigator to tease out subtle effects on the targets of the chemical probe.

A potential disadvantage of using a chemical probe for biological experiments is the penchant for so-called off-target effects,^{44,45} a problem that has also been noted for RNAi as well as CRISPR/Cas9 technologies.^{46–48} The solution to this problem of spurious inactivation of other kinases by TWS119 lies in ensuring that the genetic knockdown of the target gene produces the same phenotype as using the chemical probe to inactivate the enzyme. In our study of Tf endocytosis regulation by TbGSK3 β , only TWS119, out of several inhibitors of the enzyme tested, phenocopied the genetic knockdown and so qualifies as a chemical surrogate for the genetic loss of TbGSK3 β function in vivo. However, it is important to note that TWS119 (as well as TbGSK3 β) may impact other biological processes that were not assayed in this article. A clear extension of this work will be determining whether TbGSK3 β regulates other biological processes, especially because human GSK3 β regulates multiple pathways (reviewed in refs 49–51). Then it will be interesting to ascertain if TWS119 also affects those pathways.

New Scaffolds for Anti-Trypanosome Hit-to-Lead Drug Discovery

TbGSK3 β has been the subject of several target-based hit discovery projects^{22,32,52} that focused on libraries of human GSK3 β inhibitors or inhibitors of closely related protein kinases, for example, CDK.²² We took an alternative strategy of screening a library of 70 orally bioavailable tyrosine kinase inhibitors (Table S1) on the basis of our surprising observation that the human EGFR inhibitor, lapatinib (a 4-anilinoquinazoline), associated

with TbGSK3 β .¹⁶ By doing this, two new TbGSK3 β inhibitors were discovered: sorafenib and LY2784544. These drugs inhibited trypanosome proliferation at concentrations below 3 μ M. Because the compounds are orally bioavailable, they are excellent candidates for antitrypanosome lead discovery efforts, which would focus on modifying the scaffold to improve antiparasite activity and selectivity over host cell activity as has been demonstrated for lapatinib.⁵³

In conclusion, we have identified the first protein kinase involved in trypanosome Tf endocytosis, TbGSK3 β . A genetic knockdown of TbGSK3 β or treatment with TWS119 blocked Tf endocytosis but had no effect on BSA or HpHb endocytosis. We propose using TWS119 as a chemical tool to begin parsing mechanistic details of endocytosis in the trypanosome. Furthermore, the screen for novel TbGSK3 β inhibitors from a library of protein tyrosine kinase inhibitors identified two new orally bioavailable antitrypanosome hits that are either in clinical trials or are FDA-approved for indications other than trypanosomiasis. A medicinal chemistry initiative to improve the antitrypanosome properties of the scaffolds is warranted and will benefit from structure-guided docking of these scaffolds to structures of TbGSK3 β .^{54–57}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Knockdown of *TbGSK3β* reduces Tf endocytosis. Bloodstream form trypanosomes stably transfected with a *TbGSK3β* RNAi construct were cultured in the absence (–Tet, uninduced) or presence (+Tet, induced) of tetracycline. (a) Western blot of *TbGSK3β* protein abundance after RNAi induction. After 48 h, trypanosomes (2×10^6) were lysed and the proteins were separated by SDS-PAGE. Protein abundance in the gel was detected with stain-free reagent (Bio-Rad) (load control). Protein was then transferred to a PVDF membrane and developed using anti-*TbGSK3β* antibody. (b) The proliferation of uninduced (black line) and Tet-induced (gray line) trypanosomes was monitored over 72 h, after inoculation at 5×10^4 /mL (cells were subcultured to maintain a density of less than 1×10^6 /mL). The plotted values were mathematically derived on the basis of dilution factors. The error among three separate experiments is smaller than the data symbols. (c, d) For endocytosis assays, cells were resuspended in serum-free medium containing bovine serum albumin conjugated to Alexafluor649 (BSA-A649) ($50 \mu\text{g/mL}$) and either transferrin conjugated to Alexafluor488 (Tf-A488, $25 \mu\text{g/mL}$) or haptoglobin conjugated to Alexafluor488 in complex with hemoglobin (HpHb-A488, 38 nM). Trypanosomes were incubated for 15 min at 37°C and washed, and the amount of fluorophore uptake was measured by flow cytometry. (c) Histogram comparing Tf-A488 uptake by uninduced and induced *TbGSK3β* RNAi trypanosomes. (d) Average fluorescence uptake (Tf-A488 (gray), BSA-A649 (blue), and Hp-Hb (red)) by uninduced and induced *TbGSK3β* RNAi trypanosomes in three separate experiments. The median fluorescence in each experiment was normalized to the median fluorescence uptake of the parental trypanosome cell line that does not contain the *TbGSK3β* RNAi construct. The statistical significance is denoted with an asterisk (*), as determined by a student's *t* test ($p < 0.05$).

Figure 2.

Dose–response curves of rTbGSK3 β activity or endocytosis (Tf or BSA) to GW8510 or TWS119. Activity of rTbGSK3 β in various concentrations of (a) the known TbGSK3 β inhibitor, GW8510, or (b) the known human GSK3 β inhibitor, TWS119. Data from two separate experiments were fit to a dose–response curve using GraphPad Prism 6 software, and the 50% inhibitory concentration (IC₅₀) values were determined. The 95% confidence interval is reported. The endocytosis of Tf or BSA was determined for trypanosomes (1×10^6) treated with varying concentrations of GW8510 (c) or TWS119 (d). Trypanosomes were pretreated with inhibitor for 3 min on ice, and then Tf-Alexa488 (25 μ g/mL), BSA-Alexa649 (50 μ g/mL), or Hp-Hb (38 nM) was added. Trypanosomes were incubated (in the presence of inhibitor) at 37 °C for 15 min, and the fluorescence uptake was quantified by flow cytometry. Dose–response curves from three separate experiments were fit using GraphPad Prism 6 software.

Figure 3.

Overexpression of TbGSK3 β increases the uptake of Tf. (a) Western blot of two clones containing inducible overexpression constructs of TbGSK3 β . Trypanosomes were cultured in the presence (+Tet) or absence (-Tet) of tetracycline for 3 h, or a fraction of cells were taken immediately prior to tetracycline induction (0 h). For the Western blot, trypanosomes (1×10^6) were harvested, washed with PBS-G, and lysed in $1 \times$ SDS sample buffer. Western blotting was performed as described in Figure 1. (b) Flowchart of the experimental approach used to generate panel (c). TbGSK3 β overexpression increases Tf endocytosis, which is countered by the addition of TWS119. Trypanosomes overexpressing TbGSK3 β (+Tet) or uninduced cells (-Tet) (after 3 h) were treated with TWS119 ($10 \mu\text{M}$) or DMSO solvent control, and the amount of Tf endocytosed was determined as described in Figure 2. CA427 (control cells) are shown in both panels for reference. Clone 1 (red) and clone 2 (blue). The error from three experiments is represented as error bars; (*) statistical significance ($p < 0.05$) was determined using the student's t test.

Figure 4.

A focused screen to identify inhibitors of rTbGSK3 β . (a) A graphical depiction of the screening paradigm. The number of compounds that met each threshold are listed in parentheses. (b) Dose–response curves of the two new rTbGSK3 β inhibitors. Data from three separate experiments were fit to a dose–response curve using GraphPad Prism 6 graphing software, and the 50% inhibitory concentration (IC_{50}) values determined. Error at 95% confidence is reported.

Figure 5.

Dose–response inhibition of endocytosis for Sorafenib and LY2784544. Bloodstream form trypanosomes (1×10^6) were resuspended in serum free medium, and pretreated with varying concentrations of Sorafenib (a) or LY2784544 (b) for 3 min on ice. After pretreatment, Tf-A488 (25 $\mu g/mL$) and BSA-649 (50 $\mu g/mL$) was added. Trypanosomes were incubated at 37 °C for 15 min, and fluorescence uptake quantified by flow cytometry. Dose–response curves from three separate experiments were fit using GraphPad Prism 6 graphing software. To fit LY2784544 data, the lower baseline y-intercept was fixed to no endocytosis control values (trypanosomes incubated on ice in the presence of Tf and BSA for duration of experiment). Error is reported at 95% confidence.

Figure 6.

Chemical inhibition of HeLa cell endocytosis of transferrin. HeLa cells were grown to 75% confluence in DMEM supplemented with 10% FBS. The cells were then washed twice with 10 mL of cold DMEM (serum-free) and incubated for 3 min in the presence of each compound (10 μ M) or an equivalent volume of DMSO solvent. The cells were then incubated with Tf-Alexa488 (5 μ g/mL) for 15 min at 37 °C. The HeLa cells were washed twice with ice cold PBS and trypsinized for 3 min at 37 °C. The unadhered cells were then transferred to microcentrifuge tubes on ice, incubated with propidium iodide (5 μ M, 15 min, on ice), and analyzed by flow cytometry (as described in Materials and Methods). The median fluorescence was normalized to that of the DMSO-treated HeLa cells. Error bars represent one standard deviation in four independent experiments. The statistical significance was determined by the student's *t* test; an asterisk (*) indicates statistical significance, with *p* < 0.05.

Table 1Structures and Activities of TbGSK3 β Inhibitors^a

Name	Structure	TbGSK3 IC ₅₀ (μM)	Tf uptake EC ₅₀ (μM)	Endocytosis Selectivity Index $\left(\frac{BSA\ EC_{50}}{Tf\ EC_{50}}\right)$	<i>T. brucei</i> GL ₅₀ (μM)
GW8510		0.008 ± 0.002	0.8 ± 0.1	1.5	0.12
TWS119		0.6 ± 0.1	2.0 ± 1	>5	1.1
Sorafenib		1.7 ± 1	2.7 ± 1	2.2	3.1
LY2784544		0.6 ± 0.2	7.8 ± 2.7	0.8	1.0

^aThe IC₅₀ of each compound was determined against rTbGSK3 β by in vitro kinase assays as described in Figure 5 and the Materials and Methods section. The GI₅₀ of each compound was determined against axenic cultures of bloodstream trypanosomes as described in Materials and Methods. Tf EC₅₀ is the concentration of each compound that inhibits 50% of transferrin endocytosis. The endocytosis selectivity was calculated by dividing the BSA EC₅₀ by the Tf EC₅₀; selective inhibition of Tf endocytosis over BSA endocytosis would have a value greater than 1.