A SURVEY OF PROTOZOAN PARASITE HEXOKINASES: CHARACTERIZATION STUDIES AND POTENTIAL FOR THERAPEUTIC INTERVENTIONS

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A SURVEY OF PROTOZOAN PARASITE HEXOKINASES:
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INTERVENTIONS

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Protozoan parasites find a glucose rich environment when inhabiting the human host. As a consequence of this glucose rich environment, many parasites have evolved streamlined metabolisms that repress more complex pathways (such as the TCA cycle) in favor of high glycolytic activity, making glycolytic enzymes potential drug targets. The parasites also need to produce glycoprotein coats to shield themselves from the immune system, nucleotides for rapid proliferation, and reducing equivalents for redox homeostasis. The first enzyme of the glycolytic pathway, hexokinase (HK), is critical to the biosynthesis of all of these molecules, representing a here-to-fore underappreciated drug target. Herein I present evidence that HK is a promising target in the parasites *Trypanosoma brucei*, *Leishmania major*, and *Plasmodium falciparum*, and identify novel inhibitors of these HKs, which may have potential for drug development. *T. brucei* and *Leishmania* are neglected tropical diseases in dire need of new treatments, while *P. falciparum* is the world’s deadliest parasite, notorious for rapidly evolving drug resistance. In addition to my work on drug development, I have also investigated the unique biology of the *T. brucei* HK and its alternative cellular localization. This alternative cellular localization is driven by a unique peptide sequence found only in kinetoplastid parasites. Together, these findings demonstrate the fascinating novelty of parasite glucose metabolism, and its great potential as a drug target.
DEDICATION

For My Momma
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CHAPTER ONE
LITERATURE REVIEW

The African Trypanosome

The Parasite and the Disease

The African trypanosome, *Trypanosoma brucei*, is an extracellular protozoan parasite of the mammalian bloodstream, transmitted by the bite of a tsetse fly (*Glossina* spp.) vector. There are three subspecies of *T. brucei*, two of which infect humans and one of which does not. *T. b. gambiense* is anthroponotic (humans are its primary reservoir) and causes a chronic illness in humans that lasts for years. *T. b. gambiense* likely developed from sustained preying on humans by tsetse flies in central and West Africa (Steverding 2008). *T. b. rhodesiense* is a zoonotic parasite that causes acute human illness in south and east Africa, killing in months. *T. b. gambiense* infection is much more common than the zoonosis, accounting for ninety-eight percent of cases (Franco et al. 2014). Together the two subspecies have an annual incidence of 300,000, with around 10,000 new infections and 10,000 deaths, though the conditions in some endemic countries make accurate reporting difficult (Fevre et al. 2008).

The third subspecies of parasite, *T. b. brucei*, causes a largely asymptomatic infection in wildlife and a wasting disease nagana (from n’gana, Zulu for powerless or useless) in livestock, costing 4.5 billion dollars in agricultural losses a year (Torr et al. 2005). Humans are immune to *T. b. brucei* due to the presence of high-density lipoproteins called trypanosome lytic factors 1 and 2 (TLF1 and 2) that lyse the parasite. Both TLFs contain apolipoprotein L1 (ApoL1), which is trafficked to the lysosome after
uptake and forms an anion channel in the lysosome, leading to osmotic stress and lysis (Wheeler 2010). Both TLFs also contain haptoglobin related protein (Hpr), which binds hemoglobin that is recognized by *T. brucei* haptoglobin-hemoglobin receptor (TbHpHbR), thus increasing uptake of TLF (Wheeler 2010). There are few differences between the subspecies besides the TLF resistance mechanisms, allowing *T. b. brucei* to be used as a model in the laboratory.

The two human infectious subspecies, *T. b. gambiense* and *T. b. rhodesiense*, have separately evolved TLF resistance mechanisms. *T. b. rhodesiense* possesses a serum resistance-associated (SRA) gene whose protein product binds and inactivates ApoL1 (Oli et al. 2006). SRA is a pseudogene related to the variant surface glycoprotein (VSG) genes that *T. brucei* uses for antigenic variation. *T. b. gambiense* resistance is more complex. *T. b. gambiense* group 2 expresses less TbHpHbR and the receptor is less active due to a single amino acid mutation (Kieft et al. 2010). The gene TgsGP is essential for *T. b. gambiense* group 1 resistance (Capewell et al. 2013). TgsGP, like SRA, is a pseudo-VSG gene, though its function is less clear, as it does not directly bind ApoL1, despite localizing to the flagellar pocket where TLF uptake occurs (Wheeler 2010).

Human illness caused by African trypanosomes is known as human African trypanosomiasis (HAT), or sleeping sickness, due the parasite’s disruption of circadian rhythms once it invades the cerebral-spinal fluid. HAT has two major stages, the initial hemolymphatic stage and the later encephalitic stage. The hemolymphatic stage starts within a few weeks of being bitten by an infected fly as parasites begin to proliferate in
the blood and lymph, causing nonspecific, flu-like symptoms such as fever and headache (Kennedy 2004).

The parasites reproduce quickly in the bloodstream and are adept at evading host immunity. Parasites are shielded from the host by a dense surface coat made of a single type of variant surface glycoprotein (VSG). Rapid turnover of the parasite plasma membrane slows the accumulation of host antibodies and complement, but the immune system does eventually recognize and clear the parasites. However, by this time a small subset of the parasite population will have changed which VSG gene is expressed, avoiding detection and destruction. With over 2,000 VSG genes (and the capacity to create new ones through gene rearrangement), this process can continue *ad infinitum*, and gives HAT a nearly one hundred percent mortality rate when untreated. Eventually the parasites breach the blood-brain barrier and colonize the cerebral-spinal fluid, where a wide range of neurological pathologies can be triggered, including the classic inversion of circadian rhythms (Kennedy 2004).

The arsenal of medicines for HAT is severely limited, both in the number of drugs available and by the subspecies and disease stage each drug is effective against. Suramin or pentamidine are used against the hemolymphatic stage disease. Treatment of encephalitic stage is subspecies dependent, with eflornithine and nifurtimox used against *T.b. gambiense* and arsenical melarsoprol being the only option for *T.b. rhodesiense*. These treatments must be administered intravenously multiple times (difficult in many underdeveloped regions) and have severe side effects, particularly melarsoprol, which itself has a five percent mortality rate (Kennedy 2004). Additionally, diagnosis of the
disease is difficult, as the disease initially presents with nonspecific symptoms, and the wide range of symptoms in the later stage can be confused with other neurological disorders. Definitive diagnosis requires microscopic observation of blood samples (early stage) or lumbar puncture samples (late stage), making diagnosis challenging in rural and underdeveloped areas (Malvy and Chappuis 2011). Further frustrating diagnosis, *T.b. gambiense* and *T.b. rhodesiense* are morphologically indistinct and usually require genetic analysis (detection of SRA or TgsGP) in order to be identified. Despite the challenges presented by limited treatment options and the underdeveloped region the disease occurs in, elimination of *T.b. gambiense* has been deemed within reach (Franco et al. 2014), as the disease lacks significant animal reservoirs and the parasites have a low survival rate in the tsetse fly, with 0.1% of flies being competent for transmission (Malvy and Chappuis 2011). Elimination of *T.b. rhodesiense* is currently not feasible due the large number animal reservoirs (Franco et al. 2014). Further control of HAT will require improved screening and vector control measures, and the development of new therapeutics and diagnostic tools.

**History and Impact**

Phylogenies constructed from small subunit ribosomal RNA sequences suggest that trypanosomes have been in Africa as long as humans, and may have played a role in human evolution; humans are resistant to all trypanosomes with the exception *T.b. gambiense* and *T.b. rhodesiense*, which are believed to have evolved recently (Steverding 2008). Indeed the majority of mammals in Sub-Saharan Africa tolerate trypanosome
infection well, with the exception of domestic animals, perhaps due to their recent evolution by artificial selection or perhaps their recent introduction into Africa from other regions (Steverding 2008). The first historical recordings of trypanosomaisis are found on Egyptian papyrus dating back to 2000 BC (Steverding 2008), which report a wasting disease in cattle called *ushau*, whose description matches that of nagana. Egyptian herdsmen mixed their stock with wild animals to create resistant lines (Steverding 2008). This practice was later abandoned after a change in the course of the Nile River by dam construction destroyed tsetse fly habitats in North Africa, limiting them to their current range in Sub-Saharan Africa (Steverding 2008). The first descriptions of sleeping sickness come from twelfth century Arabian travelers in Africa, such as the historian Ibn Khaldun, who wrote of an Emperor of Mali affected by “the sleeping illness” in fourteenth century (Steverding 2008). Arab and later European slave traders learned to avoid slaves with swollen lymph nodes of the neck, a classic sign of trypanosomasis (Steverding 2008). Despite this awareness and observation of sleeping sickness throughout history, investigation into the nature of the disease did not begin in earnest until the nineteenth century, when Europeans began to explore and colonize Africa.

Three sleeping sickness epidemics have occurred in the twentieth century. The first two of these epidemics occurred at the beginning of the century as population movements (of humans and animal reservoirs) and environmental changes (ex. deforestation for cash crop production) brought on by European colonization disrupted the normally isolated foci of transmission where there was sustained contact between vectors, humans, and animal reservoirs (Malvy and Chappuis 2011). These epidemics
prompted colonial authorities to order initiatives to study and combat the disease (Steverding 2008). Sir David Bruce first identified trypanosomes in the blood of infected cattle, and established the tsetse fly as the disease vector (Bruce 1895). Later work identified the two human infective subspecies with *T.b. gambiense* identified by Dutton in 1902 and *T.b. rhodesiense* named by Stephens and Fantham in 1910. The first trypanocidal drugs were also developed at this time, most of which are still in use today. Suramin (originally Germanin) was developed by the German Bayer Company in 1917 through the screening of synthetic dyes for trypanocidal activity (Steverding 2010). The compound was originally offered to Britain for the restoration of German colonies in Africa, but the British refused. In 1924 the French pharmacist Ernest Fourneau published the structure, which was later confirmed by Bayer (Steverding 2010). The diamidine drug pentamidine was developed in the thirties. Diamidines were originally investigated due to their hypoglycemic properties and the observation that trypanosomes consume large amounts of sugar, but it was later determined that the compounds themselves were trypanocidal (Steverding 2010). Arsenic based compounds were found to be effective as early as 1858 (Steverding 2010). The most effective and safe arsenicals developed were tryparsamide (1919) and melarsoprol (1949). Ernst Friedheim developed Melarsoprol by combining melarsen oxide with dimercaprol, which was used by the British during World War II to protect against lewisite, an arsenic gas based chemical weapon (Steverding 2010). Tryparsamide use was discontinued in the sixties due to side effects, while melarsoprol remains in use today. Judicious screening and treatment campaigns by colonial authorities brought the disease largely under control by the 1960s, with less than
5,000 new infections per year (Steverding 2008). Tragically, lapses in these control programs during the transition of power in Africa from colonial authorities to independent governments led to a third epidemic that reached its peak in the 1990s (Franco et al. 2014). Better news came in the 1970s with the development of eflornithine, the first new sleeping sickness drug in decades. It is a designed inhibitor of ornithine decarboxylase (ODC) and was originally developed as an anti-cancer drug (Steverding 2010). While a potent inhibitor of the human enzyme in vitro, the high turnover rate of human ODC precluded its use against cancer. Cyrus Bacchi showed that eflornithine was effective against a mouse model of sleeping sickness in 1980, and it was later found to be effective against encephalitic stage *T. b. gambiense*. The drug targets parasite ODC, and the difference in sensitivity is the consequence of a low turnover rate of parasite ODC (Steverding 2010). Eflornithine was introduced for human use in 1990 and helped bring the surging number of infections back down after their peak in 1997 (Frano et al. 2014). Eflornithine production may have been discontinued at the turn of the century if not for negotiations between the WHO and pharmaceutical companies due to the low profitability of production (Steverding 2008). Unfortunately, melarsoprol remains the only option against the encephalitic stage and *T. b. rhodesiense*, as it has a much higher ODC turnover rate than the other sub-species.

**The Life Cycle**

*T. brucei* has a complex life cycle, with multiple stages in both the insect and mammalian hosts (Figure 1.1). The bloodstream form trypomastigote is the proliferative
form in the mammalian host and the cause of pathology, dwelling in the bloodstream and capable of invading the cerebral-spinal fluid. This long slender form can be cultured in vitro in media. An unknown factor induces a subset of the trypomastigotes to develop into short stumpy forms, which have an arrested cell cycle and are pre-adapted for life in the tsetse fly. When taken up by a tsetse fly during a blood meal, short stumpy forms differentiate into procyclic form trypomastigotes in the tsetse midgut. These procyclic forms express tsetse specific coat proteins, called procyclins, over VSG and adapt their metabolism to the proline rich midgut environment. The most notable change is the development of an active mitochondrion and TCA cycle. This is the insect stage that can be cultured in the laboratory.
Figure 1.1 The Life Cycle of *Trypanosoma brucei*

*T. brucei* has a complex life cycle (a), colonizing multiple tissues in the tsetse fly vector (b) and human host (c). Permission granted for use of the figure (Langousis and Hill 2014).

Procyclic forms exist in at least two stages, the early and late stages, which are distinct in their protein expression patterns and behavior (Imhof et al. 2014). Only the early stage procyclics express GPEET and show social motility *in vitro* when grown on
agar plates (Imhof et al. 2014). These early forms colonize the midgut and eventually penetrate the midgut wall and migrate though the fly’s alimentary canal, becoming late stage procyclics, which lack GPEET expression and social motility. The late stage procyclics reinvade the gut at the proventriculus and differentiate into proliferative epimastigotes, which infect the salivary glands, attaching to the epithelium. Meiosis and mating also occurs in the salivary glands, with compatible gametes recognizing each other via flagellar association and fusion (Peacock et al. 2014). After colonizing the salivary glands, the epimastigotes began to differentiate into metacyclic trypomastigotes, which are pre-adapted for the mammalian host and express VSGs required for successful bloodstream colonization. These infective cells are injected when the fly feeds, continuing the life cycle.

**Cell Structure**

*Trypanosoma brucei*’s elegant, undulating form possesses many novel structures and features (Figure 1.2). A member of class Kinetoplastea, *Trypanosoma brucei*’s mitochondrial DNA is packaged in a unique structure known as the kinetoplast. Mitochondrial, also called kinetoplast DNA (kDNA), plasmids form a chainmail-like structure that is attached to the flagellum through the basal body, with filaments that span the mitochondria membrane.
During cell division the movement of the basal body helps ensure proper separation of mother and daughter kDNA (Langousis and Hill 2014). *T. brucei* possess a single, tubular mitochondria that branches out in the TCA cycle active procyclic form (Imhof et al. 2014). *T. brucei* also possesses a single flagellum which is attached to the cell except for the anterior tip, spiraling around cell in a corkscrew like pattern (hence the name *Trypanosoma* – from the Greek *trypano*- borer and *soma* - body). This configuration causes the beating of the flagella to elicit an undulating movement of the cell body for locomotion.

**Figure 1.2 Cell Structure of *Trypanosoma brucei***

Schematic showing the key organelles and structures of *T. brucei*. Permission granted for use of the figure (Matthews 2005).
The flagellum is attached to the cell membrane and cytoskeleton through the flagellar attachment zone (FAZ). A crystalline paraflagellar rod (PFR) runs in parallel with the flagellar axoneme once it exits the cell. While its exact function is incompletely understood, the PFR is believed to have roles in structure and regulation, and is essential to motility (Langousis and Hill 2014). The flagellum is of central importance to the trypanosome, as motility is essential to virulence (Langousis and Hill 2014) and cell division (Ralston et al. 2009). Additionally, the site at which the flagella re-enters the cell body at the posterior end, the flagellar pocket, is the only site of macromolecule exchange with the environment, as the tight network of subpellicular microtubules of the cytoskeleton preclude exchange elsewhere (Langousis and Hill 2014). Endocytosis is essential to the parasite for nutrient acquisition and the pocket is the site of antibody clearance and VSG turnover. Finally, trypanosomes (and all other kinetoplasts) possess a unique metabolic organelle known as the glycosome.

**Glycosomes & Glycolysis**

Glycosomes are peroxisome-like organelles that house the enzymes catalyzing the first six or seven steps of the glycolytic pathway, in bloodstream forms and procyclic forms, respectively, due to life stage dependent express of cytoplasmic or glycosomal phosphoglycerate kinase (Blattner et al. 1998). In the bloodstream form, this compartmentalization results in a net balance of ATP and NADH generation and consumption, while the last three remaining steps generate two ATP in the cytoplasm (Figure 1.3).
Figure 1.3 Compartmentalization of Glycolysis in *Trypanosoma brucei*

Bloodstream form *T. brucei* compartmentalizes the first seven steps of glycolysis in modified peroxisomes known as glycosomes. The abbreviations used are as follows:

ALD: aldolase; DHAP: dihydroxyacetone phosphate; 1,3BPGA: 1,3-bisphosphoglycerate; ENO: enolase; F-6-P: fructose-6-phosphate; FBP: fructose 1,6-bisphosphate; G-3-P: glyceraldehyde 3-phosphate; G-6-P: glucose-6-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Glc: glucose; Gly-3-P: glycerol-3-phosphate; GPDH: glycerol 3-phosphate dehydrogenase; Mito: mitochondrial enzymes; PEP: phosphoenolpyruvate; 2-PGA: 2-phosphoglycerate; 3-PGA: 3-phosphoglycerate;
PGI: glucose-6-phosphate isomerase; PGK: phosphoglycerate kinase; PGM: phosphoglycerate mutase; PFK: phosphofructokinase; PK: pyruvate kinase; PYR: pyruvate; TbHK: *T. brucei* hexokinase 1 and/or 2; TPI: triose-phosphate isomerase.

Permission granted for use of the figure (Coley et al. 2011).

Despite the modest ATP gain, the abundance of glucose in the bloodstream and the compartmentalization of the pathway allow modulated flux to meet the parasite’s energy needs. As the mitochondrion is inactive in the mammalian host, glycolysis and glycosomes are essential to BSF parasites, and are promising drug targets. Glycosomes undergo remodeling in procyclic cells and are active in this stage as well, but are not essential (Bauer et al. 2014, Parsons et al. 2001). Glycosomal proteins contain peroxisome-targeting sequences one or two (PTS1 and PTS2), which are a three amino acid sequence on the carboxy terminus (Gould et al. 1990) and an eleven amino acid sequence on the amino terminus (Tsukamoto et al. 1994), respectively. There is also a less common internal PTS (Peterson et al. 1997) of undefined sequence composition. The PTS1 and 2 sequences are recognized by the chaperones PEX5 (all PTS sequences) and PEX7 (PTS2), and translocated into the glycosome by the PEX14 complex (Figure 1.4), after which PEX5 and PEX7 are released back into the cytoplasm (Coley et al. 2011).
Figure 1.4 Glycosome Targeting in *Trypanosoma brucei*

C-terminal peroxisome targeting sequences (PTS1) are recognized by the peroxin 5 (PEX5) chaperone, which docks with the membrane bound peroxin 14 to import glycosome matrix proteins. Peroxin 7 recognizes the N-terminal PTS2, and also docks with PEX14. PEX7 may reach PEX14 via interaction with PEX5. Permission granted for use of the figure (Coley et al. 2011).

Proper trafficking of glycosomal proteins is essential, as mislocalization has been shown to be lethal (Blattner et al. 1998; Helfert et al. 2001). The two ATP consuming enzymes hexokinase (HK) and phosphofructokinase (PFK) are not regulated by feedback inhibition by their products, unlike the same enzymes from many other organisms, leading to the “turbo explosion” hypothesis that the lethality of the mis-localization of glycosomal proteins is due to unregulated depletion of cellular ATP (Haanstra et al. 2008). The novelty and essentiality to the pathogenic stage of the parasite make glycolytic enzymes of particular interest.
The Hexokinases

Hexokinase catalyzes the first step of glycolysis, the transfer of the γ phosphate of ATP to glucose, yielding ADP and glucose-6-phosphate. *T. brucei* possesses two hexokinases (TbHK1 and TbHK2). The proteins are 98% identical, with only ten different amino acids, seven of which are found at the C terminus. RNAi and gene knockout studies have shown that TbHK1 is essential to both procyclic and bloodstream form parasites, while TbHK2 is essential to bloodstream forms only (Albert et al. 2005 and Chambers et al. 2008). The TbHKs have a number of novel features that distinguish them from orthologous enzymes. The enzymes are compartmentalized in the glycosome via a PTS2 sequence.

Regulation is in part conferred by pH. Under acidic pH, an ADP binding site is revealed and ADP can be a potent inhibitor of the enzyme (Dodson et al. 2011). Notably, the metabolite glycerol-3-phosphate can protect TbHK1 from ADP inhibition at low pH (Dodson et al. 2011). Other means of regulation include regulated oligomerization. TbHKs form hexamers, while in most systems HKs are monomeric or dimeric (Chambers et al. 2008). It is unknown if TbHK1 and 2 form homo- or hetero-hexamers *in vivo*, but heterohexamers have higher catalytic activity *in vitro* (Chambers et al. 2008). In addition to their novelty compared to HKs for other organisms, the TbHKs have distinct properties from one another, remarkable considering their high identity. *E. coli* produced rTbHK1 has robust catalytic activity *in vitro* assays, while attempts to purify an active rTbHK2 have been unsuccessful (Morris et al. 2006). Mutagenesis swapping of the last ten amino acids of the proteins reverses these properties, highlighting the importance of the C-
terminal tail that harbors the majority of the amino acid differences. Curiously, heterohexamers of rTbHK2 with a catalytically dead rTbHK1 have activity, and have been used to demonstrate that TbHK2 is inhibited by pyrophosphate (Chambers et al. 2008). At the genetic level, the two HK genes are in tandem on chromosome 10, and are part of the same polycistronically transcribed pre-messenger RNA. Differences in the 3’ untranslated region regulated differential expression of the TbHKs (Dodson, unpublished data). Recently Imhof and colleagues showed that TbHK1 is upregulated in early stage procyclies, while TbHK2 is upregulated in late stage procyclies (Imhof et al. 2014). This is interesting given that TbHK2 double knockout procyclies have impaired social motility, which is only present in late stage procyclies (McAlpine, unpublished data).

A TbHK2 specific antibody has also shown that TbHK2 has an alternative localization to the flagellum, challenging the dogma that extra-glycosomal TbHKs are toxic (Joice et al. 2012). Because of the lack of sufficiently specific tools, it is unclear if TbHK1 also has alternative localization. Published mitochondrial (Panigrahi et al. 2009) and flagellar proteomes (Oberholzer et al. 2011) suggest that both TbHKs have alternative localizations, but the sheer abundance of TbHK1 means that contamination cannot be ruled out. The essentiality of TbHK (making its inhibition lethal) and its low amino acid identity with host HKs (~28%, allowing inhibitor selectivity over host enzyme), make it a heretofore underappreciated drug target. A recent high throughput screening (HTS) campaign identified TbHK1 specific inhibitory small molecules (Sharlow et al. 2010). Further work is needed to characterize the differences and
relationship between TbHK1 and 2, investigate their alternative localization, and further develop the leads found in the HTS.

*Leishmania*

**The Parasite and the Disease**

Parasites in the genus *Leishmania* cause disease in an impressive spectrum of animals, including twenty-one species that affect humans. Kinetoplast relatives of *T. brucei*, these parasites are transmitted by sand flies of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World). The WHO estimates there are about 1.3 million cases of leishmaniasis annually, causing 20,000 – 30,000 deaths. *Leishmania* can be anthroponotic or zoonotic, depending on the species of parasite and vector. *Leishmania* is an intracellular parasite of host phagocytic immune cells, particularly macrophages. The parasite survives the harsh environment of the phagosome via a combination of adaptation to and modification of the compartment. Using immune cells as Trojan horses, *Leishmania* can affect a myriad of different tissues, including skin (cutaneous leishmaniasis), mucosal tissues (mucocutaneous leishmaniasis), and infections of internal organs (visceral leishmaniasis). The type of infection depends on the species and host factors, such as immune status. For example, AIDS patients are more likely to develop mucosal infections (Strazzulla et al. 2013). Old World (Eastern Hemisphere) *Leishmania* is usually cutaneous and/or visceral, and is mainly concentrated in Southern Europe, North Africa, the Middle East, India and Southeast Asia. New World (Western
Hemisphere) *Leishmania* is usually cutaneous and/or mucosal, and is mainly found in Mexico, Central America, and the Amazon basin.

The presentation of symptoms depends on the form of the disease, but all forms of leishmaniasis begin as cutaneous infections, producing a single lesion around the location of the sand fly bite. These lesions usually heal, but some species can cause disperse cutaneous lesions throughout the body (*L. amazonensis*) or have lesions metastasize to involve mucosal tissues (*L. panamensis* and *L. braziliensis*), or visceral tissues (*L. donovani* and *L. infantum*) (McGwire and Satoskar 2013).

The pathogenesis of mucocutaneous leishmaniasis is currently not well characterized. In addition to the painful, disfiguring lesions, the destruction and inflammation of the upper respiratory tract can make breathing and eating difficult, and is conducive to secondary infections. Visceral infections mainly affect the spleen, liver, and bone marrow, causing pancytopenia and compromising immunity (McGwire and Satoskar 2013). Visceral *Leishmania* is also known as kala-azar, Hindi for “black fever,” a reference to the tendency of some strains of *L. donovani* in India to cause darkening of the skin.

Relapses of *Leishmania* infections can occur after successful treatment. Reappearance of cutaneous infection at the periphery of healed lesions is known as leishmaniasis recidivans. A more severe form of relapse is Post-kala-azar dermal leishmaniasis (PKDL), characterized by diffuse dermal lesions after treatment of visceral infections. Both leishmaniasis recidivans and PKDL can occur after months or years of being asymptomatic.
The arsenal of drugs for leishmaniasis is diverse, but limited on a case-by-case basis depending on the species causing infection and the region where the infection occurred. Pentavalent antimonials are the most common treatment for leishmaniasis, but are problematic for a number of reasons: its mechanism of action is unknown, it is not orally bio-available (daily intravenous infusions can be difficult in the impoverished areas where Leishmania occurs), resistance is increasing (Kumar 2013), and there are many serious side effects, including cardiotoxicity (McGwire and Satoskar 2013).

Amphotericin B is also commonly used for visceral leishmaniasis. Originally developed as an anti-fungal, amphotericin B binds ergosterol in the parasite membrane and forms a pore, disrupting membrane integrity and causing cytoplasmic leakage. While effective, amphotericin B requires long hospital stays and can cause serious renal complications; liposomal formulations of amphotericin B are much better tolerated, but higher cost often precludes its use.

The aminoglycoside antibiotic paromomycin is sometimes used in combination with amphotericin B or alone, and works by inhibiting ribosome function by binding the 16S subunit. Paromomycin has many common aminoglycoside side effects, including ototoxicity and nephrotoxicity (McGwire and Satoskar 2013). Pentamidine is another second line anti-leishmanial, but its mode of action is unknown and it has a myriad of toxic side effects. Originally intended as a cancer drug, Miltefosine is one of the few oral leishmaniasis drugs available, and is well tolerated. Unfortunately, miltefosine is not efficacious against all species, but importantly can treat visceral leishmaniasis and PKDL (McGwire and Satoskar 2013). Cutaneous lesions can also be treated with heat or
cryotherapy. Full patient compliance for many these treatments is low, due to their toxicity, cost, and the amount of time required. Further, the number of cases of leishmaniasis recidivans and PKDL (5-10% of cases in India, 50% of Sudanese cases), show that the majority of these treatments do not provide a sterile cure (Zijlstra et al. 2003). Thus, there is a growing need for novel Leishmania therapies.

**History and Impact**

Leishmaniasis has been present throughout human history, with one of the earliest reference to cutaneous lesions appearing on Assyrian tablets dating back over 2,500 years (Cox 2002). In the new world, Leishmania lesions have been found depicted in ancient sculptures and carvings. Physicians throughout history noted these sores, but the visceral disease was often confused with malaria, with similar symptoms such as fever and enlarged spleen. Leishmaniasis was first distinguished from malaria in 1824 in Jessore, India, where there was an outbreak of malaria-like fevers that failed to respond to quinine, and lacked the periodicity of malarial fevers (Cox 2002). Many thought visceral leishmaniasis to be a new virulent malaria species, until Leishman and Donovan independently demonstrated the parasite in the spleens of patients in 1900 (hence the name) (Leishman 1903 and Donovan 1903). The Phlebotomus sand fly vector remained elusive until 1921 (Sergent et al. 1921), and transmission through biting was not demonstrated until 1941 (Adler and Ber 1941). New World leishmaniasis was assumed to be identical, but in 1911 Vianna showed that the South American parasites were a different species (Vianna 1911) and in 1922 it was found that the New World vector was
a different genus, *Lutzomyia*. Vianna also discovered that the trivalent antimonial Tartar Emetic, originally used for sleeping sickness, could be used to treat cutaneous and mucocutaneous *Leishmania*, and pharmaceutical research in the 1940’s would result in the antimonials used today (Kumar 2013). Indeed, the entire pharmaceutical arsenal for leishmaniasis consists of drugs that did not have *Leishmania* as their original target: amphotericin B (fungi), paromomycin (bacteria, amoebas), pentamidine (*T. brucei*), and miltefosine (cancer).

**Life Cycle**

*Leishmania* parasites are extracellular parasites of their vectors, female sand flies of the genera *Phlebotomus* and *Lutzomyia*, and intracellular parasites of their vertebrate hosts, including humans (Figure 1.5). When an infected sand fly feeds, motile metacyclic promatigotes are injected into the host. Immune reaction to the bite results in the promatigotes being phagocytosed by macrophages or other phagocytic immunes cells. The parasite factors prevent phagosome maturation to avoid destruction and the promastigotes differentiate into proliferative, non-motile amastigotes that thrive within the phagosome, eventually lysing the cell, releasing infective amastigotes.

When a sand fly feeds on an infected host and takes up infected host cells, amastigotes in macrophages sense the environmental change, triggering their transformation into procyclic promastigotes, which proliferate and colonize the sand fly midgut before becoming infective metacyclic promastigotes (Dostálová and Volf 2012). The details are unclear, but sexual reproduction appears to occur in the sand fly
Leishmania was long thought to be clonal, but a population genetics study of *L. braziliensis* suggests sexual reproduction (Rougeron et al. 2009), and another study has used *L. major* strains with different selectable markers to show genetic exchange (Akopyants 2009). Promastigotes secrete a proteophosphoglycan gel known as promastigote secretory gel, which obstructs the sand fly digestive track, causing regurgitation during blood meals that promotes transmission, completing the life cycle (Rodgers 2012).

**Figure 1.5 The Life Cycle of Leishmania**

*Leishmania* is both an extra- and intra- cellular parasite as it cycles through mammalian hosts and sand fly vectors. Permission granted for use of the figure (Harhay et al. 2011).
Cell Structure

As fellow kinetoplastids, *Leishmania* shares a number of structural similarities with *T. brucei*, including glycosomes, the flagellar pocket, single mitochondria, and their shared namesake the kinetoplast (Figure 1.6). In contrast to *T. brucei*, *Leishmania* has an external flagellum that is not associated with the cell membrane as a promastigote, and a severely shortened flagellum as amastigote. While the amastigote flagellum does not provide motility, it may still have an important role in sensing and cell-to-cell signaling (Gluenz et al. 2010). Additionally, *Leishmania* has an active mitochondrion in all life cycle stages.

Figure 1.6 Cell Structure of Leishmania
Diagram showing the structure of *Leishmania* promastigotes (left) and amastigotes (right). Permission granted for use of the figure (Besteiro et al. 2007).

**Hexokinases**

*Leishmania* genomes harbor two nearly identical hexokinases in tandem on chromosome 21 (89 to 99 percent amino acid identity depending on the species), which are similar to TbHK1 and 2, with 61 percent identity between *L. major* hexokinase 1 (LmHK1) and TbHK1. Unlike *T. brucei*, *Leishmania* parasites also have a glucokinase, the role of which is unclear. The glucokinase has a low affinity for glucose ($K_m = 2.55$ mM), suggesting it may serve as a sensor of glucose levels or shunt excess glucose into alternative pathways such as the pentose phosphate pathway (PPP) (Caceres et al. 2007). Both hexokinases and the glucokinase contain PTSs, with HK1 and HK2 having an N-terminal PTS2 and GK having a C-terminal PTS1. Despite having a PTS2, HK may moonlight as a hemoglobin receptor in the flagellar pocket (Krishnamurthy et al. 2004). Expression of a PTS2-truncated HK causes sugar toxicity in *L. donovani*, suggesting this alternative localization must be somehow regulated (Kumar et al. 2010).

While *Leishmania* does not share *T. brucei*’s reliance on glycolysis – its mitochondria is active in all life stages- hexokinase may still be critical. Amino sugars may be the primary carbon source in the human pathogenic stages of the parasite, and metabolism of these compounds begins with phosphorylation by HK (Naderer et al. 2010). Additionally, mannose metabolism is critical for virulence, due to the high mannose content of the parasite surface coat (Spath et al. 2000; McConville et al. 2002;
Sacks et al. 2000) and the use of mannogen as the primary energy reserve (Ralton et al. 2003). HK contributes to the biosynthesis of both, either by producing G6P to be converted to mannose-6-P (M6P), or by phosphorylating mannose directly. Further studies will be needed to validate Leishmania HK as a potential drug target.

**Plasmodium**

**The Parasite and the Disease**

Protozoan parasites in the genus *Plasmodium* cause the disease malaria in a variety of animals, including humans. Human malaria is caused by four species of protozoan parasites in the genus *Plasmodium*: *falciparum*, *vivax*, *ovale*, and *malariae*. Additionally, the primate parasite *P. knowlesi* can be zoonotically transmitted to humans. Of these species, *P. falciparum* is both the most common and the most virulent. *P. vivax* and *ovale* are also of concern, due to their ability to infect liver cells and remain quiescent, allowing recurrent infections to occur for years after the initial infection, making a sterile cure difficult. The parasites are transmitted by female mosquitoes in the genus *Anopheles*, which contains over a hundred species with global distribution.

Malaria is a global health threat endangering over half the world’s population, with 3.3 billion people living in areas where transmission can occur. According to estimates by the WHO, there were more than 149 million cases of malaria in 2010, resulting in over half a million deaths, though these figures may be underestimations due to conditions in endemic countries (Snow et al. 2005). Over ninety percent of malaria related deaths occur in Africa, mostly in children under five. While malaria is not always
fatal, it also has significant effects on quality of life and economic development, reducing the gross domestic product of some endemic countries by over one percent.

Malaria can cause a variety of symptoms, such as fever, fatigue, and vomiting. Complications such as renal failure (black water fever) and brain damage (cerebral malaria) can also occur with the more virulent *P. falciparum*.

The blood stream stage accounts for the symptoms of malaria and thus is a target for anti-malarials for the treatment of malaria. The most common anti-malarials used fall under two main categories: quinine - derived compounds (such as chloroquine) and artemisinins. Quinine based treatments, which prevent the parasite from sequestering toxic hemezoin that is produced by parasite metabolism of host erythrocyte hemoglobin, have been in use for decades and significant resistance has emerged, with several multidrug resistant strains identified (Dua et al. 2003). The more recently developed artemisinin based therapies have proven effective, but resistance has begun to emerge to these compounds as well (Amaratunga *et al.* 2012). Further, the anti-malarial mechanism of artemisinin is unknown, making the study of resistance or improvements to the drug difficult. Ever emerging resistance necessitates the development of new anti-malarials.

While mosquito nets and insecticides have reduced transmission, eradication remains elusive and is made difficult by the poor conditions and infrastructure in the third world countries where malaria is prevalent. Vaccine development has been frustrated by the intra-cellular nature of the parasite and its capacity for antigenic variation. Malaria eradication will require significant screening, vector control, and drug development and deployment campaigns in concert.
History and Impact

Mosquitoes have always been a scourge of mankind, with mosquito borne illness accounting for more deaths in human history than any other cause. The majority of these deaths have come from malaria. The human infective species *P. vivax*, *P. ovale*, and *P. malariae* originated from monkey infective species in Africa that existed in regions into which humans migrated. Malaria was found across the majority of every continent other than Antarctica at the peak of its historical distribution. *P. falciparum* is a new comer, jumping from gorillas to humans as recently as 3,000 years ago, perhaps explaining its greater virulence compared to the older human infective species (Liu et al. 2010).

References to illnesses resembling malaria are found worldwide; ancient Chinese medical books (~4,700 years ago), Hindu scriptures (~3,500 years ago), and Egyptian and Sumerian texts (~4,000 years ago) all mention diseases matching malaria (Cox 2010). Malaria reached Europe more recently; Greek and Roman texts only begin describing malaria less than 2,500 years ago, notably by Hippocrates in the third century BC. Interestingly the ancients’ notes of the fever’s periodicity and virulence allow us to infer the causative species, as each species has a characteristic periodicity: benign tertian (recurrence every two days, *P. vivax* and *ovale*), quartan (recurrence every three days, *P. malariae*), and malignant subtertian (recurrence every other day, *P. falciparum*). The association of malaria with mosquito dense areas was also made prior to germ theory and the elucidation of the disease’s means of transmission. High transmission rates in areas around marshes and swamps where mosquitos breed was attributed to the stagnant water
and foul air rather than the mosquitoes, hence the name malaria, from the Italian “malaria,” or bad air. This knowledge led to some control of malaria transmission through the avoidance and drainage of swamps and marshes. Scientific inquiry into malaria’s cause and transmission began in the 1800’s, especially after the establishment of the germ theory by Pasteur and Koch. Charles Louis Alphonse Laveran, a French army medical officer working in Algeria, discovered the blood stage parasites in 1880 through careful observation of blood samples of infected and uninfected individuals, marking the first observation of a parasitic protozoan (Laveran 1881). Though it took him years to convince the scientific community at large, his efforts were eventually recognized with the 1907 Nobel Prize. Among the first to continue Laveran’s efforts was the Italian scientist Amico Bignami and his colleagues, who together with Briton Ronald Ross proved the transmission of the parasite by mosquitoes in the late 1890’s (Ross 1898). The initial disappearance of the parasite when injected into the human host would remain a mystery until the discovery of the liver stage parasites by Henry Shortt and Cyril Garnham in 1947, and the cause of relapses of \textit{P. vivax} and \textit{P. ovale} would remain unresolved until the discovery of hypnozoites by Wojciech Krotoski in 1982 (Krotoski et al. 1982).

The development of anti-malarials also began in the 1800’s. Chinchona bark powder was among the first remedies for malaria, and in 1820 its plasmodicidal components quinine and quinidine were identified (Cox 2010). Chemist William Henry Penkinds attempted to make synthetic quinine in 1856 (a feat not accomplished until 1944), and his resulting failure was mauve, the first synthetic dye (Cox 2010). This
sparked the German dye industry, and led to the screening of dyes for anti-microbial activity and staining (the same industry that would produce suramin, noted earlier). Paul Ehrlich discovered that methylene blue was an effective stain for the parasite in 1891, and in the 1920’s the German Bayer Company began testing methylene blue derivatives for plasmodicial activity (Cox 2010). Several of these compounds would become important drugs, particularly chloroquine and primaquine. Chloroquine, once a proper dosage regimen was developed, was a potent and cost effective anti-malarial prior to the emergence of resistant strains, and primaquine remains the only drug effective against *P. vivax* and *P. ovale* hypnozoites. The widespread use of the insecticide DDT and chloroquine after World War II eliminated malaria in much of the developed world and lowered incidence drastically in India, but concerns about DDT’s effect on the environment, resistant mosquitos, and the independent emergence of four different chloroquine resistant *Plasmodium* strains ended hopes of malaria eradication elsewhere in the 1960s. In 1971 Chinese scientists isolated the plasmodicial component artemisinin from sweet wormwood, a traditional herbal remedy for malaria (Cox 2010). Artemisinin based treatments remain the most effective available today, but resistance has recently emerged in Southeast Asia (Amaratunga *et al.* 2012). Artemisinin resistance in Southeast Asia, the high preference for humans among African mosquitoes, and the lack of sufficient infrastructure and resources in many malaria endemic countries make the future of malaria control unclear, and makes the develop of new anti-malarials a priority.
Life Cycle

*Plasmodium* infects multiple host and vector tissues to perpetuate its life cycle (Figure 1.7). When an infected mosquito takes a blood meal, sporozoites in the mosquito’s salivary gland enter the bloodstream of the human host. The sporozoites travel to the liver and infect liver cells as intracellular parasites, reproducing asexually to form multi-nucleated shizonts, which rupture and release up to 40,000 merozoites that then infect erythrocytes. *P. vivax* and *ovale* form hypnozoites during the liver stage, which can lie dormant and remerge later to re-establish active disease. Once inside the erythrocyte, immature trophozoites develop into shizonts that release merozoites, continuing the asexual erythrocytic cycle. The periodic emergence (rupturing shizonts) and disappearance (invading merozoites) of the parasites from the bloodstream causes episodic fever and chills as the immune system responds to the parasites. Trophozoites can alternatively develop into gametocytes that are infective to feeding mosquitoes. Sexual reproduction occurs in the mosquito midgut via fertilization of the female gametocyte by the flagellated male gametocyte, producing an ookinete. The mobile ookinete develops into an oocyst that bursts and releases sporozoites that travel to the salivary glands, completing the cycle.
Figure 1.7 The Life Cycle of *Plasmodium*

*Plasmodium* cycles between mammalian hosts and mosquito vectors. Permission granted for use of the figure (Menard et al. 2013).

**Biology & Cell Structure**

*Plasmodium*, like most parasites in phylum Apicomplexa, possesses many unique cell structures to assist in host cell invasion. The phylum is named for the apical complex observed at the tip of invading Apicomplexan cells. The non-photosynthetic plastid organelle known as the apicoplast, was named for the only phylum in which it is found.
This unusual organelle has four membranes, the result of secondary endosymbiosis of a red alga by an Apicomplexan ancestor (Arisue and Hashimoto 2014). While the apicoplast has lost the ability to perform photosynthesis, it is essential to the parasite, performing vital functions such as lipid, isoprenoid, and heme biosynthesis. The apicoplast is housed within the apical complex, a collection of parasite specific structures that facilitate host cell invasion. The principal components of the complex are the rhoptries and micronemes, organelles which secrete proteins essential to invasion. Invasion is achieved by forming a tight junction between host cell receptors and parasite ligands, followed by invagination of the host membrane to form the parasitophorous vacuole. Like *T. brucei*, Plasmodium’s mitochondria is dormant in the mammalian bloodstream, with the electron transport chain remaining active only to reduce ubiquinone (Tanaka *et al.* 2012) while an abridged TCA cycle produces acetyl-CoA (Olszewski *et al.* 2011). The specialized parasite digestive vacuole digests host hemoglobin for amino acids while sequestering toxic ferrous heme degradation byproducts as inert hemozoin. Overall the structure of *Plasmodium* is highly specialized for invasion and rapid proliferation.

**Hexokinase**

The blood stage of *Plasmodium*, like *T. brucei*, lacks a complete TCA cycle and depends on glycolysis for ATP production (Olszewski *et al.* 2011). Several lines of evidence support this dependence on glycolysis. Knockout studies of mitochondrial proteins (Tanaka *et al.* 2012) and radiolabeled carbon studies (using U-13C-glucose, U-
$^{13}$C-$^{15}$N-aspartate and U-$^{13}$C-$^{15}$N-glutamine) (Olszewski et al. 2011) show that the mitochondria is not a significant contributor to cellular ATP levels, and Roth et al. found that the rate of glycolysis is increased up to 100-fold in infected erythrocytes (Roth et al. 1988). Additionally, knockout studies show that the hexose transporter protein responsible for importing glucose for glycolysis is essential to the parasite (Slavic et al. 2010), and inhibition of glycolysis with unmetabolizable glucose derivatives rapidly depletes parasite ATP (van Schalkwyk et al. 2008).

The pentose phosphate pathway (PPP) is also critical to the parasite. The PPP is a sister pathway of glycolysis, as it also begins with glucose-6-phosphate produced by HK, and the two pathways share other intermediates, such as fructose-6-phosphate (Figure 1.8). The pathway consists of oxidative and non-oxidative phases. The oxidative phase produces reducing equivalents in the form of NADPH. Both of *Plasmodium*’s antioxidant defense systems (one thioredoxin based and one glutathione based) are NADPH dependent, and the PPP is the main source of NADPH for the parasite (Preuss et al. 2012). The non-oxidative phase produces ribose-6-phosphate, the precursor for *de novo* nucleotide synthesis. Indeed, the importance of the generation of lipid, isoprenoid, amino acid, and nucleotide precursors by glycolysis and the PPP may be underappreciated, as the parasite must synthesize large quantities of biomolecules to divide rapidly (Salcedo-Sora et al. 2014). PPP activity is also increased in infected erythrocytes, up to 72 fold (Roth et al. 1986). The positioning of hexokinase at the top of these two essential pathways makes it an attractive drug target in *Plasmodium*. Furthermore, the *Plasmodium* genome contains only one copy of hexokinase, and this hexokinase has only ~26 percent
amino acid identity with human HKs (Preuss et al. 2012). Low identity with the host enzyme should allow selective inhibitors of the parasite enzyme to be identified. However, previous studies of *Plasmodium falciparum* hexokinase (PfHK) have been frustrated by an inability to produce soluble, active recombinant PfHK for *in vitro* studies (Olafsson et al. 1992).

Figure 1.8 Overview of Glucose Metabolism in *P. falciparum.*

The abbreviations used are as follows: 6PGD, 6-phosphogluconate dehydrogenase; DHAP, dihydroxyacetone phosphate; EPM, erythrocyte plasma membrane; GluPho,
glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase; GLUT1, glucose transporter; HK, hexokinase; HT, hexose transporter; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; TPI, triosephosphate isomerase. Permission granted for use of the figure (Preuss 2012).

**Summary**

In the following chapters I will present data showing the characterization of TbHK1 and the discovery and development of TbHK1 inhibitors that are lethal to *T. brucei* in culture. I also show that TbHK2’s alternative localization to flagellum is driven by a Kinetoplastid-specific peptide sequence in the N-terminus. Further, I explore the potential of HK as a drug target in the parasites *P. falciparum* and *L. major*, and screen the HKs from these parasites against a library of known TbHK1 inhibitors. Together, these studies will show the central role of HK in parasite metabolism, and the promise of HK inhibitors for new treatment options.

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CHAPTER TWO

EXPLORING THE MODE OF ACTION OF EBSELEN IN TRYPANOSOMA BRUCEI HEXOKINASE INHIBITION

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Abstract

Glycolysis is essential to *Trypanosoma brucei*, the causative agent of African sleeping sickness, suggesting enzymes in the pathway could be targets for drug development. 

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, EbSe) was identified in a screen as a potent inhibitor of *T. brucei* hexokinase 1 (TbHK1), the first enzyme in the pathway. 

EbSe has a history of promiscuity as an enzyme inhibitor, inactivating proteins through seleno-sulfide conjugation with Cys residues. Indeed, dilution of TbHK1 and inhibitor following incubation did not temper inhibition suggesting conjugate formation. Using mass spectrometry to analyze EbSe-based modifications revealed that two Cys residues (C327 and C369) were oxidized after treatment. Site-directed mutagenesis of C327 led to enzyme inactivation indicating that C327 was essential for catalysis. C369 was not essential, suggesting that EbSe inhibition of TbHK1 was the consequence of modification of C327 via thiol oxidation. Additionally, neither EbSe treatment nor mutation of the nine TbHK1 Cys residues appreciably altered enzyme quaternary structure.
Introduction

*Trypanosoma brucei* is the causative agent of African sleeping sickness in humans and nagana in livestock, both of which have tremendous impact on the lives of people in sub-Saharan Africa (Brun et al., 2010). Bloodstream form (BSF) *T. brucei*, the life cycle stage that grows rapidly in the blood of the mammalian host, depends solely on glycolysis for ATP production. As a consequence, glycolytic enzymes from the parasite have been considered as potential targets for therapeutic design.

The first enzyme activity in glycolysis, which transfers a phosphoryl group from ATP to glucose, is catalyzed by hexokinases (HK). *T. brucei* harbors two hexokinase genes, *TbHK1* and *TbHK2*, and both gene products are essential to BSF parasite (Chambers et al., 2008a). The TbHKs, whether purified from parasites or heterologously expressed in *E. coli*, oligomerize into hexamers (Misset et al., 1986; Chamber et al., 2008b). Due to the 98% identity of TbHK1 and TbHK2, the contribution of both to hexamer formation *in vivo* has remained elusive. Nonetheless, recombinant heterohexamers generated *in vitro* with known ratios of TbHK1 and TbHK2 have kinetic properties more similar to those reported for *T. brucei*-derived TbHK than recombinant TbHK homohexamers (Chambers et al., 2008b), suggesting that in the parasite oligomers are most likely heterohexamers. Notably, the composition of the heterohexamers *in vivo* is regulated in response to the nutritional environment in which the cells are cultured. The mechanisms behind this dynamic hexamerization, including the enzyme protein domains that participate in oligomerization, remain to be elucidated.
TbHK1 has previously been genetically and chemically validated as a potential target for therapeutic design. Further, the enzyme has been the subject of both structure-based approaches and high throughput screening (HTS) campaigns to identify compounds with potential as leads in therapeutic development. The HTS campaign included the screening of 220,233 compounds for inhibitors of TbHK1 (Sharlow et al., 2010a; Sharlow et al., 2010b). From this effort ten inhibitors, including six structurally related isobenzothiazolinone inhibitors have been identified. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, EbSe, Fig. 1A), a selenium containing variant of isobenzothiazolinones, was the most potent TbHK1 inhibitor found in the HTS campaign, with an IC$_{50}$ = 0.05 ± 0.03 µM. Notably, 2-phenyl-1,2-benzothiazol-3(2H)-one (SID 17387000, EbS, Fig. 1A), which differs from EbSe by replacement of the selenium atom with sulfur, was also identified in the HTS as a potent TbHK1 inhibitor (IC$_{50}$ = 2.0 ± 0.5 µM).
Figure 2.1 Ebselen Inhibition of TbHK1

EbSe, a known Cys-reactive compound, inhibits TbHK1 activity. (A) Structures of ebselen (EbSe) and ebsulfur (EbS), SID 17387000. (B) EbS and EbSe inhibition are irreversible by dilution. TbHK1 (32 ng) was incubated with EbSe or EbS in the assay for 15 minutes. Alternatively, inhibitor was incubated with enzyme prior to addition of other assay components, which yielded a 200-fold dilution of enzyme and inhibitor. (C) DTT
can block but not reverse TbHK1 inhibition by EbSe. TbHK1 (32 ng) was incubated with EbS or EbSe (hatched bars) followed by the addition of DTT (100 mM) prior to assay. Experiments were performed in triplicate and standard deviation is indicated.

EbSe is known to form seleno-sulfide adducts with target protein Cys residues. For example, EbSe inhibited human indoleamine 2,3-dioxygenase (IDO) through covalent modification of multiple IDO Cys residues, causing a change in enzyme conformation and inactivation (Terentis et al., 2010). This EbSe-based oxidization of critical Cys residues can also result in the generation of inappropriate disulfide linkages (Sakurai et al., 2006). Here we explore the role Cys residues have in EbSe-based TbHK1 inhibition. Through these efforts, we have found that EbSe oxidizes a single critical Cys residue, rather than promiscuously modifying Cys residues. Additionally, we have characterized the role of TbHK1 Cys residues in enzyme activity and the impact of their modification on oligomerization, finding that several of the Cys residues are essential for catalysis and can influence hexamerization.

Materials and Methods

Reagents

Tris (2-carboxyethyl)phosphine (TCEP), glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide (NAD⁺), adenosine triphosphate (ATP), and glucose were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA), while phosphoenol pyruvate (PEP), 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen, EbSe, PubChem SID 856002) and
glucosamine were obtained from VWR International (West Chester, PA). 3-(N-
maleimidopropionyl)-biocytin was purchased from Cayman Chemical (Ann Arbor, MI).

**Recombinant Enzyme Purification and Assay Conditions.**

Recombinant TbHK1 was purified as described from a culture of *E. coli* M15(pREP) harboring pQE30 (Qiagen, Valencia, CA) with TbHK1 cloned in frame of a 6-His tagging sequence (Morris et al., 2006). Briefly, a 10 mL bacterial culture was used to inoculate a 1 L culture which was grown to an OD of ~1 and then induced for 24 hr at room temperature with 250 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified as described (Morris et al., 2006).

TbHK1 Cys variants were generated using the parental pQE30 TbHK1 construct and a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). Primers used for the mutagenesis are listed in Supplementary Table 1 (Table S1) and generation of all variants was confirmed by sequencing. Protein expression and purification were performed as described above, with fractions from the purification probed by western blotting using an anti-RGS-His₆ antibody (Qiagen, Valencia, CA) to identify those harboring the recombinant protein. All variants were at least 90% pure based on coomassie blue staining of proteins resolved by SDS-PAGE.

HK assays were performed in triplicate as described using a coupled reaction to measure enzyme activity (Misset and Opperdoes, 1984; Morris et al., 2006). In short, the coupled assay uses glucose-6-phosphate dehydrogenase (G6PDH) to convert glucose-6-
phosphate (G6-P) generated by HK to 6-phosphogluconate with coincident reduction of
NADP to NADPH, which is monitored spectrophotometrically at 340 nm. Note that EbSe was found to be ineffective in a counterscreen for inhibition of G6PDH. Kinetic analyses were performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA).

**Mass Spectrometry analysis of EbSe-treated TbHK1.**

Mass spectrometry was performed to map EbSe modification on TbHK1. First, solution samples were C4 zip tipped (Millipore) following the manufacturers protocol. The desalted protein was dried down and re-suspended in 100 ng of trypsin (Sigma, proteomics grade) and digested overnight at 37°C. The digested sample was then placed in an auto sampler vial for LC-MS/MS analysis.

Enzymatically digested samples were analyzed via liquid chromatography (LC)-electrospray ionization (ESI) -tandem mass spectrometry (MS/MS) on an Orbitrap Elite mass spectrometer (Thermo) coupled to a Dionex 3000 nano LC system. A 20 cm 75 micron C-18 reversed phase LC column (packed in house, with Waters ODS C18) was utilized with a 200 nL/min flow rate and a 120 min gradient from 2% acetonitrile, 0.2% formic acid to 50% acetonitrile, 0.2% formic acid. MS data were acquired in a data-dependent strategy selecting the fragmentation events based on the precursor abundance in the survey scan (400–1700 Th). The resolution of the survey scan was 60,000 at m/z 400 Th with a target value of 1e6 ions and 1 microscan. Low resolution CID MS/MS spectra were acquired on the top 20 ions, with a target value of 1000 ions in normal CID scan mode. MS/MS acquisition in the linear ion trap was partially carried out in parallel to the survey scan in the Orbitrap analyzer by using the preview mode (first 192 ms of the
MS transient). The maximum injection time for MS/MS was 100 ms. Dynamic exclusion was 120 s and early expiration was enabled. The isolation window for MS/MS fragmentation was set to 2 Th.

MS/MS data was searched against a custom database containing the users provided sequence using the searching algorithm node Sequest HT in Proteome Discoverer 1.4 (Thermo). Variable modifications of oxidation on methionine and Cys, di-oxidation and tri-oxidation of Cys, the addition of EbSe to cysteine, the substitution of S with Se, and the conversion of Cys to dehydro-Ala were all considered.

Native Gel Analysis and Negative Stain Transmission Electron Microscopy.

For native gels, protein samples were diluted in native gel loading buffer (10% (v/v) glycerol, 2.7 mM Tris-HCL, pH 6.8, 0.1% bromphenol blue) and resolved on a 4% polyacrylamide gel (4% bis acrylamide, 375 mM Tris-Cl, pH 8.8, 0.05% (v/v) TEMED, and 0.05% ammonium persulfate) using a Tris/glycine buffer (2.7 mM Tris-HCl and 192 mM glycine, pH 6.9) (Chambers et al., 2008b). Proteins were detected by silver staining.

For electron microscopy, samples were prepared by placing a drop of protein in solution on a formvar coated copper TEM grid held in anti-capillary forceps. The protein was allowed to settle to the grid surface for 60 seconds and excess liquid wicked away with a filter paper. The grids were then stained with a drop of aqueous 2% uranyl acetate for 30 seconds after which the excess stain was wicked away. Images of the negatively stained protein particles were collected using a JEOL JEM 1200 EX transmission
electron microscopy equipped with a Gatan Orius 830 camera. The images were prepared for publication using Adobe Photoshop.

3-(N-Maleimidopropionyl)-biocytin (MPB) Assays.

TbHK1 and variants (equal amounts as determined by coomassie staining of an SDS-PAGE gel) were incubated (30 min, RT) with MPB (50 µM) in buffer (20 mM Na$_2$HPO$_4$, 5 mM glucose, 0.4 M (NH$_4$)SO$_4$), resolved by 4% native gel electrophoresis, and monomers analyzed following transfer to nitrocellulose by western blotting using an anti-biotin antibody (1:10,000; Cell Signaling Technology Danvers, MA). Relative darkness was quantitated using ImageJ software. To score the consequences of inhibitor on MPB labeling, TbHK1 was incubated with increasing concentrations of inhibitor (15 min, RT) followed by addition of MPB (50 mM), and the mixture incubated for an additional 30 min at RT.

RESULTS

In two independent screens for TbHK1 inhibitors, we identified EbSe (2-phenyl-1,2-benzisoselenazol-3(2H)-one) as a potent TbHK1 inhibitor. In the first, a LOPAC screen, EbSe inhibited TbHK1 88.1 ± 0.6% at 10 mM. Also, EbSe and five other structurally related isobenzothiazolinones were identified as TbHK1 inhibitors during a high throughput screen (HTS) of 220,233 small molecules (Sharlow et al., 2010b). While EbSe was the most potent inhibitor identified (IC$_{50}$ = 0.05 ± 0.03 µM), the finding that it can form covalent seleno-sulfide conjugates with Cys residues or act as a Cys oxidant
suggested that it was not an ideal lead for further development. Supporting this supposition, EbSe had been identified as an inhibitor of a number of different enzymes in multiple validated HTS campaigns, possibly because of its Cys-reactive nature (Sharlow et al., 2010b).

Other Cys reactive TbHK1 inhibitors have also been identified. For example, the thiol-reactive reagent 4-chloromercuribenzoic acid was identified in the LOPAC screen as a potent inhibitor of TbHK1 (84.7 ± 0.4% inhibition at 10 mM) (Sharlow et al., 2010b). Additionally, the irreversible disulfide-reactive reagent tris (2-carboxyethyl)phosphine (TCEP) inhibited TbHK1 (IC$_{50}$ = 6.6 ± 0.4 mM), supporting the possibility that a Cys residue (or multiple Cys residues) is important for catalysis.

*Neither dilution, DTT, nor excess Mg$^{2+}$ can reverse EbSe inhibition of TbHK1.*

Six isobenzothiazolinones were identified in the HTS as potent inhibitors of TbHK1, including EbS (SID 17387000), which is a structural analog of EbSe that harbors a S atom in place of the Se found in EbSe (Figure 2.1A). Inhibition by this compound or by EbSe was not reversible by 200-fold dilution of the inhibitor after pre-incubation with enzyme, suggesting a covalent modification of TbHK1 (Fig. 2.1B). Because EbSe inhibition of human IDO had been reported to be reversible by inclusion of the reducing reagent dithiothreitol (DTT) (Terentis et al., 2010), we assessed the consequences of this reagent on both EbS and EbSe inhibition (Fig. 2.1C). Alone, DTT (100 mM) had little impact on enzyme activity. Addition of DTT after either EbS or EbSe incubation only modestly rescued enzyme activity. While pre-incubation of TbHK1 with DTT prevented
EbSe inhibition (data not shown), it is likely this occurred as a result of DTT interacting directly with EbSe to block association of the small molecule with the enzyme. A report describing the formation of DTT/EbSe adducts supports this possibility (Borges et al., 2005). Last, EbSe could be inhibitory to TbHK1 as a result of causing inappropriate coordination of the essential Mg$^{2+}$ cofactor. Addition of excess Mg$^{2+}$ did not relieve EbSe inhibition, even at ~15-fold higher concentrations than are used in the standard reaction (data not shown).

**MPB as a probe for exposed Cys interactions with inhibitors.**

To further explore the role of surface-exposed Cys residues in inhibitor binding, the Cys modifying reagent 3-(N-maleimidopropionyl)-biocytin (MPB) was used to modify surface-exposed Cys residues (Bayer et al., 1985). At concentrations up to 50 mM, MPB did not impact enzyme activity; however, pre-incubation with MPB did not appreciably alter EbSe inhibition, suggesting that the residues accessible to MPB were not involved in the enzyme inhibition or that EbSe could compete with the MPB modification or interact with free MPB.

**Mass Spectrometry to identify covalent modifications of TbHK1.**

Because the MPB experiments did not clarify the role of Cys residues in EbSe inhibition, we pursued scoring the direct consequences of EbSe treatment by ESI-MS/MS. This was performed keeping in mind that Cys residues have been described in
the literature as the target of EbSe modification, either by formation of a selano-Cys bond between the enzyme and inhibitor or with the EbSe serving as a thiol oxidant.

Modifications of TbHK1 were scored using digested peptides from EbSe treated and untreated enzyme that were subjected to ESI-MS/MS (Supplemental Data set 1). Using this approach, species were identified with dioxidized (sulfone) and trioxidized (sulfonic acid) modified C103 in both untreated and treated samples. In contrast, dioxidized and trioxidized C327 and C369 modifications were identified only in the EbSe treated samples (Figure 2.2). Peptides bearing oxidized C327 and C369 were not observed in the untreated samples, suggesting that they were either not present in the untreated sample or that the modified peptides were present but simply not detected in the untreated samples. Lastly, EbSe-conjugated through a seleno-sulfide bond to TbHK1 was not detected in the treated samples.

![Figure 2.2 TbHK1 Cysteine Residues](image)

**Figure 2.2 TbHK1 Cysteine Residues**

Two Cys residues on the large lobe of TbHK1 are modified in EbSe treated samples. The predicted distribution of TbHK1 Cys residues, based on modeling to the yeast structure, with the nine Cys residues and the catalytic base (D214) included to indicate the active
site (Chambers et al., 2008c). The * indicates the Cys found by ESI-MS/MS to be oxidized in both untreated and EbSe-treated TbHK1, while the ** indicates the two oxidized Cys residues observed only in peptides from treated samples.

*Mutagenesis of individual Cys residues in TbHK1.*

The observation that EbSe modified both C327 and C369 suggested that one or both were important for catalysis and/or EbSe inhibition. Further, it hinted at the possibility of generating EbSe-insensitive enzyme by alteration of the putative target residues. To explore the role of the nine TbHK1 Cys residues (Figure 2.2) in catalysis and inhibition, variants harboring Cys to Ala changes of the residues were generated.

The consequences of these modifications on enzyme activity were varied. Three of the variants (C212A, C327A, and C386A) lacked detectable activity (Table 2.1), while three other variants (C369A, C401A, and C402A) had subtly reduced specific activities. The remaining two variants (C242A and C445A) had specific activities similar to the unaltered enzyme. EbSe and TCEP were then tested against the active variants. Both compounds were potent inhibitors of all of the active variants (including C369A), with no detectable difference in sensitivity compared to unmodified TbHK1 (Table 2.1).
<table>
<thead>
<tr>
<th>Variant</th>
<th>Specific Activity (mmol·min⁻¹·µg⁻¹)</th>
<th>EbSe IC₅₀ (µM)</th>
<th>TCEP IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.60 ± 0.03</td>
<td>0.35 ± 0.07</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>C103A</td>
<td>NA b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C103S</td>
<td>0.17 ± 0.00</td>
<td>&lt;WT c</td>
<td></td>
</tr>
<tr>
<td>C212A</td>
<td>ND d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C212S</td>
<td>0.23 ± 0.01</td>
<td>&lt;WT</td>
<td></td>
</tr>
<tr>
<td>C242A</td>
<td>1.1 ± 0.12</td>
<td>0.55 ± 0.02</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>C327A</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C327S</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C369A</td>
<td>0.24 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>C386A</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C386S</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C401A</td>
<td>0.17 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>C402A</td>
<td>0.23 ± 0.01</td>
<td>0.43 ± 0.11</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>C445A</td>
<td>0.79 ± 0.01</td>
<td>0.52 ± 0.05</td>
<td>5.4 ± 0.1</td>
</tr>
</tbody>
</table>

a Experiments were performed in triplicate and standard deviation is indicated.

b NA, protein not expressed.

c Inhibition was 100% using EbSe at a concentration that inhibited WT protein 50%.

d Not determined, as activity was not detected.

In an effort to recover activity from the inactive Cys to Ala variants (C212A, C327A, and C386A), the three Cys residues were altered by mutation to a more conserved residue, Ser. The C212S variant had reduced yet detectable activity, while the other two yielded activity barely above the threshold of the variability in the assay.

Notably, C212S remained sensitive to EbSe inhibition (Table 2.1).
Effects of Cys variants on oligomerization.

To explore the impact of Cys residue alteration on the protein tertiary structure, native gels were used to resolve hexamer and monomer abundance, which were then characterized by silver staining and western blotting, respectively (Figure 2.3A) (Chambers et al., 2008b). Hexamer abundance was not altered in most of the variants with C386A and C445A having ~2-fold increases in oligomer abundance (Figure 2.3A). Two TbHK1 variants (C327A and C369A) had slightly reduced levels of monomer (as determined by probing with the anti-six His antibody), while C445A had a ~10-fold reduction in monomer abundance, suggesting this residue influences oligomerization. The proteins were also incubated with MPB and biotinylation of monomeric TbHK1 scored in order to further assess the consequences of Cys mutation on structure. Protein was resolved by native gel electrophoresis (4%), with monomer transferred to nitrocellulose followed by western blotting with antisera to the six His tag used for affinity purification (to score relative protein concentration) or an anti-biotin antibody to determine the relative level of biotinylation.
Figure 2.3 Relative hexamer, monomer, and free Cys abundance for TbHK1 and variants. (A.) Relative hexamer and monomer abundance of TbHK1 and Cys variants.
Relative hexamer abundance (hatched bars) was determined by densitometry of appropriately sized bands visualized by silver staining of a 4% native gel followed by comparison to TbHK1 (lane 1). Relative monomer abundance (black bars) was determined by western blot analysis of the appropriate region of a 4% native gel using an anti-RGSH$_6$ antibody and comparison to TbHK1. (B.) MPB modification of the monomer was determined by incubating variants with MPB (50 µM) for 30 min and monitoring the appropriate region of the gel by western blotting using an anti-biotin antibody. Experiments were performed in triplicate and standard deviation is indicated, with representative data included above each bar graph.

Using MPB to characterize surface exposed Cys residues in the TbHK1 variants, seven of the eight variants were labeled similarly to WT protein (Figure 2.3B). However, C445A had an increased number of free Cys residues available for biotinylation by MPB per monomer as compared to TbHK1. This amino acid alteration may lead to an unstable monomer that, as a result of the modification, has many of its Cys residues exposed and available for MPB modification. Additionally, neither EbSe nor TCEP impact oligomerization of TbHK1 (data not shown).

**Oligomers of C445A are indistinguishable from TbHK1 by electron microscopy.**

To date, the oligomers of TbHK1 have been refractory to structural studies due to an inability to generate useful crystals. However, EM analysis of recombinant protein suggests that enzyme oligomers are globular and roughly symmetrical, with an average
diameter of 18.25 ± 0.19 nm (Figure 2.4). The C445A variant, which has slightly greater abundance of hexamer in the native gel analysis (Figure 2.3A), had a similar distribution of sizes (average diameter = 16.5 ± 2.8 nm), suggesting the mutation had relatively little impact on gross architecture. Notably, the overall structure of the hexamers suggests a ring-shaped tertiary structure.

![Figure 2.4](image)

**Figure 2.4  Negative stain TEM analysis of globular TbHK1 hexamers.**

Samples of WT TbHK1 (upper) and C445A variant TbHK1 (lower) were analyzed using a JEOL JEM 1200 EX transmission electron microscope after negative staining. Inset contains enlargement of four representative protein particles. Scale bar = 100 nm.
DISCUSSION

Currently there are a limited number of drugs available for the treatment of African trypanosomiasis. These agents have drawbacks – several are limited to treatment of infections before the parasite has crossed the blood-brain barrier, while others are active against one but not both human-infective subspecies of *T. brucei*. Target-based drug discovery offers the advantage of assessing drug/target interaction and allowing potential anticipation of possible resistance mechanisms.

TbHK1, which has been genetically and chemically validated as a suitable target for therapeutic development, has been the subject of several screening campaigns for small molecule inhibitors (Chambers et al., 2008a; Dodson et al., 2010; Sharlow et al., 2010b). The small molecule EbSe, the sulfur derivative EbS, and several structural analogs of EbS were identified in these campaigns, suggesting that the compounds could prove useful for probing the enzymology of the protein. Further analysis revealed that both EbSe and EbS were mixed inhibitors of TbHK1 (with respect to ATP) and both were toxic to parasites (Sharlow et al., 2010c). While the potency against recombinant enzyme and parasites was different (with IC\textsubscript{50} values of 0.05 ± 0.03 and 2.0 ± 0.5 for EbSe and EbS compared to EC\textsubscript{50} values of 2.9 ± 0.28 and 0.030 ± 0.067 for the two compounds, respectively), both were able to reduce parasite G6-P levels after acute exposure, suggesting that at least one possible *in vivo* targets was TbHK1 (Sharlow et al., 2010a; Sharlow et al., 2010b). Recently, trypanothione reductase has been identified as a second potential target (Lu et al., 2013). With both targets being essential to the parasite,
it is difficult to resolve which (if either) are responsible for the toxic action of the molecules.

EbSe had previously been characterized as a mimic of glutathione peroxidase, utilizing reduced glutathione to reduce hydrogen peroxide and lipid hydroperoxides. Additionally, EbSe has also been identified as a potent electrophile, facilitating inter- and intramolecular disulfide linkages (Sakurai et al., 2006). For example, EbSe was found to have reacted with up to 8 Cys residues of human IDO, causing a change in conformation that led to IDO inactivation (Terentis et al., 2010). Here, we have pursued a further dissection of the mode of action of TbHK1 inhibition by this group of compounds with a particular focus on covalent Cys modification. The results of these efforts suggest that the inhibitory activity of EbSe against TbHK1 is likely the result of thiol oxidation of C327.

Initially, we found that TbHK1 inhibition by EbSe was not relieved by enzyme/inhibitor dilution, which supported the idea that the compound was indeed irreversibly modifying the enzyme. This was in stark contrast to inhibition of a HK from Plasmodium falciparum (PfHK), which we have recently characterized (Harris et al., 2013). While EbSe and EbS were ~10-fold more potent inhibitors of PfHK, inhibition by both was completely relieved by dilution (Harris et al., 2013).

While the dilution experiments suggested that EbSe was irreversibly modifying TbHK1, the finding that EbSe inhibition was not reversible on treatment with DTT supported the notion that the inhibition was not due to the canonical promiscuous seleno-sulfide formation that has been observed previously with EbSe. This supposition was supported by the mass spectrometry analysis, which revealed oxidized Cys species
including sulfone and sulfonic acid functionality at C103, C327, and C369, with the latter
two sites of oxidations detected only in the EbSe treated samples. Mutational analysis
revealed that C327 is essential while C369 could be altered without notable consequence
on either catalysis or EbSe sensitivity, suggesting that inhibition of TbHK1 is a
consequence of oxidation of the former Cys. Lacking a solved structure for either
monomeric or heteromeric TbHK1, it is difficult to resolve the reasons for the observed
inhibition as both C327 and C369 are predicted (based on modeling) to be on the large
lobe of the enzyme at some distance from the active site.

EbSe has been described as a thiol oxidant in other studies (Schewe, 1995;
Sakurai et al., 2006) suggesting a model for TbHK1 thiol oxidation (Figure 2.5). In the
model, the Se atom of EbSe is attacked by the Cys SH followed by a proton transfer. The
resultant S-Se conjugate is then hydrolyzed, with a subsequent proton transfer followed
by deprotonation of the OH to yield the initial Cys sulfoxide. This functionality was not
observed by MS/MS analysis, and is expected to undergo rapid in situ oxidation to the
corresponding sulfone and sulfonic acids that were observed by MS. In principle, the
initial conjugation with EbSe would be reversible prior to hydrolysis, accounting for the
finding that promiscuous Cys oxidation was not observed. This mechanism would also
potentially explain the reduced potency of EbS. EbS is structurally nearly identical to
EbSe but the Se-bearing molecule would likely be a more potent oxidizing agent.
Despite its potentially promiscuous nature, EbSe is non-toxic to humans and is currently deployed in phase III clinical trials for treatment of ischemic stroke (Stroke Trials Registry Home Page, as of 08/16/2013) and was found to improve the outcome of patients suffering stroke (Yamaguchi et al., 1998). These studies and others that indicate that EbSe has antibacterial properties as a consequence of inhibition of bacterial thioredoxin reductases (Lu et al., 2012) suggest that the benzisoselenazol derivatives could prove useful for therapeutic development.

**Acknowledgements**

* This work was supported in part by the US National Institutes of Health 1R15AI075326 to JCM. We thank Jennifer Bethard (Department of Cell and Molecular Pharmacology,
Mass Spectrometry Facility, Medical University of South Carolina) for the mass spectrometry analysis.

The abbreviations used are: BSF, bloodstream form; EbS, 2-phenyl-1,2-benzisothiazol-3(2H)-one; EbSe, ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one); G6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Gly3P, glycerol-3-phosphate; GK, glycerol kinase; HK, hexokinase; PF, procyclic form; rTbHK1, recombinant Trypanosoma brucei hexokinase 1; TbHK, T. brucei hexokinase.

References


Morris, J.C., 2010a. Identification of Inhibitors of *Trypanosoma brucei* Hexokinases, Probe Reports from the NIH Molecular Libraries Program, Bethesda (MD).


CHAPTER THREE

STRUCTURE-ACTIVITY STUDIES OF THE BENZAMIDOBENZOIC ACID SCAFFOLD FOR TRYPANOSOMA BRUCEI HEXOKINASE INHIBITION

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Abstract

Hexokinase (HK) is the first enzyme in the glycolytic pathway and is essential to the survival of Trypanosoma brucei in the human host, where it relies on glycolysis exclusively for ATP production. A high throughput screen for TbHK1 inhibitors identified several potential scaffolds for further consideration. Several inhibitors developed from a benzamidobenzoic acid scaffold have demonstrated potent inhibition of rTbHK1 in vitro, leading to the development of a probe inhibitor (Probe, SID 99437306, IC$_{50}$ = 976 nM). However, this compound lacked activity against parasites, perhaps due to poor cell permeability. Modifications to Probe to improve permeability have been explored, yielding compounds with improved anti-parasitic activity against bloodstream form parasites (LD$_{50}$ = 1.88 µM). Mechanistic studies show that these inhibitors are not competitive inhibitors, and presumably act through an allosteric binding site. Further, these inhibitors have little impact on a human cell line (IMR90) and on similar host enzymes, with >40 fold selectivity. Continued development of this scaffold could lead to a novel trypanocidal drug.
Introduction

Trypanosoma brucei is the causative agent of African sleeping sickness in man and the wasting disease nagana in livestock. Together, these two diseases cause thousands of deaths (Fevre et al. 2008) and cost billions of United States dollars in agricultural losses (Torr et al. 2005) to the under-developed region of sub-Saharan Africa. T. brucei is an extra-cellular parasite of the mammalian bloodstream and cerebrospinal fluid transmitted by tsetse fly vectors. The hemolymphatic stage presents fever and flu-like symptoms, while the encephalitic stage can cause a variety of neurological malaises, including an inversion of circadian rhythms. Current treatment options are inadequate, as they require multiple transfusions, have toxic side effects, and many are only effective against one of the two subspecies and only a select few can treat the encephalitic stage of the disease. New therapeutics must be developed in order to further control this disease.

When inhabiting the mammalian host, T. brucei represses mitochondrial function and relies completely on glycolysis for ATP production, making the enzymes of the glycolytic pathway potential drug targets. Genetic studies have validated the first enzyme in the pathway, hexokinase (HK), as a drug target (Chambers et al. 2008). Hexokinase catalyzes the transfer of the γ-phosphate of ATP to the 6 carbon of glucose, yielding glucose-6-phosphate (G6P) and ADP. T. brucei has two HKs, TbHK1 and TbHK2, which share 98% amino acid identity and both of which are essential to the bloodstream form (BSF) of the parasite. The enzymes share low identity with human hexokinases (30–33%), suggesting they could be selectively targeted. Curiously, only TbHK1 is active
when produced from recombinant *E. coli*, making TbHK1 the primary subject of drug screens (Morris et al. 2006).

Recently, a high throughput screening (HTS) campaign has identified novel TbHK1 inhibitors (Sharlow et al. 2010a). Several isobenzothiazolinones were identified in the screen, but this scaffold was found to act through a promiscuous cysteine interaction (Joice et al. 2013), and a structure-activity relationship (SAR) could not be established beyond the need for the cysteine reactive sulfur (Sharlow et al. 2010b). A singleton from the HTS, benzamidobenzoic acid, was more amenable to optimization, and after a generation of 104 analogues was produced, SID99437306 was identified with a 10-fold improvement in TbHK1 inhibition and named “Probe” (Figure 3.1 (Sharlow et al. 2010b)).

The first generation of analogues showed that modifications to the amide linker were not tolerated, while the addition of bromine to the 5 carbon of the benzoic acid and substituting a branched lipophilic group for the 4-methoxy group on the parental 4-methoxyphenyl moiety was found to enhance TbHK1 inhibition (Sharlow et al. 2010b). Probe represents the synergy of the latter two modifications (Figure 3.1). Probe was shown to be non-toxic to human cell lines (including A549, IMR-90, HeLa, and MDA-MB-231 (Sharlow et al. 2010b)), have 40-fold selectivity for TbHK1 over the similar human enzyme glucokinase (hGK), and had minimal promiscuity in a screen against a panel of human kinases (Sharlow et al. 2010b). Additionally, Probe was a mixed inhibitor with respect to both substrates, suggesting it may bind an allosteric site unique to the parasite enzyme. Unfortunately, both the original benzamidobenzoic acid HTS hit and
Probe lacked significant activity against BSF parasites (LD$_{50} > 25$ µM), necessitating further development of the scaffold.

Figure 3.1 First Generation Development of the Benzamidabenzoic Acid Scaffold

(A) Initial HTS hit, SID24797131. (B) Probe identified from first generation analogues, SID99437306. (C) Scaffold for second-generation analogues. Numbers indicate modification sites.
Materials and Methods

Reagents

Glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide (NAD$^+$), adenosine triphosphate (ATP), and glucose were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Inhibitors were synthesized at the University of Kansas Specialized Chemistry Center (KUSCC). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from EMD Millipore (Darmstadt, Germany).

Recombinant Protein Production

Recombinant TbHK1 and hGK was purified as described from a culture of E. coli M15(pREP) harboring pQE30 (Qiagen, Valencia, CA) with TbHK1 or hGK cloned in frame with a 6-His tagging sequence (Morris et al., 2006). Briefly, a 10 mL bacterial culture was used to inoculate a 1 L culture which was grown to an OD600 of ~1 and then induced for 24 hr at room temperature with 500 mM IPTG and purified as described (Morris et al., 2006).

Hexokinase Assays

HK assays were performed in triplicate as described using a glucose-6-phosphate dehydrogenase (G6PDH) coupled reaction to measure enzyme activity in a 96 well plate (Misset and Opperdoes, 1984; Morris et al., 2006). In short, the coupled assay uses G6PDH to convert G6P generated by HK to 6-phosphogluconate with coincident
reduction of NADP to NADPH, which is monitored spectrophotometrically at 340 nm. For inhibition assays, inhibitors dissolved in DMSO are added to the reaction mix and incubated for 10 minutes prior to the addition of glucose to start the reaction. Inhibitors were counter-screened for inhibition of G6PDH. Data analysis was performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA).

**Live/Dead Cell Assays**

To determine the impact of TbHK1 inhibitors on cell growth, $5 \times 10^4$ BSF parasites (cell line 90–13, a 427 strain) were added into 96-well clear-bottomed polystyrene plates in 200 µl HMI-9 (Hirumi and Hirumi 1989) supplemented with 10% fetal bovine serum and 10% Serum Plus (Sigma-Aldrich, St. Louis, MO) and grown in the presence of inhibitor (1 µl) or equivalently diluted DMSO for 3 days in 5% CO$_2$ at 37°C. CellTiter Blue (Promega, Madison WI) was added (20 µl) and the plates incubated an additional 3 hours under standard culture conditions. Fluorescence emission at 585 nm was then measured after excitation at 546 nm. DMSO solvent was maintained at or below 1%, with 1% causing a 16% reduction in cell number at the end of the three-day assay.

**Results**

*Second generation analogues are active against parasites.*

The second generation of inhibitors primarily explored different lipophilic groups on the phenyl group, a substitution that was well tolerated in first generation analogues (Figure 3.2; Table 3.1). It was believed that the lack of *in vivo* activity for Probe was due
to low cell permeability, and that the addition of a hydrophobic group(s) may improve permeability. A benzene ring was also added to the 5 carbon of the benzoic acid, a modification omitted in the previous generation. Studies with the previous generation showed that substituting the carboxylic acid for a tetrazole was acceptable, but not an improvement (Sharlow et al. 2010b), but this modification was made to the Probe scaffold to determine if there was any synergy or permeability improvement. Two of the second-generation compounds had trypanocidal activity, SID144241590 and SID144241595 (Table 3.1). SID144241590 had superior activity against TbHK1 *in vitro* and BSF parasites *in vivo* compared to SID144241595 and was named “Lead.”

Table 3.1 Second Generation Inhibitors

<table>
<thead>
<tr>
<th>Structure</th>
<th>PubChem SID</th>
<th>TbHK1 IC₅₀ (µM)</th>
<th>BSF LD₅₀ (µM)</th>
<th>% BSF Growth Inhibition (10 µM)</th>
<th>% hGK Inhibition (10 µM)</th>
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<td>144241588</td>
<td>4.8 ± 0.38</td>
<td>n.d.²</td>
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<td>Structure</td>
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<td>BSF LD₅₀ (µM)</td>
<td>% BSF Growth Inhibition (10 µM)</td>
<td>% hGK Inhibition (10 µM)</td>
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<td>82.1 ± 2.3</td>
<td>55.5 ± 13.4</td>
</tr>
</tbody>
</table>

¹ Probe included for comparison.

² Not determined.
Figure 3.2 Lead with Modification Sites

Lead was the most potent second-generation inhibitor and the scaffold for the third generation analogues. Numbers indicate modification sites for third generation inhibitors.

*Third generation compounds show limited improvement over Lead*

Third generation compounds mainly explored modifications to the benzene ring added in Lead to see if trypanocidal activity could be improved (Figure 3.2). In general, addition of electronegative groups was well-tolerated and produced some small improvements in LD$_{50}$ values (Table 3.2). The addition of nonpolar groups reduced or ablated activity, and the replacement of the benzene ring with other lipophilic groups was also not tolerated.
Table 3.2 Third Generation Inhibitors

<table>
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<tr>
<th>Structure</th>
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<th>TbHK1 IC(_{50}) (µM)</th>
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<th>% BSF Growth Inhibition (10 µM)</th>
<th>% hGK Inhibition (10 µM)</th>
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<td>% BSF Growth Inhibition (10 µM)</td>
<td>% hGK Inhibition (10 µM)</td>
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<td>% BSF Growth Inhibition (10 µM)</td>
<td>% hGK Inhibition (10 µM)</td>
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<td>TbHK1 IC$_{50}$ (µM)</td>
<td>BSF LD$_{50}$ (µM)</td>
<td>% BSF Growth Inhibition (10 µM)</td>
<td>% hGK Inhibition (10 µM)</td>
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1Not determined

2Value may be artificially high due to stability issues.
Discussion

The second generation of analogues led to the identification of SID144241590, dubbed Lead, which was more potent than Probe both in vitro (IC$_{50}$ = 0.28 µM) and in vivo (LD$_{50}$ = 1.88 µM). Lead has a benzene ring attached at the 4 carbon of the benzoic acid (Figure 3.2). This may add a lipophilic element to the inhibitor that aides in membrane permeability, explaining the compound’s trypanocidal activity. Importantly, like Probe, Lead is non-toxic to IMR90 human cell lines and had limited promiscuity against a human kinase panel from Luceome Biotechnologies (Tucson, AZ). While Lead represented a significant improvement over Probe, the aim was a compound with an LD$_{50}$ under 1 µM, prompting the synthesis of third generation analogues, which explored the effect of modifications to the benzene ring.

The third generation analogues lacked the leap in effectiveness seen with Lead. A handful of inhibitors showed slight LD$_{50}$ improvement (SID162211070, SID162211073, SID162211075, SID162211078, SID162211080, SID162211084, and SID163631669), but none of these inhibitors LD$_{50}$ values fell below 1 µM. Interestingly, all of these compounds modify the ring with electronegative groups (Table 3.2). The added bulk of the benzene ring may place it next to a basic residue in the binding pocket. Replacing the benzene with other rings or nonpolar groups completely ablated trypanocidal activity, despite some nitrogenous ring substitutions having very low IC$_{50}$ values versus TbHK1 (Table 3.2). It seems that the benzene and not merely a hydrophobic residue is needed for in vivo activity. The ring may facilitate binding with a transporter or other cofactor needed for entry to the glycosome, but an off target interaction cannot be ruled out.
Further work is needed to optimize the scaffold. The small improvements found in the third generation may be synergistic. Work is currently under way to solve the crystal structure of TbHK1. If successful, in silico docking studies with Lead and the structure could provide the clues to finding the binding site of these inhibitors, which would accelerate scaffold development. Overall, the Lead scaffold represents an exciting drug candidate, and necessitates further development.

References


CHAPTER FOUR

INTERROGATING A HEXOKINASE-SELECTED SMALL-MOLECULE LIBRARY FOR INHIBITORS OF PLASMODIUM FALCIPARUM HEXOKINASE

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ABSTRACT

Parasites in the genus *Plasmodium* cause disease throughout the tropic and sub-tropical regions of the world. *P. falciparum*, one of the deadliest species of the parasite, relies on glycolysis for the generation of ATP while it inhabits the mammalian red blood cell. The first step in glycolysis is catalyzed by hexokinase (HK). While the 55.3 kDa *P. falciparum* HK (PfHK) shares several biochemical characters with mammalian HKs, including being inhibited by its products, it has limited amino acid identity (~26%) to the human HKs, suggesting that enzyme-specific therapeutics could be generated. To that end, interrogation of a selected small molecular library of HK inhibitors has identified a class of PfHK inhibitors, isobenzothiazolinones, some of which have IC$_{50}$ values < 1 mM. Inhibition was reversible by dilution but not by treatment with a reducing agent, suggesting that the basis for enzyme inactivation was not covalent association with the inhibitor. Last, six of these compounds and the related molecule ebselen inhibited *P. falciparum* growth in vitro (0.6 ≤ EC$_{50}$ < 6.8 µM). These findings suggest that the chemotypes identified here could represent leads for future therapeutic development against *P. falciparum*.
INTRODUCTION

Malaria is a global health concern, infecting over 200 million people a year. While four members of the genus Plasmodium commonly cause human malaria, *P. falciparum* infection is responsible for the most mortality annually. In addition to disease, the burden of malaria has global economic consequences, with an impact in developing nations that results from reduced worker productivity and markedly increased disability adjusted life years (DALYs), a measure of disease burden as a consequence of mortality and morbidity (World Health Organization, life cycle.who.int).

The parasite has a complex life cycle, with the intra-erythrocytic stage being primarily responsible for pathology. This stage of *Plasmodium* lacks a complete TCA cycle and knockout and inhibitor studies of mitochondrial proteins have suggested that the mitochondrion is not a significant contributor to cellular ATP levels (Vaidya and Mather 2009; Olszewski et al. 2010). Notably, glucose consumption was found to be increased up to 100-fold in infected erythrocytes (Roth et al. 1982) and lactate levels were ~20-100 times higher than that from uninfected cells (Pfaller et al. 1982; Vander et al. 1990). These observations suggested that glycolysis was playing a key metabolic role for the parasite during the erythrocytic infection. Supporting this supposition, knockout studies revealed that the hexose transporter responsible for importing glucose was essential to the parasite, and inhibition of glycolysis with glucose analogs rapidly depleted parasite ATP (van Shalkwyk et al. 2008; Slavic et al. 2010).

The first committed step in glycolysis, catalyzed by hexokinase (HK), is the transfer of the γ-phosphoryl group from ATP to glucose. This reaction yields glucose-6-
phosphate (G6P), a metabolite with multiple potential fates. First, it can be consumed in glycolysis. Alternatively, if funneled into the pentose phosphate pathway, the metabolite can serve in the generation of NADPH, which is a key component in the *P. falciparum* antioxidant defense and *de novo* nucleotide triphosphate biosynthesis pathways (Muller 2004).

The importance of this glycolysis to the malaria parasite and the observation that the single *P. falciparum* HK (PfHK) is predicted to share limited (24%) identity with human glucokinases (HsGlk, or HK IV), suggested that this enzyme could serve as a suitable target for therapeutics (Figure 4.1). Here, we describe the characterization of recombinant PfHK. Further, we have interrogated a small molecule library of known HK inhibitors to identify potential lead compounds that inhibit PfHK. Last, we have assessed the anti-parasitic activity of these molecules against *P. falciparum* erythrocytic stage parasites and have found that several are potent anti-parasitic compounds.
Figure 4.1 Alignment of PfHK, TbHK1, and HsGlk

Alignments of PfHK, *Trypanosoma brucei* HK1 (TbHK1, Tb427.10.2010), and human glucokinase (HsGlk, ABS31137.1). Sequences were aligned using CLUSTAL 0 (1.1.0).

The * indicated completely conserved residues, while the colons (:) mark residues that share properties (>0.5 in Gonnet PAM250 matrix). Periods (.) indicated residues that fall at <0.5 on the Gonnet PAM250 matrix, indicating weak similarity. The boxed residue is the conserved catalytic base (Asp241 in PfHK).
MATERIALS AND METHODS

Chemicals and Reagents

Glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide (NAD\(^+\)), adenosine triphosphate (ATP), and glucose were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA), while phosphoenolpyruvate (PEP), 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen, Eb, PubChem SID 856002) and glucosamine were obtained from VWR International (West Chester, PA). Isobenzothiazolinones and benzamides in Table 1 were obtained from the University of Kansas Specialized Chemistry Center (KUSCC).

Recombinant Enzyme Purification and Assay Conditions

The open reading frame PF3D7_0624000 (PlasmoDB) for the *P. falciparum* hexokinase (UniProt Q02155) was synthesized for codon optimization (Genescript, Piscataway, NJ), sequenced, and cloned into pQE30 (Qiagen, Valencia, CA). Recombinant PfHK, a ~55.3 kDa protein, was purified following a protocol developed for heterologous expression and purification of a HK from the African trypanosome. Briefly, a 10 mL bacterial culture of *E. coli* M15(pREP) harboring pQE30PfHK with PfHK cloned in frame of a 6-His sequence (Morris et al. 2006) was used to inoculate a 1 L culture which was grown to an OD\(_{600}\) of ~1 and then induced for 24 hr at room temperature with 500 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified as described (Morris et al. 2006).
HK assays were performed in triplicate as described using a coupled reaction to measure enzyme activity (Morris et al. 2006; Misset and Opperdoes 1984). In short, the coupled assay uses glucose-6-phosphate dehydrogenase (G6PDH) to convert glucose-6-phosphate (G6P) generated by HK to 6-phosphogluconate with coincident reduction of NADP to NADPH, which is monitored spectrophotometrically at 340 nm. Kinetic analyses were performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA).

Hexokinase Inhibitor Library

The collection of small molecules described here as a "selected small molecular library of HK inhibitors" was originally developed, identified, and characterized as part of a HTS campaign targeting hexokinase 1 (TbHK1) from the African trypanosome, Trypanosoma brucei. This screen was completed at the Pittsburgh Molecular Libraries Screening Center (PMLSC) using a 220,233 compound library that was made available as part of the NIH Molecular Libraries Roadmap Initiative. Analog development of lead compound scaffolds, including the isobenzothiazolinones and benzamidobenzoic acid derivatives used here, resulted from an "Extended Characterization Chemical Probe Development Plan" described in a Probe Report from the NIH Molecular Libraries Program (Sharlow et al. 2010b).

P. falciparum viability assay

*Plasmodium falciparum* 3D7 parasites were grown in O⁺ red blood cells at 1% hematocrit cultured in RPMI (plus L-glutamine, without NaHCO₃) supplemented with
0.5% albumax, 0.37 mM hypoxanthine, 27 mM NaHCO$_3$, 11 mM glucose, and 10 µg/ml gentamicin under 5% CO$_2$, 5% O$_2$, and 90% N$_2$, 37°C. All assays were performed in triplicate in a total volume of 100 µL with DMSO-only incubations as a negative control. Parasitemia was determined following 72 hours of incubation by flow cytometry using acridine orange staining (1.5 µg/ml in PBS, 5 min, RT, FACSCanto II flow cytometer (BD Biosystems, Franklin Lakes, NJ).

The dose response curves for compounds that caused > 50% growth inhibition at 10 µM were pursued in a 96 well plate format using an asynchronous parasite culture. The starting parasitemia was 1%. IC$_{50}$ values were determined by scoring parasite growth in three separate wells over a two-fold dilution series starting at 40 µM for each compound. Percent inhibition was calculated by comparison to parasites grown in the DMSO controls from each plate. Chloroquine was screened as a positive control starting at 1 µM. Averages of the triplicates were calculated and dose-response curves fitted and IC$_{50}$ values determined using Prism (GraphPad Software).

RESULTS

While the importance of glycolysis to P. falciparum during mammalian infection has been established (Roth et al. 1982; Pfaller et al. 1982; Vander et al. 1990), the enzymes involved in the process have not been subject to in-depth characterization. This is likely due to the difficulty of classically purifying the parasite components from the complex milieu of the red blood cell contents (which includes many related host
activities). Additionally, the A-T rich nature of the *Plasmodium* genome has historically made production and analysis of recombinant enzymes difficult.

Indeed, our initial forays into expression of PfHK in *E. coli* were unsuccessful despite repeated efforts using a battery of expression vectors and bacterial cell lines. Expression was finally achieved using a synthetic PfHK gene optimized with a codon bias that reflected those codons employed by *E. coli*. The recombinant enzyme, which was purified to ~95% homogeneity (as determined by Coomassie staining of an SDS-PAGE gel), displayed Michaelis-Menten kinetics when substrate levels were increased (Figure 4.2A), and had an apparent $K_m$ values for glucose and ATP (0.62 ± 0.06 and 0.66 ± 0.08 mM, respectively) similar to those from yeast Hxk and *T. brucei* TbHK1 (Table 4.1, (Morris et al. 2006)). The PfHK $k_{cat}$ value was $1.2 \times 10^4$ min$^{-1}$, similar to that reported for a recently characterized *T. brucei* HK1 ($2.9 \times 10^4$ min$^{-1}$) (Morris et al. 2006). PfHK had an affinity for MgCl$_2$ (apparent $K_m = 2.7 \pm 0.4$ mM) similar to that of TbHK1 (apparent $K_m = 0.92 \pm 0.21$ mM). PfHK lacked detectable activity when CaCl$_2$ or MnCl$_2$ was included in place of MgCl$_2$ in the standard assay. The optimal pH for enzyme activity was found to be about pH 7.4, with enzyme activity reduced ~50% by a 1.2 pH unit change in either the basic or acidic direction (Figure 4.2B).
Figure 4.2 Effects of glucose concentration and pH on PfHK activity.

(A) PfHK (200 ng) activity in response to increasing glucose concentration. (B) The pH profile of PfHK (200 ng) activity. Three different buffers with buffering capacity in the range of pH tested were used for the assay: PIPES (pH 6.2, 6.5, 7.0), TEA (pH 7.4), and Tris (pH 8.0, 8.8). Reactions were performed in triplicate and standard deviation is indicated.
Table 4.1 Kinetic characterization of rPFHK

<table>
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<th>$k_{cat} \text{ (min}^{-1})$</th>
<th>Apparent $K_m \text{ (mM)}$</th>
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<tr>
<td></td>
<td></td>
<td>Glucose</td>
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<tr>
<td>rPFHK</td>
<td>$1.2 \times 10^4$</td>
<td>$0.62 \pm 0.06$</td>
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<tr>
<td>rTbHK1</td>
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<td>$0.09 \pm 0.02$</td>
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</tbody>
</table>

$^2$Previously published (Morris et al. 2006)

The enzyme lacked detectable activity when alternative phosphoryl donors, including UTP, GTP, CTP, TTP, ITP, and P Pi, were tested. Notably, ATP at concentrations greater than 3.5 mM was inhibitory to the reaction (data not shown). Products also inhibited the enzyme, with inclusion of ADP (10 mM) in the reaction inhibiting the enzyme ~94% (Figure 4.3). P Pi was also inhibitory, while AMP only modestly (~22%) inhibited PfHK at 10 mM. G6P, another product of PfHK, inhibited the enzyme ~80% at 10 mM.

Figure 4.3 Effects of phosphoryl-bearing compounds on PfHK activity.

PfHK (200 ng) activity was assessed in the presence of 5 mM ATP supplemented with ADP, P Pi, or AMP (all at 5 mM). Assays were performed in triplicate as described in the Materials and Methods and standard deviation is indicated.
Identification of small molecule inhibitors of PfHK.

The completion of a HTS for inhibitors of *T. brucei* hexokinase 1 (TbHK1), yielded several scaffolds that have since been further elaborated to resolve structure activity relationships against that enzyme (Sharlow et al. 2010a). We have taken advantage of the reagents and assays developed therein to interrogate this selected small molecule library of HK inhibitors for PfHK inhibitors. These compounds have been rigorously characterized. For example, they do not inhibit the coupled reporter enzyme, are typically poor inhibitors of human glucokinase, have variable activity against TbHK1, and are minimally toxic to a number of mammalian cell lines (including A549, IMR-90, HeLa, and MDA-MB-231 (Sharlow et al. 2010a).

Of the 16 hits obtained from the HTS effort against TbHK1, two groups were pursued as scaffolds for analog development against TbHK1. Our interest in the first analog group, which was based on five representative isobenzothiazolinones found in the TbHK1 HTS, was particularly piqued because one analog, SID 92126115, had been previously identified in a screen performed by GlaxoSmithKline of more than two million compounds for molecules with anti-*Plasmodium* activity (http://life cycle.ebi.ac.uk/chemblIntd, (Gamo et al. 2010)). SID 92126115, which inhibited parasite growth 97% at 2 mM, was a potent PfHK inhibitor (IC$_{50}$ = 0.16 ± 0.04 mM, Table 4.2). Other isobenzothiazolinones lacking the halogen substituents (SID 85747718 and SID 17387000) had slightly increased IC$_{50}$ values, though substitution of the sulfur atom with a selenium atom (to yield SID 856002, ebselen, Eb), increased potency of inhibition, a trend similar to that seen when Eb inhibition of TbHK1 was compared to that seen with
SID 17387000. Interestingly, Eb and SID 17387000 inhibition were reversible by dilution (data not shown, and Figure 4.4), which is in contrast to the irreversible inhibition observed with TbHK1 (Harris et al., unpublished observation). The reducing reagent DTT was able to prevent, but not reverse, Eb inhibition of PfHK, likely as a consequence of the preventative DTT binding directly to Eb to block its interaction with the enzyme (Borges et al. 2005). Last, the presence of the sulfur or selenium atom was important for inhibition of the enzyme, as substitution with either a carbon or oxygen atom at that position (SID 87225568 and 85752767, respectively) ablated inhibition.

Table 4.2 PfHK Sensitivity to TbHK1 inhibitors

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<tr>
<th>PubChem SID</th>
<th>Structure</th>
<th>PfHK IC₅₀ (µM)</th>
<th>TbHK1 IC₅₀ (µM)¹</th>
<th>P. falciparum EC₅₀ (µM)</th>
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<td>0.16 ± 0.04</td>
<td>3.8 ± 0.40</td>
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<sup>1</sup>Previously published in (Sharlow et al. 2010a and B)

<sup>2</sup>Identified in a GSK screen as inhibiting 3D7 P. falciparum growth 97% at 2 µM

(http://life cycle.ebi.ac.uk/chemblntd, (Gamo et al. 2010)).

<sup>3</sup>ND, not determined.
Figure 4.4 Characterization of PfHK inhibition by SID 17387000.

(A) Dilution after inhibition with SID 17387000 recovers enzyme activity. PfHK (200 ng) was incubated with SID 17387000 (0.5 mM) or buffer (10 minutes, on ice) before addition of other assay components, resulting in a ~100-fold reduction in SID 17387000 concentration. (B) Addition of the reducing agent DTT (100 mM) before, but not after, addition of SID 17387000 (0.5 µM) prevented PfHK inhibition. Standard deviation is indicated.

We also explored inhibition of PfHK with members of a second scaffold identified in the TbHK1 HTS, based around the benzamidobenzoic acid identified in the screen (SID 93575727). Further development of this class of compounds has yielded improved inhibitors of TbHK1, including the probe ML205 (SID 99437306, Table 3.1)
(Sharlow et al. 2010b). However, against PfHK, representatives of this class were either poor inhibitors (ML205 was ~40% inhibitory at 10 mM) or lacked detectable activity against the enzyme.

*PfHK inhibitors are toxic to* *P. falciparum.*

Glycolysis is important to *P. falciparum* during the erythrocytic infection, suggesting that inhibitors of the enzyme could be promising lead compounds for therapeutic development. To explore this possibility, we grew cultured *Plasmodium* parasites in the presence of compound and monitored cell density after 72 hr. Both the isobenzothiazolinones and benzamidobenzoic acid-based compounds were tested in this assay, with EC\textsubscript{50} values determined for those that inhibited cell growth >50% at 10 µM (Figure 4.5, and Table 4.2). Compounds from the isobenzothiazolinones group included two of the most potent anti-malarial compounds, SID 92126115 and SID 85747718. While all of the compounds that inhibited PfHK were toxic to parasites, one molecule (SID 85742034) was toxic to parasites while not inhibiting PfHK. With the exception of SID 87225569, the anti-parasitic compounds had EC\textsubscript{50} values that were 4 to 680 fold higher than the PfHK IC\textsubscript{50} values. This discrepancy could result from the inability of the molecules to reach their target as a consequence of limited RBC and/or parasite cell permeability or trafficking. The identified PfHK inhibitors have minimal impact on human cell lines with EC\textsubscript{50} values >12.5 µM (Sharlow et al. 2010a), suggesting at least 20-fold greater toxicity toward parasites for the most potent compound (Table 4.2).
Figure 4.5 Isobenzothiazolinones inhibited the growth of \emph{P. falciparum} 3D7 parasites in a dose dependent manner.

A two-fold dilution series of compounds SID 92126115 and 17387000 were incubated with parasites and percent inhibition was calculated by comparison to parasites grown in the DMSO controls from each plate. Chloroquine was included as a positive control. Averages of experiments performed in triplicate were calculated and EC_{50} values determined using Prism (GraphPad Software), with standard deviation indicated.

**DISCUSSION**

The intra-erythrocytic malaria parasite requires extracellular glucose for survival, largely due to the significant consumption of the hexose as a consequence of ATP
generation by glycolysis. Inhibitors of this pathway have demonstrated potent antimalarial activity. These include small molecules that interfere with hexose transport (Saliba et al. 2004), as well as glucose analogs like 2-deoxyglucose (van Schalkwyk et al. 2008; Udeinya and Van Dyke 1981).

PfHK, the first enzyme in glycolysis, has heretofore been considered an interesting potential target but has not been exploited due to an inability to produce active recombinant protein for in vitro characterization (Olafsson et al. 1992). Using an E. coli codon optimized gene, we have for the first time generated active PfHK. Characterization of PfHK revealed it to share a number of features with other HKs, including the finding that the reaction products G6P and ADP were inhibitors of the enzyme, suggesting the Plasmodium protein may be regulated via the feedback mechanism described for vertebrate HKs (Easterby and Qadri 1982). This observation was somewhat surprising given that an HK from the related apicomplexan parasite Toxoplasma gondii (which is 44% identical to PfHK) is not sensitive to substrate inhibition (Saito et al. 2002). PfHK is also kinetically similar to other HKs, having glucose, ATP, and MgCl₂ Kₘ values similar to HKs from other systems, including T. brucei and H. sapiens. The PfHK Kₘ value for glucose was similar to that described by Roth from activity associated with infected RBC lysates (620 μM and 431 μM, respectively) (Roth 1987). These values are ~5-fold higher than the Kₘ reported from HK activity associated with uninfected RBC lysates, suggesting that the host enzyme should outcompete the parasite enzyme for glucose under limiting conditions. However, glucose transport in the intra-erythrocytic parasite is
highly efficient, with the concentration of substrate inside the parasite rapidly reaching levels higher than that found in the external medium (Saliba et al. 2004).

The second PfHK substrate, ATP, is required for activity but can also be an inhibitor of the enzyme. PfHK has a $K_m$ value for ATP well below the intra-erythrocytic ATP concentration (~3 mM) and is inhibited at ATP levels approaching those found in the host cytoplasm. While we anticipate that PfHK utilizes a pool of ATP distinct from that found in the host cytoplasm, there could be conditions under which the parasite has generated sufficient ATP to satisfy its needs. To continue to synthesize ATP could be damaging to the host cell, either directly as a consequence of usurping host glucose, or by depletion of host purines required for ATP synthesis, as the parasite cannot synthesize purines and must salvage them from the host. The finding that ATP is a regulator of HK activity is not novel amongst HKs (Dodson et al. 2011), though its role in the malaria parasite remains unclear.

Taking advantage of a pre-existing library of small molecules designed around two scaffolds known to inhibit a trypanosome HK, we searched for inhibitors of PfHK. The two enzymes share limited sequence identity (28%, Figure 3.1), but nevertheless we found that members of one of the two classes of analogues, the isobenzothiazolinones, were more potent against PfHK than TbHK1 (Table 3.2). Additionally, the isobenzothiazolinone analog Eb, was extremely potent against PfHK ($IC_{50} = 0.01 \pm 0.00$). Eb can promiscuously modify cysteine residues, and this non-specific interaction is known to be the mechanism of its inhibition of some enzymes, such as human indoleamine 2,3-dioxygenase (Terentis et al. 2010). However, site directed mutagenesis
of TbHK1 cysteines, including the two conserved in PfHK (residues 273 and 398 in PfHK) did not alter TbHK1 sensitivity to Eb inhibition, indicating that either cysteine residues are not involved in Eb inhibition, or that multiple cysteines must be bound in order for inhibition to occur. Unlike the trypanosome enzyme, PfHK inhibition by Eb and the isobenzothiazolinone SID 17387000 was reversible by dilution, suggesting that the inhibitors interact with the two enzymes differently.

Previous research has demonstrated that Eb is toxic to *P. falciparum*, with EC$_{50}$ values similar to those found in our study (14 µM and 6.8 µM, respectively) (Hunter et al. 1989). Eb may have polypharmacological impact on the parasite, given the likely reactivity of the selenium atom with cellular cysteines. However, it is non-toxic to humans and is currently in phase III clinical trials for the treatment of stroke survivors (Stroke Trials Registry Home Page, as of 02/27/2013), suggesting that the consequences of off-target effects in the human host may be tolerable.

The isobenzothiazolinone SID 92126115 was previously identified in a screen performed by GlaxoSmithKline of more than two million compounds for molecules with anti-*Plasmodium* activity, inhibiting parasite growth 97% at 2 mM (Gamo et al. 2010). SID 92126115 was a potent PfHK inhibitor with an IC$_{50}$ value of 0.16 ± 0.04 mM. Isobenzothiazolinone inhibitors of PfHK were also effective at inhibiting the growth of cultured parasites, with EC$_{50}$s below 10 µM. The EC$_{50}$ values were higher that IC$_{50}$s found for PfHK, likely due to issues with cell permeability and off target interactions. With the exception of SID 85742034, compounds that did not inhibit PfHK did not kill parasites.
In conclusion, several observations support the value of PfHK as a therapeutic target. First, the single copy gene product shares limited (26%) identity with the human equivalent enzyme. Second, the enzyme is responsible for catalyzing the first reaction in two critical pathways, glycolysis and the PPP, and is likely indispensable for parasite viability. Last, inhibitors of the enzyme can inhibit parasite proliferation, as demonstrated here. Together, these findings suggest the value of further consideration of the isobenzothiazolinone scaffold, as well as identification of additional new chemotypes, as a potential lead in therapeutic development.

ACKNOWLEDGMENTS

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The abbreviations used are: Eb, ebselen; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Gly3P, glycerol-3-phosphate; HK, hexokinase; HsGlk, human glucokinases; PfHK, Plasmodium falciparum hexokinase; RBC, red blood cell. TbHK1, Trypanosoma brucei hexokinase 1.
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halo derivatives of D-glucose on glycolysis and on the proliferation of the human
CHAPTER FIVE

KINETIC CHARACTERIZATION AND INHIBITION STUDIES OF *LEISHMAINA* MAJOR HEXOKINASE

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ABSTRACT

The protozoan parasite *Leishmania major* is the causative agent of cutaneous leishmaniasis in North Africa, the Middle East, and Asia. Mannose metabolism is critical to macrophage infective stages of the parasite, with mannogen serving as the primary energy storage molecule while the surface glycocalyx of the parasite contains high mannose content. The glycolytic enzyme hexokinase contributes to mannose metabolism by catalyzing the phosphorylation of glucose to glucose-6-phosphate (which can be converted to mannose-6-phosphate) or by phosphorylating mannose directly, making it a potential drug target. Here we have expressed and purified recombinant *L. major* hexokinase 1 (LmHK1). The enzyme shares kinetic characteristics with previously characterized *Trypanosoma brucei* hexokinase 1 (TbHK1), and two classes of compounds identified as TbHK1 inhibitors were found to inhibit LmHK1. These data suggest that LmHK1 is a bonafide drug target, and that compounds with dual efficiency against *T. brucei* and *Leishmania* could be developed.
INTRODUCTION

*Leishmania major* is a protozoan parasite distributed in Northern Africa, the Middle East and in areas of China and India and is the etiological agent responsible for the majority of cases of human cutaneous leishmaniasis. The life cycle of the parasite is complex, with distinct stages in both the sand fly vector and the mammalian host. Coincident with these stages are differences in the array of expressed surface glycoconjugates and metabolic strategies, with each process being specialized for life in its particular environment.

Surface molecule biosynthesis and hexose metabolism are connected by a requirement for phosphorylated hexoses including glucose-6-phosphate and mannose-6-phosphate (Glc6P and Man6P, respectively). These sugars have a number of potential fates, including being metabolized through glycolysis for ATP production, being directed to the pentose phosphate pathway for DNA and RNA precursors, or being used to generate sugar-nucleotides for glycoconjugate biosynthesis.

Given the diverse set of pathways in which phosphorylated sugars participate, it is not surprising that hexose uptake and phosphorylation are important to the parasite. Glucose uptake, for example, is critical for promastigote stage infection of macrophages (Burchmore et al. 2003). Mannose metabolism is important for pathogenic promastigote and amastigote forms, likely due to its role in mannogen biosynthesis; mannogen makes up more than 80% of the carbohydrate in these life cycle stages, suggesting it is the primary energy reserve for the parasite. (Ralton et al. 2003; Sernee et al. 2006). Through
a salvage pathway, mannose is converted to Man6P for the biosynthesis of the sugar-nucleotide GDP-Man needed for mannogen production (Turnock and Ferguson, 2007).

Generation of phosphorylated hexoses from environmental sugars is largely the result of sugar kinases. The enzyme hexokinase (HK) is responsible for the transfer of the γ-phosphoryl group of a nucleoside triphosphate (typically ATP) onto the 6 position of hexoses (generally Glc). The genome of L. major harbors two nearly identical HKs, LmHK1 (LmjF.21.0240) and 2 (LmjF.21.0250), in tandem on chromosome 21. The two 471 amino acid proteins are predicted to be >99% identical at the amino acid level with a single residue difference (residue 468 is an Ala or Val in LmHK1 and 2, respectively). Predicted HK1s from other Leishmania species are also highly conserved with the LmHKs, with identity ranging from 98% in L. infantum (LinJ.21.0300) and L. donovani (LdBPK_210300.1) to 89% in L. braziliensis (LbrM.21.0310). This is greater identity than LmHK1 shares with the previously characterized T. brucei HK1 (TbHK1, 61% identical) and P. falciparum HK (PfHK, 32% identical). The human HKs (1-4) share limited identity to LmHK1, with human HK 1 and 3 being 36% identical. In addition, the human HKs are predicted to be almost twice as large as LmHK1, being 917 and 923 residues, respectively, as a consequence of the fusion of two HK domains into a single polypeptide. Human glucokinase (human HK 4) shares identity levels (32%) similar to those of the other human hexokinases but is more similarly sized to LmHK1, being composed of 465 amino acids. Here we show the results of characterization and inhibition studies of purified recombinant LdHK1 from E. coli, showing the enzyme’s
distinct allosteric regulation compared to human and T. brucei HKs, and potential as a drug target.

MATERIALS AND METHODS

Chemicals and Reagents

Glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide (NAD\(^+\)), adenosine triphosphate (ATP), and glucose were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA), while phosphoenol pyruvate (PEP), 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen, Eb, PubChem SID 856002) and glucosamine were obtained from VWR International (West Chester, PA). Isobenzothiazolinones and benzamides in Table 1 were obtained from the University of Kansas Specialized Chemistry Center (KUSCC). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from EMD Millipore (Darmstadt, Germany).

Recombinant Enzyme Purification and Assay Conditions

The open reading frame LmjF.21.0240 (TriTryp DB) for L. major hexokinase 1 was amplified from genomic DNA, sequenced, and cloned into pQE30 (Qiagen, Valencia, CA). Recombinant LmHK1, a ~51.7 kDa protein, was purified following a protocol developed for heterologous expression and purification of a HK from the African trypanosome (Morris et al. 2006). Briefly, a 10 mL bacterial culture of E. coli M15 (pREP) harboring pQE30: LmHK1 with LmHK1 cloned in frame of an N-terminal
6-His sequence was used to inoculate a 1 L culture which was grown to an OD$_{600}$ of ~1 and then induced for 24 hr at room temperature with 500 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacteria were then harvested, resuspended in lysis buffer, and incubated with lysozyme (1mg/ml) and DNase (7.5 units/ml) prior to sonication. Soluble recombinant enzyme was then purified as previously described (Morris et al. 2006). The protein was purified to 99% homogeneity (as determined by Coomassie Blue staining of an SDS-PAGE gel).

HK assays were performed in triplicate as described using a coupled reaction to measure enzyme activity (Morris et al. 2006; Misset and Opperdoes 1984). In short, the coupled assay uses glucose-6-phosphate dehydrogenase (G6PDH) to convert glucose-6-phosphate (G6P) generated by HK to 6-phosphogluconate with coincident reduction of NADP to NADPH, which is monitored spectrophotometrically at 340 nm. Kinetic analyses were performed using Kaleida Graph 4.1 (Synergy Software, Reading, PA).

RESULTS

The recombinant enzyme had a high affinity for both substrates, with apparent $K_m$ values for glucose and ATP of 0.13 ± 0.01 and 0.16 ± 0.04 mM, respectively. These values are similar to those for yeast Hxk and *T. brucei* TbHK1 (Table 5.1).
### Table 5.1: Kinetic characterization of LmHK1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Glucose (mM)</th>
<th>ATP (mM)</th>
<th>Mannose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LmHK1</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.04</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>TbHK1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09 ± 0.02</td>
<td>0.28 ± 0.1</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Yeast HK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18 ± 0.1</td>
<td>0.25 ± 0.04</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>HK assays were performed in triplicate and standard deviation is shown.

<sup>b</sup>Data compiled from (Morris et al. 2006) for comparative purposes.

<sup>d</sup>ND, Not determined.

Using glucose as a substrate, the LmHK1 $k_{cat}$ value was $3.6 \times 10^4$ min$^{-1}$, similar to that reported for the previously characterized TbHK1 ($2.9 \times 10^4$ min$^{-1}$) (Morris et al. 2006). Interestingly, the enzyme could utilize mannose as an alternative to glucose, with an apparent $K_m$ value indistinguishable from that of glucose ($0.11 \pm 0.02$ mM), and a higher $K_{cat}$ than glucose ($5.4 \times 10^4$ min$^{-1}$). LmHK1 required a divalent cation, being most active with MgCl$_2$ and having no detectable activity when MnCl$_2$ or CaCl$_2$ were substituted for MgCl$_2$ in the standard assay. The enzyme functioned across a broad pH range, with values between 6.2 and 8.0 supporting at least 50% of the activity found at the optimal pH of ~7.4 (Fig. 5.1B). Basic pH above 8.0 was strongly inhibitory.
Figure 5.1 Effects of glucose concentration and pH on LmHK1 activity.

(A) LmHK1 (~200 ng) activity in response to increasing glucose concentration. (B) The pH profile of LmHK1 activity. Three different buffers with buffering capacity in the range of pH tested were used for the assay: PIPES (pH 6.2, 6.5, 7.0), TEA (pH 7.4), and Tris (pH 8.0, 8.8). Reactions were performed in triplicate and standard deviation is indicated.
In addition to ATP, LmHK1 could also use the purine nucleoside triphosphate GTP as a phosphoryl donor, though the enzyme affinity was lower for this substrate (with an apparent $K_m$ value for GTP of $2.13 \pm 0.10$ mM). The pyrimidine nucleoside triphosphates CTP and UTP also supported activity with apparent $K_m$ values of $3.74 \pm 0.59$ and $1.07 \pm 0.08$ mM. TTP, but not ITP or PP$_i$, was also a competent substrate (Figure 5.2A). While the broad spectrum of nucleoside triphosphates used as substrates differed from that used by the previously characterized TbHK1 (Morris et al. 2006), both enzymes were similarly inhibited by high concentrations of ATP (LmHK1 $K_i$ value of $9.78 \pm 0.97$ mM, Figure 5.2B). Additionally, both enzymes were inhibited by ADP and not by AMP, although LmHK1 ADP inhibition (84 ± 5.1% at 10 mM) was observed under pH conditions distinct from the acidic ones required for ADP inhibition of TbHK1 (Dodson et al. 2011a). The other product, Glc6P, had minimal impact on LmHK1 activity inhibiting the enzyme 22 ± 5.1% at 10 mM.
Figure 5.2 Effects of phosphoryl-bearing compounds on LmHK1 activity.

(A) LmHK1 activity with 5 mM of different phosphoryl-donors. (B) High concentrations (>5 mM) of ATP inhibit LmHK1 activity. In the insert, LmHK1 activity was assessed in
the presence of 5 mM ATP supplemented with ADP, or AMP (all at 5 mM). Assays were performed in triplicate and standard deviation is indicated.

Testing molecules found to inhibit *T. brucei* hexokinase 1 (TbHK1) (Sharlow et al. 2010a, Dodson et al. 2011b), several LmHK1 inhibitors with varied potency have been identified. These include modest inhibitors like the flavonoid quercetin (32.1 ± 0.48% inhibition at 10 mM) and the indazole-3-carboxylic acid derivative lonidamine, an anti-cancer drug that targets tumor HKs (73.7 ± 5.2% inhibition at 10 mM). More potent inhibitors were found following interrogation of a panel of analogs based on scaffolds found to be inhibitors of TbHK1 by high throughput screening (HTS) efforts. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Eb, PubChem SID 856002, Table 5.2), an inhibitor of both TbHK1 and the *P. falciparum* HK (PfHK, (Harris et al. 2013)), inhibited LmHK1 with an IC$_{50}$ value of 2.2 ± 0.2 µM, very similar to that of TbHK1 (IC$_{50}$ value of 0.97 ± 0.03 µM). Likewise, structurally related isobenzathiazolinone inhibitors of TbHK1 (SID17387000, for example) were similarly potent against LmHK1 (100% inhibition at 10 µM). We also explored inhibition of LmHK1 with members of a second scaffold, benzamidobenzoic acid (SID 93575727), which was also identified in the TbHK1 HTS. Further development of this class of compounds has yielded improved inhibitors of TbHK1, including the compound named “Probe” (SID99437306, Table 2) (Sharlow et al. 2010b). These benzamidobenzoic acid derivatives were similarly inhibitory to both TbHK1 and LmHK1 with SID99437306, for example, yielding nearly identical IC$_{50}$ values for both enzymes (Table 2). Against PfHK, however, representatives of this class
were either poor inhibitors (Probe was ~40% inhibitory at 10 µM) or lacked detectable activity (Harris et al. 2013).

Table 5.2 LmHK1 Sensitivity to TbHK1 inhibitors

<table>
<thead>
<tr>
<th>PubChem SID</th>
<th>Structure</th>
<th>LmHK1 IC$_{50}$ (µM)</th>
<th>TbHK1 IC$_{50}$ (µM)</th>
<th>PfHK IC$_{50}$ (µM)</th>
<th>L. amazonensis EC$_{50}$ (µM)</th>
<th>T. brucei EC$_{50}$ (µM)</th>
<th>P. falciparum EC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>856002</td>
<td><img src="image1.png" alt="Image" /></td>
<td>2.2 ± 0.2$^3$</td>
<td>0.97 ± 0.03$^3$</td>
<td>0.01 ± 0.00</td>
<td>4.10 ± 0.40</td>
<td>2.9 ± 0.28</td>
<td>6.8 ± 1.25</td>
</tr>
<tr>
<td>99437306</td>
<td><img src="image2.png" alt="Image" /></td>
<td>1.1 ± 0.07</td>
<td>0.98 ± 0.07</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>60210941</td>
<td><img src="image3.png" alt="Image" /></td>
<td>1.7 ± 0.11</td>
<td>0.28 ± 0.002</td>
<td>&gt;10</td>
<td>13.60 ± 0.50</td>
<td>1.9 ± 0.71</td>
<td>N.D.$^4$</td>
</tr>
</tbody>
</table>

$^1$2-phenyl-1,2-benziselenazol-3(2H)-one (ebselen, Eb, PubChem SID 856002) was obtained from VWR International (West Chester, PA) while the benzamide was obtained from the University of Kansas Specialized Chemistry Center (KUSCC).

$^2$Previously published in (Sharlow et al. 2010a and B; Harris et al. 2013).

$^3$Value established using commercially available compound. Previously published TbHK1 value, using synthetic compound purified from contaminants after synthesis was 0.05 ± 0.03 µM (Sharlow et al. 2010a).

$^4$Not Determined.

Eb has been found to be both an inhibitor of HKs from a number of parasite species including TbHK1 and PfHK (Table 5.2). The compound is also a potent anti-parasitic compound, with EC$_{50}$ values of 2.9 ± 0.28 and 6.8 ± 1.25 µM against T. brucei BSF parasites and P. falciparum red blood cell stages, respectively (Sharlow et al. 2010a; Harris et al. 2013). Against L. major promastigotes, Eb was found to have an EC$_{50}$ value of 4.1 ± 0.4 µM (Sharlow et al. 2010b), supporting the merit of assessing additional HK
inhibitors as potential anti-\textit{Leishmania} lead compounds. SID99437306, which lacks activity against the \textit{Plasmodium} parasite, was also ineffective against \textit{Leishmania} parasites (EC\textsubscript{50} value >10). However the third generation benzamidobenzoic acid derivative, SID60210941, did have moderate activity against \textit{Leishmania} parasites (EC\textsubscript{50} value = 13.6 ± 0.5 \textmu M). Notably, members of both scaffolds, the isobenzathiazolinone and benzamidobenzoic acid derivatives, are minimally toxic to a number of mammalian cell lines (including A549, IMR-90, HeLa, and MDA-MB-231 (Sharlow et al. 2010a).

\textbf{DISCUSSION}

While glycolysis appears to be nonessential for the survival of \textit{Leishmania} parasite, several lines of evidence suggest the glycolytic enzyme LmHK1 could be a viable drug target. Amino sugars are major carbon source for amastigotes (Naderer et al. 2010), which LmHK1 can phosphorylate (0.33 ± 0.1 mmol/min at 20 mM glucosamine), and inhibition of another glycolytic enzyme, pyruvate kinase, is toxic to the parasite (Sandoval et al. 2008). Furthermore, the importance of mannose, which LmHK1 has a high affinity for (K\textsubscript{m} = 0.11 ± 0.02 mM), has been well documented for both energy storage as mannogen (Ralton et al. 2003; Sernee et al. 2006) and for producing the parasite’s glycoprotein coat (Spath et al. 2000; McConville et al. 2002; Sacks et al. 2000).

The kinetics of LmHK1 lends credence to the idea that glucose is not the enzyme’s primary substrate. Like TbHK1, LmHK1 is insensitive to G6P, unlike TbHK1, LmHK1 is inhibited by ADP at physiological pH. Perhaps insensitivity to G6P and inhibition by ADP allow LmHK1 to readily phosphorylate mannose while not depleting
ATP. LmHK1’s ability to utilize other NTPs may also allow it to metabolize mannose without affecting cellular ATP.

Thus it is not surprising that LmHK1 inhibitors are toxic to parasites (Table 5.2). While Eb and isobenzathiazolinones in general are promiscuous cysteine binders that could kill *Leishmania* parasites through off-target effects, the third generation benzamidobenzoic acid derivative SID60210941 has been selected for hexokinase inhibition and is not considered promiscuous. Previous work with TbHK1 has shown that the benzamidobenzoic acids are mixed inhibitors with respect to both glucose and ATP, suggesting an allosteric binding site (Sharlow et al. 2010b). The 61% amino acid identity and shared inhibition by benzamidobenzoic acids between LmHK1 and TbHK1 suggests a shared binding site. The ten-fold increase in EC$_{50}$ for SID60210941 against *Leishmania* compared to *T. brucei* could be the result of differences between the binding site, uptake and metabolism of the compound, or compensatory activity by *Leishmania*’s glucokinase. It may be possible to identify subsets of the benzamidobenzoic acid derivative library that are selective for LmHK1. Future *in vivo* studies will be needed to elucidate the contribution of LmHK1 to parasite metabolism and verify LmHK1 as a drug target. Additional screening is also needed to identify more potent LmHK1 inhibitors.

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Cell. 6, 1450-1463.
CHAPTER SIX

PROCESSING OF *TRYPANOSOMA BRUCEI* HEXOKINASE

REVEALS SECONDARY TARGETING SEQUENCES

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ABSTRACT

Trypanosoma brucei relies on glycolysis exclusively for ATP production when infecting the human host, housing most of the pathway in glycosomes, a kinetoplast specific organelle related to peroxisomes. Compartmentalization of glycolytic enzymes has been suggested to be a means to avoid unregulated ATP consumption by the first two kinases in glycolysis, hexokinase (TbHK) and phosphofructokinase (PFK). However, the unanticipated extra-glycosomal distribution of T. brucei HK2 (TbHK2) suggests alternative or additional functions for the protein, particularly when it is localized proximal to the flagellum (in bloodstream form parasites) or basal bodies (in insect stage parasites). TbHK2 is targeted to glycosomes via a N-terminal peroxisome targeting sequence (PTS2), which is recognized by the peroxin targeting and import machinery. The PTS2 consists of ~11 amino acids which have been found in other higher eukaryotes to be removed after delivery to peroxisomes. Here we have shown that a 25 amino acid sequence immediately downstream of the PTS2 (named N-terminal domain 25, NTD25) serves as a flagellar targeting sequence. Using a fluorescently-tagged reporter system, we have found that the N-terminal end of TbHK2 is proteolytically processed; suggesting that removal of the PTS2 through regulated cleavage may serve as a means by which the NTD25 signal becomes exposed. Further, mutagenesis of conserved amino acids in these two domains has revealed residues critical to TbHK2 processing and localization. Elucidation of this alternative localization pathway may facilitate the identification of new drug targets and further our understanding of the unique role of HKs in T. brucei and potentially other kinetoplastid parasites.
INTRODUCTION

Trypanosomes that infect mammals, including the African and American trypanosomes (*Trypanosoma brucei* and *T. cruzi*) and some *Leishmania spp.*, harbor organelles called glycosomes that compartmentalize a number of important metabolic processes, including most of glycolysis, for which the organelle is named. *T. brucei*, the causative agent of African sleeping sickness, is notable for its dependence on glycolysis, relying exclusively on the pathway in the mammalian blood to generate ATP.

The compartmentalization of glycolytic enzymes in the glycosome has been suggested to be a means to avoid unwanted ATP consumption by the first two kinases in glycolysis, hexokinase (TbHK) and phosphofructokinase (PFK), as neither enzyme is regulated by feedback inhibition at physiological pH (Nwagwu and Opperdoes 1982). However, the unanticipated extra-glycosomal distribution of TbHK2 (Joice et al. 2013) suggests alternative or additional functions for the protein, particularly when it is localized proximal to the flagellum (in bloodstream form (BSF) parasites) or basal bodies (in insect stage (PF) parasites).

Proteins destined for glycosomes frequently bear either N-terminal or C-terminal targeting sequences that are similar to those found on polypeptides targeted to peroxisomes in other systems. These sequences, peroxisome targeting sequence 2 (PTS2) and PTS1, are located on the N-terminus and C-terminus respectively. These signal peptides are recognized by targeting and import machinery (PEX proteins) that are largely conserved from yeast to man (Galland and Michels 2010). The PTS2 consists of
~11 amino acids which has been found in plant and animals to be removed after targeting to peroxisomes (Flynn et al. 1998). In constrast, the PTS1 is rarely removed.

Removal of the PTS2 has been attributed to the activity of a serine protease, DEG15 in plants (Schuhmann et al. 2008) and TYSND1 in mammals (Kurochkin et al. 2007). Similar processing has not been described in lower eukaryotes, including the yeast S. cerevisiae (Flynn et al. 1998; Helm et al. 2007). Here, we describe the processing of the PTS2 of TbHK2 in the early-branching eukaryote T. brucei, finding that proteins destined for the glycosome can be targeted to an alternative destination as a consequence of removal of the PTS2.

MATERIALS AND METHODS

Reagents and Chemicals

Antibodies to Arginine-Glycine-Serine (Histidine)_6 (RGS (His)_6) and GFP (GF28R, which detects GFP and variants including eYFP) were purchased from Qiagen (Valencia, CA) and Thermo Scientific Pierce (Rockford, IL), respectively. Antisera to T. brucei hexokinase (αTbHK, at 1:100,000) and T. brucei glycerol kinase (αGK, 1:100,000) were the kind gifts of Dr. Paul Michels. For western blotting, primary antibodies were detected with appropriate secondary antibodies conjugated to horseradish peroxidase (Rockland Immunochemicals) and were visualized using the ECL Western Blotting System (Pierce). Restrict enzymes were purchased from Promega (Madison, WI).
Parasite growth and transformation

BSF parasites were cultured in HMI-9 medium (Hirumi and Hirumi 1989) containing 10% heat-inactivated fetal calf serum at 37°C under 5% CO₂. PF trypanosomes were grown in SDM-79 medium (Brun and Shonenberge 1979) supplemented with 15% fetal calf serum at 28°C with 5% CO₂. Cultures were always harvested prior to entering stationary phase, *i.e.*, at densities lower than 2 x 10⁶ cells/mL for BSFs or 2 x 10⁷ cells/mL for procyclic cells, by centrifugation.

To explore the subcellular localization of TbHK2 truncations and assess PTS2 processing, constitutively expressed eYFP-tagged versions of the TbHK2 PTS2 (residues 1-11), PTS2NTD25 (N-Terminal Domain 25, residues 1-36), or NTD25 (residues 12-36) were cloned into pXS6 or pXS2 expression vectors. These vectors drive transgene expression from the *T. brucei* rRNA promoter, typically yielding robust expression (Alexander et al., 2002). Constructs were linearized with restriction enzymes (MluI for pXS2 and NotI for pXS6) and transfected into BSF strain Lister 427 cells (4 x 10⁷ cells) using the Amaxa Human T Cell Nucleofector Kit (Lonza, Basel, Switzerland) as described (Burkard et al., 2007). For PF transfections, restriction enzyme linearized constructs were introduced by electroporation as described (Wang et al., 2000). Variants were generated using the parental pXS2 or pXS6 PTS2NTD25eYFP constructs and a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). Mutagenesis primers were prepared according to the manufacturer’s directions with average size of ~35-45 base pairs bearing a one to three nucleotide change from the native sequence. Generation of all variants was confirmed by sequencing.
Fluorescence microscopy and subcellular fractionation

For live cell imaging, parasites were washed in PBS, immobilized in CyGel prepared according to manufacturer’s directions (Abcam, Cambridge MA), and applied to glass coverslips and visualized on a Zeiss Axiovert 200M using Axiovision software version 4.6.3 for image analysis. Immunofluorescence (IF) assays on fixed parasites was performed as described (Joice et al. 2013).

Fractionation of BSF parasite cell compartments was performed using increasing concentrations of digitonin as described (Joice et al. 2013). Briefly, BSF parasites (10^8 cells) were washed in ice-cold buffer (25 mM HEPES, pH 7.4, 250 mM sucrose and 1 mM EDTA) and then resuspended in 0.25 mL of the same buffer. The cell suspension was divided into aliquots containing ~50 µg of protein and digitonin added (0.01, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 mg digitonin per mg of protein). Cells were incubated for 4 min (RT) followed by centrifugation (12,000 × g for 2 min), with the supernatant (released fraction) assayed by western blotting. Untreated cells and those permeabilized by incubation in 0.5% Triton X-100 (total release, TR) were included.

RESULTS

Hexokinases from trypanosomes have conserved N-terminal ends.

A comparison of the N-terminal sequences of HKs from members of the order Trypanosomatida, including African trypanosomes (T. brucei brucei, T. brucei gambiense), the American trypanosome (T. cruzi) and Leishmania spp. (including L.
major and L. infantum), revealed that first 36 residues are highly similar (Figure 6.1A). This similarity is not limited to trypanosomes that infect mammals, as Angomonas deanei, a parasite of insects, shared 41% identity and 64% conserved residues with TbHK2 for the first 36 residues. The PTS2 sequence at the N-terminal end of the polypeptides is similar to that found on many glycosome-resident proteins and not exclusive to the HKs, as T. brucei aldolase (TbALD) and others possess a similar sequence. However, the similarity of the HK sequences to the glycosome-targeted TbALD is limited to the first 11 residues. Beyond the PTS2 is a 25-residue domain (the NTD25) that is not found on HKs from other systems (including human, Plasmodium, or plant) and appears to be unique to the Trypanosomatida HKs.

The NTD25 harbors a flagellar targeting sequence.

The PTS2 is a targeting sequence that drives fully-folded proteins to peroxisomes, organelles that share many features with glycosomes. The trypanosome PTS2 has been likewise found to traffic proteins to glycosomes (Blattner et al., 1995), but the dual localization of TbHK2 (Joice et al. 2013) to glycosomes and the flagellum suggested that perhaps the TbHK2 ORF harbored multiple targeting sequences. To assess the role of the PTS2NTD25 in protein localization, parasites were stably transformed with expression vectors harboring truncations and variants of the PTS2NTD25 fused to the N-terminus of eYFP. Fluorescence of these variants was then observed to determine the impact on localization.
Expression of eYFP without an N-terminal addition yielded cytoplasmic distribution, as has been previously described (Bauer et al. 2014). Addition of the TbHK PTS2 sequence targeted the eYFP to glycosomes in both BSF and PF parasites (Figure 6.1B, left column). Fusion of the PTS2NTD25 onto the N-terminus of eYFP yielded a more complex localization pattern, with distribution in glycosomes and in the flagellum (Figure 6.1B, middle column). Expression of eYFP bearing only the NTD25 yielded expression that was limited to the flagellum in PFs. The phenotype in BSF parasites expressing the construct was more complex, with the fluorescence also detectable in the flagellum. Perhaps the more startling observation was that the majority of the cells expressing detectable fluorescence had a partially detached flagellum, suggesting the expression of the fusion protein was altering flagellar physiology (Figure 6.1B, right column).
Figure 6.1 Sequence and Localization of PTS2NTD25

(A) Alignment of the PTS2 NTD25 from T. brucei with other kinetoplast HKs. The NTD25 region is conserved in other kinetoplast HKs, but not other glycosomal proteins, such as T. brucei aldolase (TbALD). Tbg = T. brucei gambiense, Tc = Trypanosoma cruzi, Lm = Leishmania major, Lin = Leishmania infantium. (B) Localization of PTS2NTD25eYFP fusion constructs. Immunofluorescence analysis of localization and phenotypic consequence of expression of PTS2NTD25eYFP fusions in PF (top row, pXS2) and BSF (bottom row, from stably integrated pXS6).
The N-terminus of the TbHKs is altered by proteolysis.

The unexpected finding that the NTD25 harbors a competent flagellar localization signal led us to explore the mechanism that may be behind extra-glycosomal localization of the TbHKs. To determine if the N-terminal end of the TbHKs is processed to remove the PTS2 signal and thereby allow function of the NTD25 signal, recombinant TbHK1 epitope-tagged with a C-terminal RGS (His)_6 (rTbHK1, which is 98% identical to TbHK2 and shares an identical PTS2NTD25 at the N-terminus) was incubated with BSF lysate followed by SDS-PAGE and western blotting for the RGS (His)_6. Incubation with BSF lysate led to slightly altered mobility of the ~51 kDa protein, a difference that was absent following incubation with heat-denatured lysate (Figure 6.2A). The subtle change in mobility of the rTbHK1 suggested an N-terminal processing event.

To further explore this processing event, BSF parasites expressing PTS2NTDeYFP were lysed and the lysate incubated with or without a protease inhibitor cocktail (Figure 6.2B). Probing the resulting proteins with an antibody to eYFP revealed that the full-length fusion protein (indicated as α) was processed to two species (β and γ) with the more subtle processing step to β altering the mobility of the protein by ~1 kDa while γ appeared to be ~4 kDa less than α. During the course of incubation, α was lost with the coincident accumulation of β and γ. In PF cells, a similar pattern of products was generated (not shown). The processing event impacted the N-terminal end, as the signal from α was lost over time when the sample was probed with anti-sera reactive to TbHK PTS2NTD25 (Figure 6.2B, lower row). While it remains unresolved if β is an
intermediate to γ formation, neither species maintains sufficient antigenic similarity to the TbHK PTS2NTD25 epitope to be detected.

Figure 6.2 Processing of TbHK1 and PTS2NTD25eYFP

(A) Western blot with RGS (His)_6 antibody shows a reduction in the size of rTbHK1 when incubated with 90-13 BSF trypansome lysate, but not when the lysate is boiled prior to mixing. Possible mechanism behind the western blot results on the right. (B) Time course shows processing of the PTS2NTD25eYFP construct. Cell lysates of T. brucei expressing PTS2NTD25eYFP were incubated at 37°C for 0, 1, or 3 hours in the presence or absence of protease inhibitor cocktail (PIC). New, smaller bands appeared
when the lysates were probed with an antibody to eYFP (α-FP, top panel) while signal faded when the lysates were probed with an antibody to the PTS2 (α-PTS2, bottom panel). (C) Cell lysates of *T. brucei* expressing PTSeYFP, PTS2NTD25eYFP, or NTD25eYFP were incubated at 37°C for 1 hour in the presence or absence of PIC and probed with α-FP.

*Variants of the N-terminus can be processed but are toxic to *T. brucei*.*

PTS2NTD25 fusions bearing alterations introduced by point mutation were used to identify residues that are critical for function of the processing and targeting. Serine 13 of the TbHKs is an alanine in the same position in the HKs from *T. cruzi* and *Leishmania* spp. Altering this residue to an alanine to yield the variant S13A had no impact on either processing or cellular distribution (Figure 6.3A and B, respectively). Mutation of the conserved arginine 23 to alanine (R23A) also had no effect on processing, but instead had a catastrophic impact on cell morphology in both BSF and PF cells (Figure 6.3B). In BSF parasites, expression of the R23A eYFP fusion led to failure to properly divide, with fluorescence largely distributed to foci in the cell. PF cells also had a flagellar defect, with multiple flagella emanating from individual cells.
Figure 6.3 Processing and Localization of PTS2NTD25eYFP Point Variants

(A) Overview of site-directed mutagenesis of PTS2NTD25eYFP construct residues to alanine and their effects on processing and localization. + = behaves like wildtype, β = not processed beyond β form, C = cytoplasmic; defect in localization, FD = flagellar defect; multiple flagella and/or defective cell division. *flagellar defect only present in double variant N6/7A. (B) Cell lysates of *T. brucei* expressing PTS2NTD25eYFP point variants were incubated at 37°C for 1 hour (unless otherwise indicated) and probed with α-FP. (C) Subcellular fractionation of 427 BSF expressing PTS2NTD25eYFP wild type and N7A fusions. Trypanosomes were incubated with increasing concentrations of digitonin and release of eYFP and glycerol kinase (GK, a glycosome resident protein).
monitored by western blots. Total release (TR) corresponds to cells incubated with 0.5% Triton X-100 to permeabilize all membranes. (D) Immunofluorescence analysis of distribution and phenotypic consequence of expression of PTS2NTD25eYFP point variants in BSF 427s (from stably integrated pXS6). Scale bars are 5 µM.

Altering asparagine 7 to alanine blocked the production of γ, even after 6 hours of incubation in cellular lysate (Figure 6.3A). Interestingly, localization by microscopy was not detectably altered (not shown). However, digitonin subcellular fractionation suggested a difference in distribution of the N7A variant from the unaltered fusion protein (Figure 6.3C). The α (full length) species from both unaltered and N7A variant PTS2NTD25eYFP was released at concentrations of detergent required to liberate cytosolic components, not surprising given that biosynthesis likely occurs on cytoplasmic ribosomes. The β species from cell lines harboring the N7A and unaltered fusions also behaved similarly, being released upon addition of sufficient digitonin to release glycososomal contents including the glycosome marker glycerol kinase (GK). This finding suggests that the β species may be a glycosome-resident molecule or that the processing activity may be glycosome-associated and released only upon detergent addition. The main difference between the N7A and the unaltered eYFP fusion was the absence of the γ species in the N7A samples. Cells expressing the unaltered fusion released γ after solubilization with 0.5% TX-100 (total release, TR), suggesting the species may reside in a digitonin-insensitive compartment. Because the N7A variant does not yield γ, it was predictably absent from the total release fraction.
Altering the aspartate 18, which is completely conserved in the NTD25s, to alanine (D18A) disrupted processing by blocking the formation of γ, but had no impact on cell growth. Likewise, changing the conserved residue glutamic acid 20 to alanine (E20A) prevented accumulation of γ but again was tolerated without ill effect by the parasites.

**DISCUSSION**

Glycosomes share similarities with peroxisomes, including the protein import mechanisms required for the introduction of glycosome resident proteins to the matrix of the organelle. While proteolytic processing of the PTSs is well documented in higher eukaryotes (Kurochkin et al. 2007; Helm et al. 2007), our study is the first example of PTS2 processing in lower eukaryotes. Further, the NTD25 flagellar targeting sequence identified appears to be specific to kinetoplasts, making this flagellar targeting pathway a potential drug target, as mislocalization of TbHK is lethal to parasites (Bakker et al., 2000; Haanstra et al., 2008).

Both the PTS2 and NTD25 appear to be cleaved in this localization pathway, resulting the production of 3 forms, which have been labeled as α, β, and γ (Figure 6.4). Point variants of the PTS2NTD25eYFP construct support this theory and show potential binding and cleavage sites of proteases (Figure 6.3). The processing of α to β appears to be required to expose the binding site for the protease responsible for the β to γ cleavage. Mutation of the N7 residue prevents recognition by this enzyme, while variants further downstream (D18A, E20A) are proposed to impact processing because they are near the
The R23A variant is interesting because although it is processed and localized normally, expression of PTS2NTD25eYFP R23A appears to interfere with flagellar function in cell division, suggesting that arginine 23 has a critical role in TbHK’s flagellar function.

**Figure 6.4 Proposed Model of TbHK2 Processing**

The PTS2 directs TbHK2 to glycosomes via PEX proteins. Processing and export from the glycosome reveals the NTD25, which directs TbHK2 to the mitochondria/basal bodies in PFs and the flagellum in BSFs, where the NTD25 is removed. Question marks indicate the action of 2 proteases yet to be identified.
Another curiosity is the difference in NTD25eYFP localization between life cycle stages (Figure 6.1). More studies are needed to determine if these localizations represent distinct functions or if the flagellar activities of TbHK are not used in PFs and the basal bodies are a reserve location. The discovery of TbHK processing has revealed a novel pathway for study and an exciting new potential drug target.

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The abbreviations used are: BSF, bloodstream form; eYFP, enhanced yellow fluorescent protein; GK, glycerol kinase; HK, hexokinase; IF, immunofluorescence; NTD25, N-terminal domain 25 amino acids found downstream of the peroxisome targeting sequence 2 in *T. brucei* hexokinases; PF, procyclic form; PTS2, peroxisome targeting sequence type 2; PTS2, peroxisome targeting sequence type 2; PTS2NTD25, peroxisome targeting sequence type 2 and N-terminal domain 25 amino acids from *T. brucei* hexokinas; TbHK1, *T. brucei* hexokinase 1; TbHK2, *T. brucei* hexokinase 2; Tet, tetracycline repressor gene; vPBS, Voorheis’s modified PBS.
References


Protozoan parasites continue to be a problem in the developing world, affecting the most vulnerable populations with the least resources to respond. The treatments for these diseases are decades old, and many are losing their effectiveness in the wake of emerging resistance and many also have toxic side effects. Thus there is a dire need for new therapeutics. These parasites have evolved to be highly specialized to suit the environment of their human host. One common adaptation by parasites is the repression of mitochondrial activity and increased glycolytic flux to take advantage of the abundant glucose in the host. This makes parasite glycolytic enzymes attractive targets for drug development. Hexokinase is of particular interest, because it catalyzes the first step of glycolysis, typically a rate-limiting step, and because it is also connected to a number of other biosynthetic pathways. Consequently, the hexokinases of *Trypanosoma brucei*, *Plasmodium falciparum*, and *Leishmania major* have been investigated as drug targets.

*Trypanosoma brucei*

*T. brucei* is an extracellular parasite of the mammalian bloodstream and cerebrospinal fluid, transmitted by the bite of tsetse flies in sub-Saharan Africa. Fatal if untreated, African trypanosomiasis causes 10,000 deaths a year, and current treatments are inadequate due to the need for multiple intravenous infusions, toxic side effects that kill 5% of patients, and growing resistance. *T. brucei* is dependent on glycolysis for ATP production in the mammalian host, and its two hexokinase enzymes (TbHK1&2) have
been validated as drug targets (Chambers et al. 2008). A recent high throughput screen (HTS) has identified small molecule inhibitors of TbHK1 (Sharlow et al. 2010a). Two hits from the screen have been pursued as scaffolds for drug development: isobenzothiazolinones and benzamidobenzoic acid. Additionally, new immunofluorescence studies have shown that TbHK2 has an alternative localization to the flagellum, challenging the previous dogma that TbHKs were found only in the parasite’s glycosomes, specialized peroxisomes that house most of the glycolytic enzymes.

*Ebselen Mechanism of Action*

The most potent TbHK1 inhibitor identified in the HTS was ebselen, and several similar isobenzothiazolinone compounds were also identified as inhibitors. These inhibitors were shown to be non-competitive with substrates and did not effect enzyme oligomerization. Ebselen is known to promiscuously bind cysteine residues. To determine if cysteine interaction was the means of TbHK1 inhibition, TbHK1 incubated with ebselen or empty vehicle was submitted for mass spectrometry, and site-directed mutagenesis was used to create alanine variants at TbHK1 cysteine residues. Mass spectrometry showed that cysteines C327 and C369 in TbHK1 treated with ebselen had been irreversibly oxidized, while the mutagenesis experiments showed that C327, but not C369, was essential for TbHK1 activity. It appears that ebselen and presumably other isobenzothiazolinone compounds inactivate TbHK1 by oxidizing C327. While non-toxic to humans and currently in clinical trials for stroke victims (Stroke Trials Registry Home
Page, as of 01/26/2015), the promiscuous nature of ebselen and the inability to establish a structure activity relationship for the isobenzothiazolinone scaffold make further drug development unlikely.

*Development of the Benzamidobenzoic Acid Scaffold*

The benzamidobenzoic acid scaffold has shown remarkable improvement with the first and second generations of compounds. The best TbHK1 inhibitor identified from the first generation of compounds, SID99437306 (dubbed “Probe”), was 10-fold more potent than the initial HTS hit ($IC_{50} = 0.98 \pm 0.07 \mu M$). It was also non-toxic to human cell lines and 40-fold selective for TbHK1 over the equivalent human enzyme (Sharlow et al. 2010b). However, Probe still had the initial HTS hit’s fatal flaw of being ineffective against parasites in culture. A lack of cell permeability was believed to be the cause of Probe’s inability to kill parasites. Subsequent efforts were aimed at modifying the scaffold with hydrophobic residues to improve permeability. The best compound identified in the second generation of compounds, SID60210941, or Lead, is both more effective against TbHK1 ($IC_{50} = 0.28 \pm 0.02 \mu M$), and effective against parasites in culture ($LD_{50} = 1.88 \pm 0.71 \mu M$). The third generation of inhibitors, which explored additions to the benzene ring added in Lead, has led to only small improvements in $IC_{50}$ and $LD_{50}$, but these changes may be additive. Further work is also needed to identify the mechanism of action of the benzamidobenzoic acids. While it is known that the compounds are mixed inhibitors with respect to glucose and ATP, the presumed allosteric binding site has not been identified.
Identification of TbHK’s Flagellar Targeting Sequence

The discovery that TbHK2 has extra-glycosomal localization to the flagellum prompted a search for a secondary targeting sequence within the protein. A 25 amino acid sequence in the N-terminus of TbHK2 (NTD25) immediately downstream of the peroxisome targeting sequence 2 (PTS2, which targets proteins to the glycosome) was found to have high conservation amongst only kinetoplastids. Immunofluorescence studies with PTS2, NTD25, and PTS2NTD25 fusions to eYFP showed that NTD25 is indeed a flagellar targeting sequence. Western blots of T. brucei lysate with PTS2NTD25eYFP show that two distinct processing events occur that alter the mobility PTS2NTD25eYFP. Site-specific alterations to PTS2NTD25eYFP have identified important residues for processing. Attempts at elucidating the cleavage site by mass spectrometry have been frustrated by technical problems. Nevertheless, these studies have revealed a kinetoplastid-specific pathway for further study and as a potential drug target. More work is needed to determine the exact sites of processing and identify the other proteins involved.

Leishmania major Hexokinase 1

L. major is one of Leishmania species that causes cutaneous leishmaniasis in the Old World, and is related to the more deadly Leishmania species that cause visceral disease. Leishmania species are intracellular parasites of mammalian macrophages, transmitted by sand fly vectors. While Leishmania is not dependent on glycolysis like its
kinetoplastid relative *T. brucei*, mannose, another hexokinase substrate, is important for *Leishmania* virulence. *Leishmania* uses mannogen as its primary energy store (Ralton et al. 2003) and its glycoprotein coat also has high mannose content (Davis et al. 2004).

To characterize *L. major* hexokinase 1 (LmHK1), the gene was cloned from *L. major* genomic DNA into an *E. coli* expression vector to produce recombinant LmHK1. Kinetic characterization showed that LmHK1 was distinct from both TbHK1 and human HKs. While LmHK1 is insensitive to inhibition by its product glucose-6-phosphate like TbHK1, ADP does inhibit the enzyme, suggesting that the “turbo explosion” hypothesis (Haanstra et al. 2008) may not apply to *Leishmania*. LmHK1 also has an affinity and efficiency for mannose (*K_m* = 0.11 ± 0.02 mM, *K_cat* = 5.4 x 10^4 min^-1) on par with glucose (*K_m* = 0.13 ± 0.03 mM, *K_cat* = 3.6 x 10^4 min^-1), supporting the idea that it may be vital due to a role in mannose metabolism. Known TbHK1 inhibitors also inhibited LmHK1, and parasites were sensitive to some benzamidobenzoic acid compounds (EC_{50} = 13.6 ± 0.5 µM). These findings suggest that LmHK1 may be a viable drug target, but further work is needed to prove that LmHK1 is essential.

*Plasmodium falciparum* Hexokinase

Malaria is the most deadly parasitic disease in the world (>1 million deaths per year), and is caused by five species in the genus *Plasmodium* that are spread by female *Anopheles* mosquitos. *P. falciparum* is the most virulent species and causes the majority of deaths. *Plasmodium* species are intracellular parasites of red blood cells, causing fever, anemia, and in severe cases renal and brain damage. *Plasmodium* has repressed
mitochondrial activity in the mammalian host, and relies on glycolysis for ATP, with infected erythrocytes having up to a 100 times more glycolytic activity (Roth et al. 1982). Infected erythrocytes also have up to a 72-fold increase in activity in the pentose phosphate pathway, which the parasites rely on for producing reducing equivalents and nucleotide precursors (Roth 1987). HK catalyzes the first step in both of these pathways, making it an excellent drug target.

Recombinant *P. falciparum* HK (PfHK) was produced from *E. coli* bearing an expression vector containing an *E. coli* codon-optimized version of PfHK. PfHK has kinetic properties typical of an HK, and unlike the kinetoplastid HKs, is subject to feedback inhibition by its products. PfHK is insensitive to the benzamidobenzoic acid compounds, suggesting that the compounds may interact with a kinetoplastid-specific feature. PfHK was 10-fold more sensitive to ebselen (*IC*₅₀ = 0.01 ± 0.00 µM) than TbHK1 (*IC*₅₀ = 0.05 ± 0.03 µM), and *P. falciparum* parasites were also sensitive to ebselen (*EC*₅₀ = 6.8 ± 1.25 µM). Despite the conservation of TbHK1 C327 (as C398) in PfHK, ebselen may act on PfHK via a different mechanism, as ebselen inhibition of PfHK, but not TbHK1, can be rescued by dilution.

The central role of hexokinase in *Plasmodium* metabolism and these early studies show that PfHK is a promising drug target - hexokinase inhibitors are toxic to parasites. A HTS campaign is currently being prepared to identify more PfHK inhibitors. The screen will identify PfHK-specific inhibitors (PfHK and TbHK1 share only 29% amino acid identity), which will serve as scaffolds for further drug development. Another priority will be *in vivo* studies of PfHK. While studies of parasite metabolism and related
protein suggests PfHK is essential, this needs to be genetically validated. This future work will confirm PfHK as a drug target and provide starting points for drug development.

References


