A GPI-PHOSPHOLIPASE C AND PROTEIN TYROSINE KINASES IN TRYPANOSOMA BRUCEI

by

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(Under the Direction of Kojo Mensa-Wilmot)

ABSTRACT

Trypanosoma brucei causes human African trypanosomiasis (HAT). The disease threatens over 60 million people in 36 African countries. Chemotherapy is the only method of treatment available against HAT. Generally, the drugs are toxic and difficult to administer. Today, the need for safer, orally administered drugs is urgent.

T. brucei is covered with a glycosyl phosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG). VSG is essential for the viability of *T. brucei* in the host's bloodstream. The parasite also expresses a GPI-specific phospholipase C that is able to cleave the GPI-anchor of VSG. Interestingly, little VSG is released in non-differentiating bloodstream cells. The intracellular localization of GPI-PLCp is not well characterized and the function(s) of the enzyme are just beginning to be elucidated. To gain better perspective of the functions of GPI-PLCp, it is important to determine the intracellular location of the enzyme.

Tyrosine kinase activity is present in *T. brucei*. Preliminary investigations suggest that drugs that inhibit protein tyrosine kinases (PTKs) kill the parasite. Given their role in cancer, PTKs have become the focus of an enormous effort to discover new drugs against the disease. Several drugs that inhibit PTKs have been developed and are being used to treat some cancers.

The goal of this dissertation is two-fold. First, to further understand the function of GPI-PLCp in *T. brucei*, we aimed to determine the intracellular location of the enzyme. Towards this, we used two approaches; (i) fluorescence microscopy, and (ii) iodixanol density-gradient centrifugation and found GPI-PLCp to be a glycosome protein. In a second project, we wanted to determine if PTK-specific drugs used to treat cancer could kill *T. brucei*. To this end, we treated *T. brucei* with the drugs Canertinib, PKI166 and AEE788 and found that all kill cultured trypanosomes. Further we found AEE788, a pyrrolopyrimidine, to be the most potent drug tested and within 90 minutes of exposure it: (i) reduces tyrosine phosphorylation, (ii) blocks endocytosis of transferrin, (iii) causes a morphological change, and (iv) triggers cell death in *T. brucei*. Finally, to understand which processes may be influenced by tyrosine phosphorylation in bloodstream *T. brucei*, we identified tyrosine-phosphorylated proteins by pTyr-affinity chromatography and LC-MS/MS. Using this technique, we identified 132 putative pTyr proteins and two proteins with confirmed pTyr residues. Collectively, these data validate PTKs as targets for drug discovery in *T. brucei* and introduce pyrrolopyrimidines as a lead scaffold for anti-trypanosome drug discovery.

INDEX WORDS: *Trypanosoma brucei,* Human African trypanosomiasis, Variant surface glycoprotein, Glycosyl phosphatidylinositol, GPI-PLC, Glycosome, Tyrosine kinase, Tyrosine phosphorylation, pTyr, Pyrrolopyrimidine, 4-anlinoquinazoline, Canertinib, PKI-166, AEE788

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DEDICATION

To my Family

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1.1 Trypanosomiasis

Human African trypanosomiasis (HAT or sleeping sickness) is caused by the protozoan parasite *Trypanosoma brucei.* The disease threatens over 60 million people in 36 African countries. The parasite is introduced to the mammalian bloodstream by the bite of an infected tsetse fly from the genus *Glossina*. Incidence of the disease is limited by the ecology of the insect vector, which is only found in sub-Saharan regions of Africa. There are many species of trypanosome, two sub-species belonging to the species *brucei,* infect humans. *Trypanosoma brucei rhodesiense* is mostly found in eastern and southern Africa and is responsible for an acute, more virulent form of infection. *Trypanosoma brucei gambiense* causes a more chronic form and is primarily found in west and central Africa. The chronic form of the disease accounts for more than 90% of the reported cases (for review [1-3]).

After being bitten by an infected tsetse fly, a painful skin lesion called a trypanosomal chancre may form at the bite site. It is here that initial parasite multiplication occurs. Anywhere from 1-3 weeks after the bite, the trypanosomes are detected in the host's bloodstream, lymph nodes and systemic organs in the early or *hemolymphatic* stage of the disease [4]. This stage is defined as the period prior to parasite migration into the central nervous system (CNS). The early stage is marked by general signs of infection, including but not limited to, malaise, fever

accompanied by rigors and vomiting, itching, weight loss, headache, cardiac/liver/endocrine dysfunction, and ocular disturbances. If the early stage is left untreated, the parasites will cross the blood-brain barrier into the central nervous system (CNS) in as little as a few weeks (*rhodesiense* infection) or months (*gambiense* infection). This marks the late or *encephalitic* stage of the disease. During this stage, the infected individual may suffer from sleep disturbances such as reversal of sleep/wake cycle, insomnia, or daytime somnolence. Other symptoms may include sensory and motor disturbances or physiological symptoms such as memory loss, dementia, depression, anxiety and irritability. If still untreated, the patient will progressively deteriorate, symptoms will become more severe and death may occur [4].

1.2 Treatment

Chemotherapy is the only method of treatment available against HAT. If the disease is diagnosed early, the chances of a cure are high. Treatment depends on the phase of infection, early or late. Early phase treatments include suramin and pentamidine: late phase treatments include melarsoprol and eflornithine (the only compound registered to combat HAT within the last 50 years). Nifurtimox, either alone or in combination with other drugs, particularly eflornithine, is being considered an option to treat the late stage of the disease [2, 5]. Generally, these drugs are toxic (sometimes severe) and difficult to administer in poor countries (due to the need for parenteral administration in a hospital). These considerations aside, new strains of the parasite are beginning to emerge that are resistant to some of the drugs [6]. Today, the need for newer, safer, orally administered drugs is urgent. Research aimed at understanding the biological and biochemical processes of the parasite may reveal new targets against which new drugs can be directed in an effort to kill the parasite *in vivo*.

1.3 Life Cycle

Trypanosomes are extracellular parasites that reside in the circulatory system of infected individuals. A fly introduces metacyclic trypanosomes into the host by injecting saliva into the puncture wound produced during feeding. Upon entering the mammalian bloodstream metacyclics differentiate into long slender trypomastigotes and replicate. At peak parasitemia, a proportion of the parasites differentiate into "short stumpies" that are pre-adapted for transmission into the tsetse fly (Fig. 1.1). During a blood meal from an infected individual, the tsetse fly ingests the short stumpy trypomastigotes that can differentiate into procyclic form that multiply in the mid-gut of a fly. Procyclics migrate to the salivary glands differentiating into epimastigotes. Epimastigotes attach to the salivary gland epithelium, continue to multiply and ultimately develop into infective, non-dividing metacyclic trypomastigotes. The life cycle is complete when metacyclic trypanosomes are transmitted to a mammalian host [2].

2 Antigenic Variation

In the bloodstream, *T. brucei* is constantly exposed to the host immune system. The parasite population survives the host's antibody-mediated defenses by a process known as "antigenic variation" [7, 8]. Each bloodstream trypanosome expresses a single variant surface glycoprotein (VSG) gene from a repertoire of an estimated 1000 genes. Upon establishing infection, the host develops antibodies against the parasite's VSG coat and kills a large proportion of the population. However, a small fraction of the population (estimated at 10^{-5}/generation, [8]) express an antigenically different VSG. This small population of trypanosomes remains undetected by the host's immune system, survives the immune response, and proliferates to become the new dominant population continuing the cycle.

3 Glycosomes

In the bloodstream, *T. brucei* is completely dependent on glycolysis for ATP generation [9]. Glycosomes are intracellular, peroxisome-related microbodies that contain the first 7 enzymes involved in the conversion of glucose to 3-phosphoglycerate in kinetoplastids [10, 11]. Glycosomes are essential for survival of the parasite within its mammalian host [12].

Glycosomes are related to peroxisomes of other eukaryotes. They are surrounded by a single phospholipid bilayer, and do not contain DNA. Both organelles contain enzymes involved in fatty acid β-oxidation, ether-lipid biosynthesis and pyrimidine biosynthesis. Their biogenesis and protein import pathways are also similar. Membrane and matrix proteins are synthesized on free ribosomes in the cytoplasm and either post-translationally inserted (into the lipid bilayer) or imported, into the organelles, respectively [13-16]. Similar sequences are used in routing polypeptides to these organelles (see below) [13, 17, 18]. Together, these studies suggest that both organelles may have been derived from a single ancestral peroxisome-like organelle [19].

Peroxisomes, PEX genes and their products (peroxins) have been studied extensively. To date, 32 PEX genes have been identified. Nine peroxin orthologues have been identified in trypanosomatids; PEX2, 5, 6, 7, 10, 11, 12, 14 and 19 [12, 13, 16, 20-23] which are involved in three processes of peroxisome biogenesis: (i) import of matrix proteins, (ii) membrane biogenesis (insertion of membrane proteins into the peroxisome bilayer), and (iii) proliferation of peroxisomes (for review [24-26]). Most studies on the biogenesis of these organelles have been done on peroxisomes of yeast and mammalian cells [16]. Despite several species-specific differences (see below), the biogenesis of these organelles are thought to have been conserved throughout evolution [16].

3.1 Peptide Signals for Glycosome Targeting

The nature of the targeting sequence dictates whether a protein is imported into the glycosome lumen (matrix proteins) or inserted into the glycosomal membrane (membrane proteins). Matrix proteins are targeted to peroxisomes by either of the following: (i) PTS1, a C-terminal tripeptide SKL (single letter amino acid code) or a variation thereof; (ii) PTS2, an N-terminal nonapeptide motif RLXXXXX(Q/H)L where X may be any amino acid; (iii) an internal targeting sequence [27]; or (iv) by associating with a protein that contains a PTS [28]. The latter method is referred to as "piggyback import" into the peroxisome [29]. The first three targeting mechanisms have been described in *T. brucei* (reviewed in [26]).

Peroxisomal membrane proteins (PMPs) localize to the organelle independent of PTS1 or PTS2. Each PMP contains a membrane peroxisomal-targeting sequence (mPTS). Most mPTSs contain at least two elements: (i) a PEX19-binding site, which may target the PMP to the peroxisome, and (ii) an adjacent transmembrane domain (TMD), which anchors the PMP into the peroxisome membrane [30]. The PEX19-binding region generally consists of approximately 11 hydrophobic and positively charged amino acids [30]. Several PMPs have been identified in *T. brucei* (PEX2p, 10, 11, 12 and 14). To date, PEX19-binding motifs have been discovered in PEX2p, PEX10p and PEX11p [31].

Peroxisome proteins are recognized by one of three cytoplasmic receptors, PEX5p, PEX7p or PEX19p. These receptors and their cargo dock at the peroxisome membrane and interact with a host of other PEX proteins that facilitate import of matrix proteins or insertion of membrane proteins (for review [32]).

PEX5p is the receptor for PTS1. Upon binding to proteins bearing PTS1, PEX5p translocates to the peroxisome membrane where it interacts with several PMPs (e.g. PEX13, 14

and 17) before being imported into the lumen of the peroxisome. In the lumen, cargo is released and PEX5p is recycled back to the cytoplasm for additional rounds of import (see Fig. 1.2A for more detail) [32, 33]. PEX7p is the receptor for PTS2. PEX7p requires a species-specific auxiliary protein for PTS2 import: in *S. cerevisiae* PEX7p requires PEX18p or PEX21p [34]; PEX20p in *Yarrowia lipolytica* and *Neurospora crassa* [35, 36]; or in mammals, the longer of two splice isoforms of PEX5p [37]. Like PEX5p, PEX7p shuttles between the cytoplasm and the peroxisome lumen during cargo translocation [38]. PEX19p binds newly synthesized PMPs and facilitates their insertion into the peroxisome membrane [39, 40]. The molecular machinery required for inserting PMPs is different from that needed for import of PTS1 or PTS2 proteins (Fig. 1.2B). After binding newly synthesized PMPs in the cytoplasm, PEX19p then transports its cargo to the peroxisome membrane where it docks to the membrane via interaction with PEX3p [41, 42]. After associating with the peroxisome membrane the mPTS-bearing PMP is inserted into the membrane. The mechanisms underlying this process are not well characterized. In *T. brucei*, all three receptors (PEX5p, 7 and 19) have been identified and are essential for the viability of the parasite [13, 16, 21, 31, 43, 44]. RNAi of each peroxin severely reduces the growth of the parasite and causes mislocalization of proteins bearing the respective targeting sequences [16, 31].

4 The Endocytic Pathway

Endocytosis is a process by which cells internalize materials from their surroundings by formation of membrane vesicles at the plasma membrane. The function of endocytosis varies depending on the cell. Endocytosis serves to recover lipids and proteins from the plasma membrane, regulate activities such as neuronal transmission, metabolism, proliferation, acquisition of nutrients, and mount a defense against invading organisms.

Endocytosis may be divided into two general categories: phagocytosis (cell eating) and pinocytosis (cell drinking). Phagocytosis refers to the internalization of large particles (> 0.5 μ diameter) and pinocytosis refers to the formation of smaller vesicles (< 0.2 μ diameter) [45, 46]. Pinocytosis occurs via four basic mechanisms: macropinocytosis, calveolae-mediated endocytosis, clathrin-mediated endocytosis and calveolae/clathrin-independent endocytosis.

4.1 Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis (CME) is the best-characterized mechanism of the pinocytocytic pathways. It occurs in virtually all eukaryotic cells and is the major pathway by which cells internalize nutrients, growth factors, antigens, pathogens, signaling molecules and recycling receptors [47-50]. During this process, receptors and their bound ligands are internalized in clathrin-coated pits (CCPs) that pinch off the plasma membrane as clathrin-coated vesicles (CCVs). Soon after CCVs pinch off the membrane, the clathrin coat is rapidly disassembled and endocytosed material is transported through a series of endocytic compartments where it may be recycled to the plasma membrane or transported to the lysosome to be degraded [46].

Clathrin-mediated endocytosis requires several major proteins: adaptor proteins (APs), clathrin, dynamin, and actin. The most abundant proteins involved in the formation of CCPs are APs and clathrin [51]. Adaptor proteins play a central role in CCP formation via their ability to bind phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2, see below) and clathrin, and to recruit other components for CCV formation machinery (e.g. dynamin, actin). Cargo internalization is initiated by the association of APs with PtdIns(4,5)P_2 and the internalization signals on the cytoplasmic tails of transmembrane proteins [52]. Adaptor proteins on the plasma membrane eventually lead to the recruitment of clathrin and actin, nucleating vesicle formation.

The assembly unit of clathrin, a triskelion, consists of three heavy chains and three light chains that radiate out from a central hub [49]. The association of clathrin with APs at the plasma membrane is important for membrane invagination and the formation of a clathrin coat around the vesicle (for review [51]).

Deeply invaginated coated pits are characterized by the presence of dynamin [51], a GTPase that binds to adaptor proteins containing Src homology 3 (SH3) domains via its COOH-terminal proline/arginine-rich domain (PRD) [53]. Dynamin self-assembles around the neck of the coated pit to form a helical collar. This neck structure connects the forming vesicle to the plasma membrane and must be constricted to bring the opposing sides of the membrane within the neck close enough to facilitate fusion and ultimately formation of a free clathrin-coated vesicle [51].

Actin is essential for endocytosis in yeast [54]. Mutations of actin cause internalization defects more pronounced than those observed when clathrin is mutated [55]. In mammalian cells, actin polymerization occurs at endocytic sites during the invagination of CCVs [56, 57]. Furthermore, actin dynamics are required for pit invagination and vesicle scission (reviewed in [58]). Several accessory proteins that interact with the endocytic machinery and the actin cytoskeleton have been identified [45] (for review [59]), and inhibition of their function can disrupt clathrin-mediated endocytosis [58, 60-62]. Together these data suggest that actin is directly involved in clathrin-mediated endocytosis. [45, 57, 58, 63].

4.2 Lipid Regulation of Endocytosis

Phosphoinositides play key roles in organization of cytoskeleton, vesicle transport and cell signaling. Phosphatidylinositol-(4,5)-bisphosphate regulates clathrin-mediated endocytosis. First, several adaptor proteins involved in the formation of CCPs, such as AP2, epsin and

AP180/CALM, contain PtdIns(4,5)P_2-binding domains. Disruption of the binding ability of these proteins impairs endocytosis [64-67]. Second, alteration of phosphoinositide kinases and phosphatases involved in PtdIns(4,5)P_2 metabolism, such as the genetic inactivation or overexpression of synaptojanin, knockdown of PtdIns(4)P 5-kinase type Iβ or the addition of rapamycin, disrupts endocytosis [68-71]. Finally, PtdIns(4,5)P_2 stimulates actin assembly, which is important for endocytosis, by binding to proteins such as profilin and WASP (Wiskott-Aldrich syndrome protein) (reviewed in [72, 73]).

4.3 Regulation of Endocytosis by Kinases

Protein phosphorylation regulates many interactions in cells including division, metabolism, migration, apoptosis and endocytosis of CCVs. Kinase regulation of clathrin-mediated endocytosis entails phosphorylation of many of the core and accessory proteins of the endocytic machinery.

Tyrosine phosphorylation of proteins may be important for regulation of clathrin-mediated endocytosis. Activation of epidermal growth factor receptor (EGFR) activates Src tyrosine kinases [74]. Src facilitates endocytosis by phosphorylating clathrin heavy chain, AP-2, dynamin and cortactin [75-79]. Inhibition of Src kinase blocks or attenuates endocytosis of the neural cell adhesion molecule and the β2-adrenergic, c-Kit and cholecystokinin receptors [80-83] (reviewed in [76]). Furthermore, the tyrosine kinase inhibitor genistein attenuates the internalization of EGFR, B-cell antigen receptor and asialoglycoprotein receptor [84-86], and partially inhibits endocytosis of the transferrin receptor [87].

5 Endocytosis in T. brucei

The endocytic system of *Trypanosoma brucei* is highly polarized; endocytosis and exocytosis events are concentrated at the flagellar pocket [88]. The flagellar pocket is a deep,

flask-shaped invagination at the base of the flagellum that is located at the posterior end of *T. brucei*. Cell surface receptors for the uptake of macromolecules are retained in the flagellar pocket whereas VSG is dispersed throughout the plasma membrane [89, 90].

Clathrin-mediated endocytosis is essential for the viability of *T. brucei* [91]. It is a very important process in the parasite for several reasons. First, endocytosis of extracellular fluid is important to maintain cell volume. Second, *T. brucei* is dependent on the uptake of essential nutrients (including low density lipoprotein (LDL) and iron [92]) from the host's bloodstream. LDL is an essential source of lipids, and iron is required for growth [93, 94]. Third, as an extracellular parasite, *T. brucei* is exposed to the host's immune system and can be killed by antibodies and complement. However, the parasite is protected from antibody-mediated killing because antibody-bound VSG is rapidly internalized [95] and directed to lysosomes for degradation [96] (for review [88]).

5.1 Protein Components of Clathrin-Mediated Endocytosis in T. brucei

Clathrin heavy chain in *T. brucei* (TbCLH) is approximately 60% similar to human clathrin heavy chain [49]. Clathrin is essential for the viability of both bloodstream and procyclic *T. brucei*. RNAi of clathrin in bloodstream *T. brucei* results in the accumulation of several endocytosed markers (concanavalin A and FM4-64) at the flagellar pocket, which are normally directed to the lysosome, and also causes an enlargement of the flagellar pocket [91].

Two dynamin-like proteins (TbDLPs) (97% identical) are found in *T. brucei*. RNAi of TbDLP in procyclic *T. brucei* resulted in an enlargement of the flagellar pocket, similar to that caused by the loss of TbCLH [91, 97] and actin ([98], see below). Further, endocytosis of fluorescently tagged surface proteins was reduced 40%. Interestingly, ablation of TbDLP also

inhibits mitochondrial fission suggesting that the protein is also involved in mitochondrial division [97]. RNAi of TbDLP has not yet been studied in bloodstream *T. brucei*.

Actin localizes to the endocytic pathway of *T. brucei*, as indicated by the colocalization of the protein with endocytosed tomato lectin [98] that binds to an oligosaccharide moiety specifically found in the endosomal and lysosomal compartments of *T. brucei* [99, 100]. Ablation of actin mRNA by RNAi in bloodstream *T.brucei*, resulted in a rapid arrest of cell growth, massive enlargement of the flagellar pocket, and ultimately cell death. Furthermore, endocytosis of transferrin was reduced to less than 50% than that observed in wildtype cells in 24 h. After 48 h, endocytosis was completely blocked although cells remained intact for up to 3 days before lysing. Interestingly, actin is uniformly distributed throughout the cell in procyclic form *T. brucei* and the loss of actin by RNAi did not affect growth and was not lethal. These data suggest that in procyclic cells, clathrin-mediated endocytosis of transferrin does not involve an actin cytoskeleton [98].

Adaptins AP1, AP3 and AP4 have been identified in *T. brucei* [49, 99]. However, little is know about their function in the parasite. In mammalian cells, these adaptor proteins are not involved in the formation of endocytic vesicles. Instead, AP1 mediates vesicle traffic from the trans Golgi network (TGN) to endosomes. AP3 and AP4 function in TGN and endosome trafficking [101]. A homologue for AP2 in *T. brucei* has not been identified [99]. Thus, the endocytic adaptin is not yet known.

6 Transferrin Endocytosis in T. brucei

T. brucei requires iron for growth [93, 94]. In bloodstream *T. brucei* transferrin, which is taken up by a receptor, mediates iron uptake. The transferrin receptor (TfR) of trypanosomes is unlike any other in nature [102]. It is a heterodimeric complex consisting of ESAG6 and ESAG7

subunits. The ESAG6 subunit is glycosylphosphatidylinositol (GPI) anchored to the plasma membrane, whereas ESAG7 is a peripheral membrane protein [103-105]. Following endocytosis, the TfR-transferrin complex dissociates and transferrin is degraded in the lysosome freeing up iron for utilization by the parasite. The TfR is recycled back to the flagellar pocket with a half-life of approximately 40 min [102, 104]. In bloodstream cells, transferrin almost completely colocalizes with TbCLH suggesting that GPI-anchored proteins are endocytosed by a clathrin-dependent process [49].

7 Protein Kinases

Phosphorylation by protein kinases is a covalent modification that occurs in response to internal or external stimuli. Phosphoproteins regulate various aspects of cell function such as growth, division, metabolism, movement and apoptosis (for review [106]). Most kinases have a similar catalytic core that contains a glycine-rich N-terminal ATP-binding pocket and a conserved aspartic acid residue required for catalytic activity [107] (reviewed in [108]).

There are different classes of protein kinases. The two largest groups are classified by the amino acids they phosphorylate: serine/threonine kinases (phosphorylate serine and threonine residues) and tyrosine kinases (phosphorylate tyrosine residues). A third class of kinases phosphorylates serine, threonine and tyrosine; these are termed dual-specificity kinases. Most have a similar catalytic core that contains a glycine-rich N-terminal ATP-binding pocket and a conserved aspartic acid residue required for catalytic activity [107] (reviewed in [108]).

7.1 Protein Tyrosine Kinases (PTKs)

Protein tyrosine kinases are the largest kinase subgroup in humans with approximately 90 members [108, 109]. Common to all tyrosine kinases is a kinase domain (Fig. 1.3A) that catalyzes the transfer of the γ-phosphate of ATP to the hydroxyl group of a tyrosine of the

substrate. The kinase domains of PTKs all have a similar two-lobed structure with three main parts: an N-terminal lobe that consists primarily of β-sheets, a C-terminal lobe of α-helices and a cleft between the two lobes that binds ATP. The adenosine group of ATP forms hydrogen bonds with the hinge region that connects the two lobes with the phosphate groups directed away from the hinge (Fig. 1.3B). Protein substrates bind to a platform on the C-terminal lobe with the hydroxyl of the tyrosine to be phosphorylated directed toward the γ-phosphate of ATP. An activation loop on the C-terminal lobe makes up part of the substrate platform and, in most PTKs, is stabilized by phosphorylation of one or more tyrosine residues (Fig. 1.4A); most PTKs are regulated by tyrosine phosphorylation [110]. Dephosphorylation of the activation loop results in a conformational change that sterically hinders the binding of ATP and protein substrate, thus rendering the PTK inactive (Fig. 1.4B) [108, 111]. Although different PTKs have a similar general structure, the ATP-binding sites contain sufficient differences that enable small molecule inhibitors to specifically bind one subclass of PTKs without affecting others (for a detailed review of the ATP-binding site see [111]). These ATP-competitive molecules inhibit kinases by binding at or near the ATP binding domain (Fig. 1.3).

Tyrosine kinases can be divided into two classes: receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). Genes encoding RTKs are found only in vertebrates and choanoflagellates [112], while NRTKs are found in all eukaryotes including trypanosomatids [113].

Receptor tyrosine kinases include epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR), among others. RTKs contain three domains; an extracellular ligand-binding domain, a transmembrane domain and an intracellular catalytic kinase domain. Ligand binding induces

oligomerization of the receptor that results in autophosphorylation of the cytoplasmic domain activating tyrosine kinase activity. Phosphotyrosine (pTyr) sites on PTKs are binding sites for adaptor proteins or enzymes with SH2 (Src homology 2) or PTB (phosphotyrosine binding) domains (e.g., PI3K (phosphatidylinositol-3 kinase), PLC-γ (phospholipase C), Grb2 (growth factor receptor-bound protein 2) and IRS-1 (insulin receptor substrate-1) [114-118]. Recruitment of these proteins couples RTKs to multiple cytoplasmic signaling pathways which may reach the nucleus, culminating in the regulation of DNA synthesis and cell division, as well as effects on cell proliferation, differentiation, migration and apoptosis [119].

Non-receptor tyrosine kinases, such as the Jak, Abl and Src families, lack an extracellular ligand-binding domain and are cytoplasmic [111, 120]. Some NRTKs localize to the plasma membrane through N-terminal myristoylation and palmitoylation. In addition to a tyrosine kinase domain, NRTKs also contain domains that interact with other proteins (e.g. Src homology 2 (SH2) and SH3 domains), lipids (Pleckstrin homology domain (PH)) and/or DNA (for review [111]). The most common protein-interaction domains in NRTKs are SH2 and SH3 domains.

The SH2 domain binds phosphorylated tyrosine residues by recognizing between 3 to 6 residues C-terminal to the phosphorylated tyrosine. However, being the largest class of phosphorylated tyrosine-recognition domains, SH2 domains are not identical [121, 122]. For example, the SH2 domain of Grb2 preferentially binds phosphorylated YXN sequences [123] whereas the SH2 domain of Src binds YEEI [124]. An SH3 domain associates with proline-rich (PXXP, where X is any amino acid, [125]) motifs in the ligand [126]. There is no evidence that SH3 domains bind phosphorylated tyrosine. Some NRTKs such as Jak lack SH2 or SH3 domains but contain unique protein-interaction domains (Jak homology) (reviewed in [127]) or other domains such as focal adhesion and integrin binding domains (reviewed in [128]. Non-

receptor PTKs are activated (via tyrosine phosphorylation, dephosphorylation or conformational change) by upstream signaling factors such as: G protein-coupled receptors, and RTKs (see [111] for review).

Dual-specificity kinases (DSK) are a small class of protein kinases that phosphorylate serine and/or threonine and also tyrosine residues. Examples of DSKs are DYRKs (dual-specificity tyrosine-phosphorylated and regulated kinase, [129]), CLKs (*cdc2*-like kinase, [130]) and STE7 ([131].

7.2 Tyrosine Kinases as Targets for Drug Discovery in Oncology

Tyrosine kinase activity is tightly regulated in normal cells. Failure to control these kinases (due to mutations, over-expression, deletions, viral infection and/or failure in down-regulation (reviewed in [132]) may result in constitutive activation that might lead to malignancy. Some PTKs are associated with proliferation of solid tumors [133].

The importance of tyrosine kinases in cancer has made them the focus of an enormous effort to discover new drugs against the disease. Most of the drug discovery programs are focused on small molecules that bind to the ATP-binding region (see above) and are commonly referred to as ATP mimics. Of the ATP mimics, most are based on the following chemical platforms; anilinoquinazolines, anilinoquinolines and anilino-pyridopyrimidines.

EGFR activation is associated with the most common human solid tumors [134]. EGFR has therefore become a major target for drugs. The 4-anilinoquinazoline AG1478 (Fig. 1.5A) is a competitive inhibitor of the ATP binding site and a very potent inhibitor of EGFR kinase [135-137]. However, poor solubility in aqueous solution with physiological salt concentrations led to its failure as a drug [136]. Subsequently, analogs of AG1478 with improved solubility such as

Gefitinib (ZD 1839, Iressa®) and Erlotinib (OSI-774, Tarceva®) (Fig. 1.5A) were developed as drugs [138, 139].

Over time, cancer patients who initially respond well to tyrosine kinase inhibitors may develop resistance to the drugs. Resistance can be caused by mutations of the protein kinases, amplification of the oncogenic protein kinase gene or other mechanisms [140]. In these instances, patients will need to be treated with different drugs. In support of these ideas, resistance mutations have been reported in the kinase domains of BCR-ABL, Kit and PDGFR in tumor cells of patients treated with Imatinib [141]. Likewise, patients treated with Gefitinib or Erlotinib have acquired EGFR mutations that render them resistant to these drugs [142]. These and other data have led to development of inhibitors that target multiple protein tyrosine kinases (PTKs). Several of these inhibitors have been developed and shown to be effective against PTKs that have acquired resistance [140]. AEE788 (Fig. 1.5B) is pyrrolopyrimidine that concurrently inhibits EGFR and VEGFR kinases [143-146].

7.3 Evidence of PTKs in Trypanosoma brucei

Tyrosine kinase activity is present in *T. brucei*. *T. brucei* cell lysates phosphorylate poly($Glu^{80}Tyr^{20}$) [147] a synthetic polymeric substrate for some tyrosine kinases. Furthermore, tyrosine phosphorylation was reduced 7-fold by genistein, which is a tyrosine kinase inhibitor. In the same study, genistein inhibited growth of *T. brucei*. Interferon-γ (IFN-γ) is a cytokine that activates the Jak1 and Jak2, which phosphorylate substrates that regulate transcription [148-150]. IFN-γ is a growth-stimulating factor for *T. brucei* [151, 152] and increases tyrosine (Tyr) phosphorylation of proteins in the parasite. The tyrosine kinase inhibitor tyrphostin A47 reduced Tyr phosphorylation in the parasite [153, 154].

Tyrosine phosphorylated proteins have been identified in *T. brucei*. Western blot analysis using anti-phosphotyrosine (pTyr) antibodies led to the discovery of Nopp44/46 [155]. In another study, western analysis detected a doublet with masses of 200 and 220 kDa; immunoelectron microscopy localized the proteins to the glycosome [156]. Most recently, in procyclic form *T. brucei*, 34 Tyr-phosphrylated proteins were identified, 19 of which were protein kinases (including kinases from the dual-specificity CLK (*cdc2*-like kinase) and DYRK (dual-specificity tyrosine-phosphorylated and regulated kinase) kinase families), by pTyr-affinity chromatography and LC-MS/MS [157].

Finally, tyrphostin A47 blocks endocytosis of transferrin in *T. brucei*. This data suggests that endocytosis of transferrin may involve a tyrosine kinase (*S. Subramanya, PhD Thesis, University of Georgia*). Furthermore, the EGFR inhibitor tyrphostin AG1478 kills *T. brucei* (*K. Mensa-Wilmot, unpublished*). Collectively, these data imply that (i) tyrosine kinases exist in *T. brucei*, and (ii) PTKs are important for viability of the trypanosome.

7.4 Genomic Analysis of the T. brucei Kinome

Bioinformatic searches of the *T. brucei* genome for eukaryotic protein kinases (ePK) and atypical kinases (aPK) identified 176 protein kinases (PKs) (reviewed in [113]). Using this approach, no tyrosine kinases (TKs) were identified in *T. brucei*. However, several kinases of the dual-specificity kinase families (STE7, CLK and DYRKs) are present in the trypanosome genome [113]. No tyrosine kinase has been purified from *T. brucei* and characterized biochemically to confirm or dispute these bioinformatics predictions.

8 Glycosyl phosphatidylinositol (GPI)

Glycosyl phosphatidylinositols (GPIs) contain a conserved glycolipid Manα1-4GlcNα1-6*myo*-phosphatidylinositol (PI). There are three types of GPIs in *T. brucei*: (i) protein-linked

GPIs (ii) polysaccharide-linked GPIs and (iii), unlinked GPIs (free GPIs). In *T. brucei*, GPIs are very abundant; bloodstream form parasites have approximately 10^7 molecules of GPI-anchored VSG on their plasma membrane [158].

GPI biosynthesis begins on the cytoplasmic side of the endoplasmic reticulum and is completed in the lumen of the organelle [159]. It is a process in which monosaccharides and ethanolamine are sequentially added to phosphatidylinositol (PI) to generate a GPI precursor to which a protein can be added. The addition of VSG to the GPI precursor is thought to occur in the lumen of the ER [160] (for more detail see Fig. 1.6).

9 GPI-specific Phospholipase C (GPI-PLCp)

Glycosyl phosphatidylinositol-specific phospholipase C (GPI-PLCp) is a developmentally regulated enzyme expressed in bloodstream *T. brucei* [161-164]. GPI-PLCp is an integral membrane protein [161-164], however the intracellular localization is controversial. By immuno-electron microscopy, GPI-PLCp was detected on the membrane of uncharacterized "intracellular vesicles" [165]. GPI-PLCp is not essential in the parasite for growth and differentiation, however the enzyme influences parasitemia in mice and is considered a virulence factor [166, 167].

Three functions of GPI-PLCp have recently been discovered in two stages of development. First, GPI-PLCp plays a minor role in the release of VSG during differentiation [165, 168]. However, this is most likely an indirect function of the enzyme because significant amounts of the protein are not detected on the plasma membrane [169]. Furthermore, during differentiation VSG is released from GPI-PLCp null cells [170]. Second, GPI-PLCp stimulates endocytosis of transferrin in bloodstream *T. brucei* [171]. This observation may explain, in part, why *GPI-PLC* null mutants have reduced parasitemia in a mouse model of HAT [166]. Because

iron is essential for growth of the parasite [94, 172] and is acquired by the endocytosis of transferrin [173], parasites that lack GPI-PLCp may not acquire iron (or other nutrients) as quickly as wild type cells (in competition with host cells) which may reduce the growth rate and reduce parasitemia. Finally, GPI-PLCp contributes to the cleavage of GPIs in *T. brucei* treated with mild alkaline or hypo-osmotic buffer [169]. Because parts of the tsetse fly digestive tract are mildly alkaline [174], the cleavage of GPIs may be a valuable process in the insect. The cleavage of GPI by GPI-PLCp produces diacylglycerol and inositol glycans both of which are second messengers in mammalian cells (reviewed in [175, 176]). Therefore, GPI-PLCp may also be involved in a stress-induced signaling mechanism in *T. brucei* [169].

There are three potential substrates of GPI-PLCp *in vivo:* (i) GPI-anchored proteins (e.g. VSG), (ii) phosphatidylinositol and (iii) GPI-intermediates (Fig. 1.6). VSG is anchored to the exoplasmic leaflet of the plasma membrane: GPI-intermediates are synthesized on the cytoplasmic side of the endoplasmic reticulum [159, 177]. In unstressed, non-differentiating trypanosomes, there is no evidence that VSG or free GPIs are cleaved [178]. The mechanisms by which GPI-PLCp is prevented from digesting intracellular GPIs are only recently emerging. Preliminary studies suggest that GPI-PLCp may be glycosomal [169]. If verified, it is possible to hypothesize that sequestration of GPI-PLCp to the glycosome prevents it from cleaving its substrates at the ER and plasma membrane. In addition, biochemical activity of GPI-PLCp may be regulated in *T. brucei*. First, *in vitro* studies indicate that tetramers of GPI-PLCp are 4-fold more active than the monomer: *in vivo* GPI-PLCp is monomeric [179]; therefore its activity may be suppressed. Second, GPI-PLCp is reversibly myristoylated *in vivo* [180]. *In vitro* studies indicate that demyristoylation decreases enzyme activity up to 30-fold, indicating that

myristoylation may also regulate the activity of GPI-PLCp *in vivo* [181]. Together, these data may explain why little or no GPI-PLCp substrate is cleaved *in vivo*.

Figure 1.1: ***Trypanosoma brucei*** **Life Cycle** (from [182]). During a blood meal, an infected tsetse fly transmits metacyclic trypanosomes (covered with VSG) to a mammalian host. After infection, the parasites differentiate into long slender blood stream forms (BSF). Long slender BSF differentiate into the short-stumpy, non-dividing forms. Feeding on an infected host, a tsetse fly ingests the short stumpies that differentiate into procyclic form (PCF) (covered with PARP, procyclic acidic repetitve protein), replicate, and migrate to the fly midgut. Here PCF differentiate into epimastigotes, replicate and migrate to the fly salivary glands. In the salivary glands, the epimastigotes differentiate into the infective, non-dividing metacyclics, pre-adapted for survival in the mammalian host. The cycle is complete when metacyclics are reintroduced to the mammalian host.

Figure 1.1

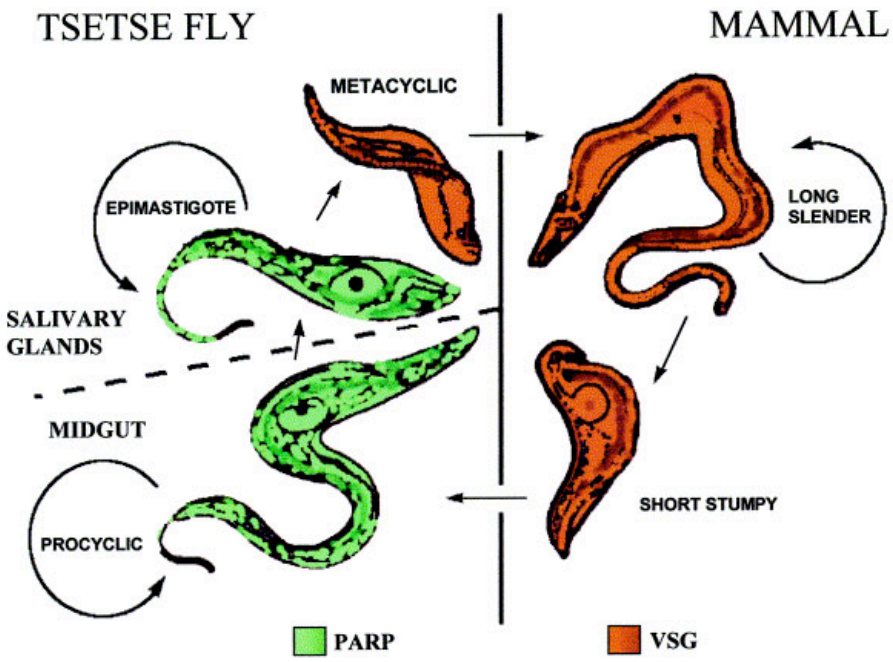

TSETSE FLY
MAMMAL
METACYCLIC
EPIMASTIGOTE
LONG SLENDER
SALIVARY GLANDS
MIDGUT
SHORT STUMPY
PROCYCLIC
PARP
VSG

Figure 1.2: Overview of Peroxisomal Protein Targeting Import (adapted from [25]). **(A)** Matrix proteins containing either a peroxisomal targeting sequence (PTS)1 or PTS2 associate with a cytoplasmic receptor (with or without a co-receptor) PEX5 or PEX7 which target the proteins to the docking complex (PEX13, PEX14 and PEX17 in yeast) in the peroxisome membrane (1). The receptor/cargo complex translocates through the peroxisome membrane with help of a RING-finger complex (PEX2, PEX10 and PEX12) that is associated with the docking complex via PEX8 (2). In the lumen, the receptor and cargo dissociate. PEX5 and PEX7 recycle to the cytoplasm (3) to repeat the process (4). PEX5 must be mono-ubiquitinated by PEX4 before being released from the membrane. PEX4 (an E2 conjugating enzyme) associates with PEX10 and is anchored to the membrane by PEX22. PEX1 and PEX6 are AAA-proteins also involved in receptor release from the peroxisome membrane; they are anchored to the membrane by PEX15 in yeast (and PEX26 in mammals). Little is known about PEX7 recycling [25]. **(B)** Membrane proteins bearing an mPTS associate with PEX19 in the cytoplasm and are then targeted to the peroxisome membrane where PEX19 and its PMP cargo dock with PEX3 and PEX16 (1). Following the association with PEX3 and PEX16, the PMP is inserted into the membrane by mechanisms that are not yet characterized (2) (reviewed in [32]).

Figure 1.2

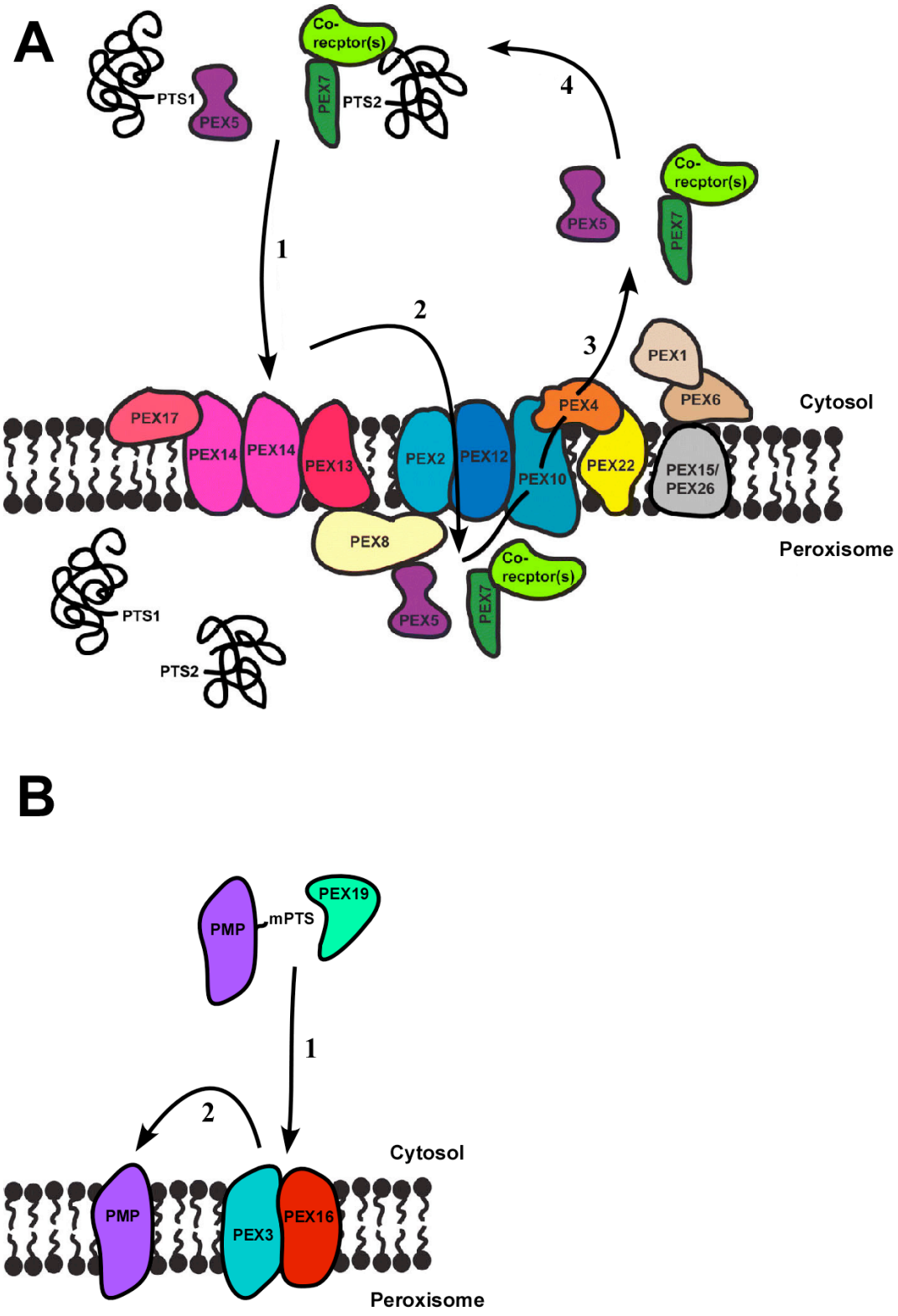

A
Co-recptor(s)
PEX7
PTS2
PTS1
PEX5
4
1
2
3
PEX1
PEX6
Cytosol
PEX17
PEX14
PEX14
PEX13
PEX2
PEX12
PEX4
PEX10
PEX22
PEX15/
PEX26
PEX8
Peroxisome
B
PEX19
PMP
mPTS
1
2
Cytosol
PMP
PEX3
PEX16
Peroxisome

Figure 1.3: Three-Dimensional Structure of the Tyrosine Kinase Domain of the EGFR in Complex with AEE788. **(A)** (adapted from [111]) α-Helices and β-sheets are shown in *red* and *blue,* respectively. The tyrosine kinase inhibitor AEE788 is shown in *yellow* and the hinge region connecting the N-and C-terminal lobes is shown in *blue*. The activation or Mg^{2+}-binding loop (*green*) contains a conserved amino-terminal aspartate, phenylalanine, glycine (DFG) motif that coordinates a magnesium ion, which positions the phosphates of ATP for transfer. The P-loop (*purple*) or nucleotide-binding loop also plays a role in coordinating the phosphate groups of ATP. The catalytic loop (*orange*) contains conserved aspartate and asparagine residues. The aspartate coordinates the hydroxyl group of the substrate tyrosine residues and may catalyze nucleophilic attack of the γ-phosphate of ATP by abstracting the hydrogen atom from tyrosine [108, 183] (for a review see [111]). **(B)** (adapted from [184]) A portion of the N-terminal lobe (top lobe) β-sheets (blue) was removed for viewing purposes. The pyrrolopyrimidine nucleus of AEE788 forms hydrogen bonds (*black dotted lines*) with residues in the hinge region (*light blue*). The N3 atom associates with the hydroxyl group of Thr854 via a water molecule. The 4-phenylethylamine moiety extends into a hydrophobic pocket consisting residues Thr790, Leu788, Lys745 and Met766. The 6-phenyl substituent is arranged between Leu718 above and Gly796 below (*not shown*). Finally, the ethylpiperazine group extends toward solvent near Asp800 and Glu804 [184]. The protein database (PDB) file 2J6M was rendered with PyMOL [185].

Figure 1.3

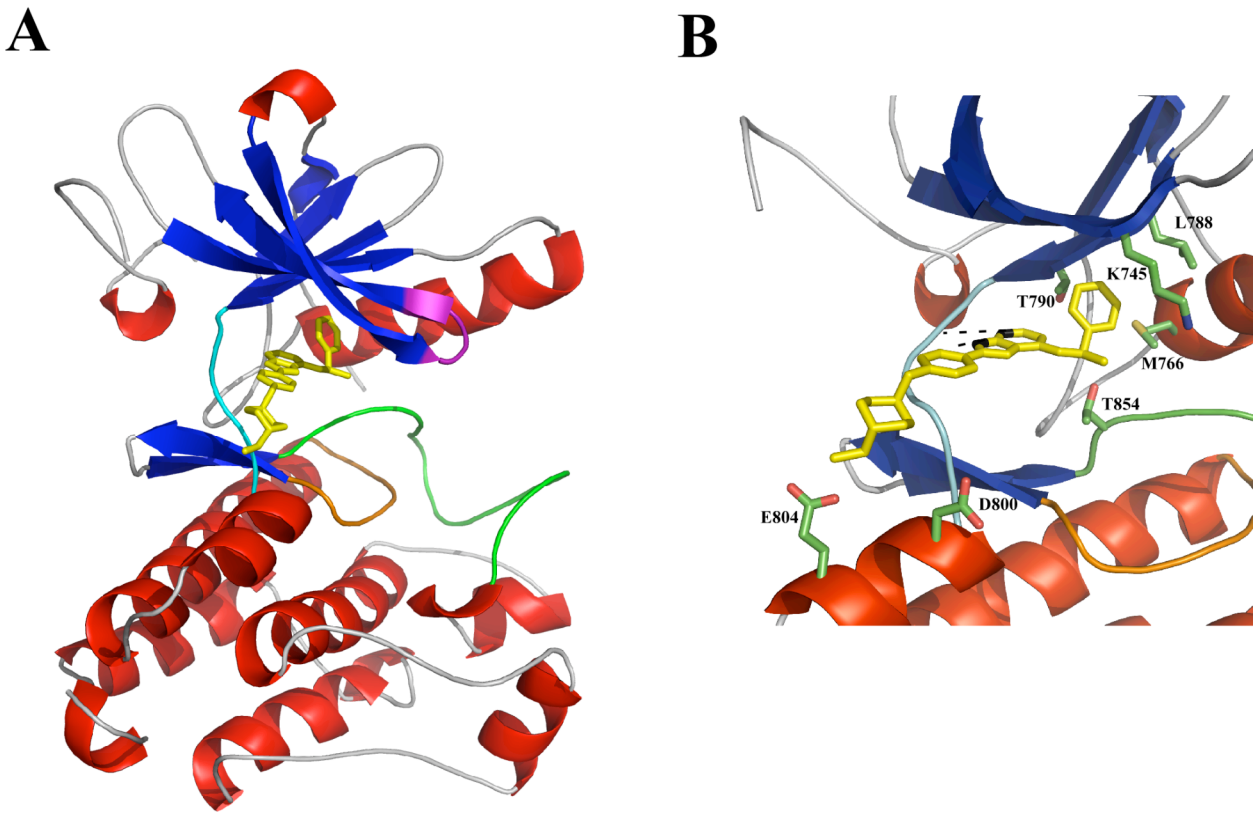


Figure 1.4: Activation Loop Tyrosine Phosphorylation Regulates Most Protein Tyrosine Kinases (modified from [186]). The activation loop (green) adopts distinct conformations associated with inhibited and activated states of the kinase domain. When inactive, the activation loop folds into the active site of the kinase, sterically inhibiting the binding of ATP and substrate. Activation of the kinase by phosphorylation of tyrosine residues within the activation loop induces a conformational change in which the activation loops adopts an extended conformation. This extended conformation allows binding of ATP and serves as a platform for incoming substrate. **(A)** The active conformation of the tyrosine phosphorylated kinase domain of the Src-family kinase Lck (PDB entry 3LCK). **(B)** The inactive conformation of the unphosphorylated c-Src kinase domain (Protein Database (PDB) entry 2SRC). The P-loop or nucleotide-binding loop is shown in *purple*; the catalytic loop in *orange*. The PDB files were rendered with PyMOL [185].

Figure 1.4

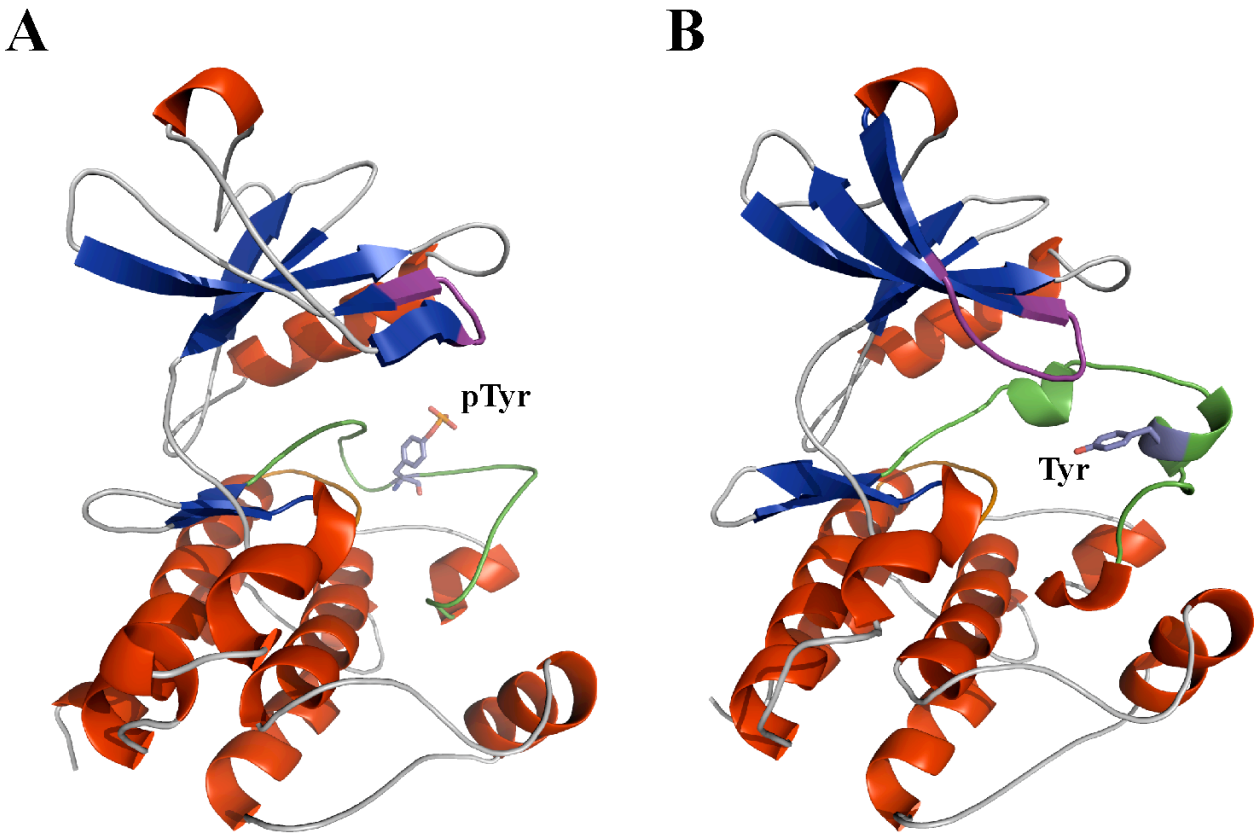
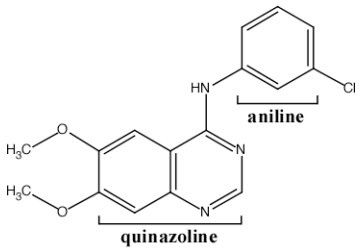


Figure 1.5: **Structures of Some EGFR inhibitors**. Structures represent two of the major chemical scaffolds found in potent tyrosine kinase inhibitors. **(A)** The 4-anilinoquinazolines, AG1478 (N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine), Erlotinib (*N*-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine) and Canertinib (*N*-[-4-[(3-chloro-4-fluorophenyl)amino]-7-[3-(4-morpholinyl)propoxy]-6-quinazolinyl]-2-propenamide dihydrochloride). **(B)** The pyrrolopyrimidine, AEE788 (6-[4-[(4-ethylpiperazin-1-yl)methyl]phenyl]-N-(1-phenylethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine).

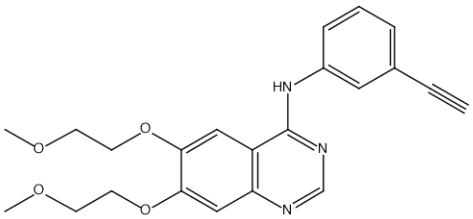
Figure 1.5

A

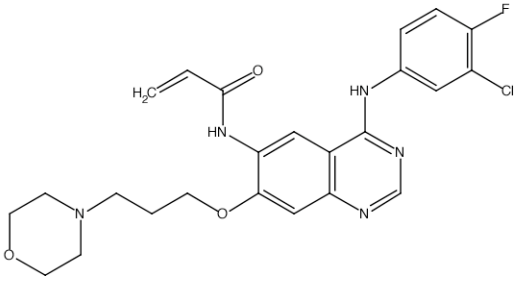
AG1478



Erlotinib



Canertinib



B

AEE788

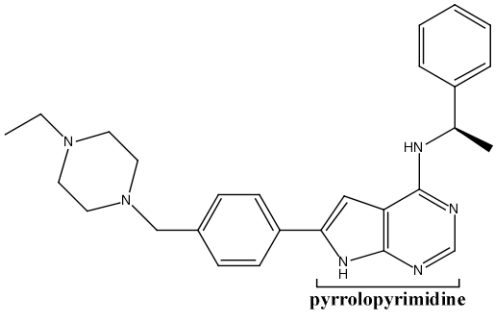
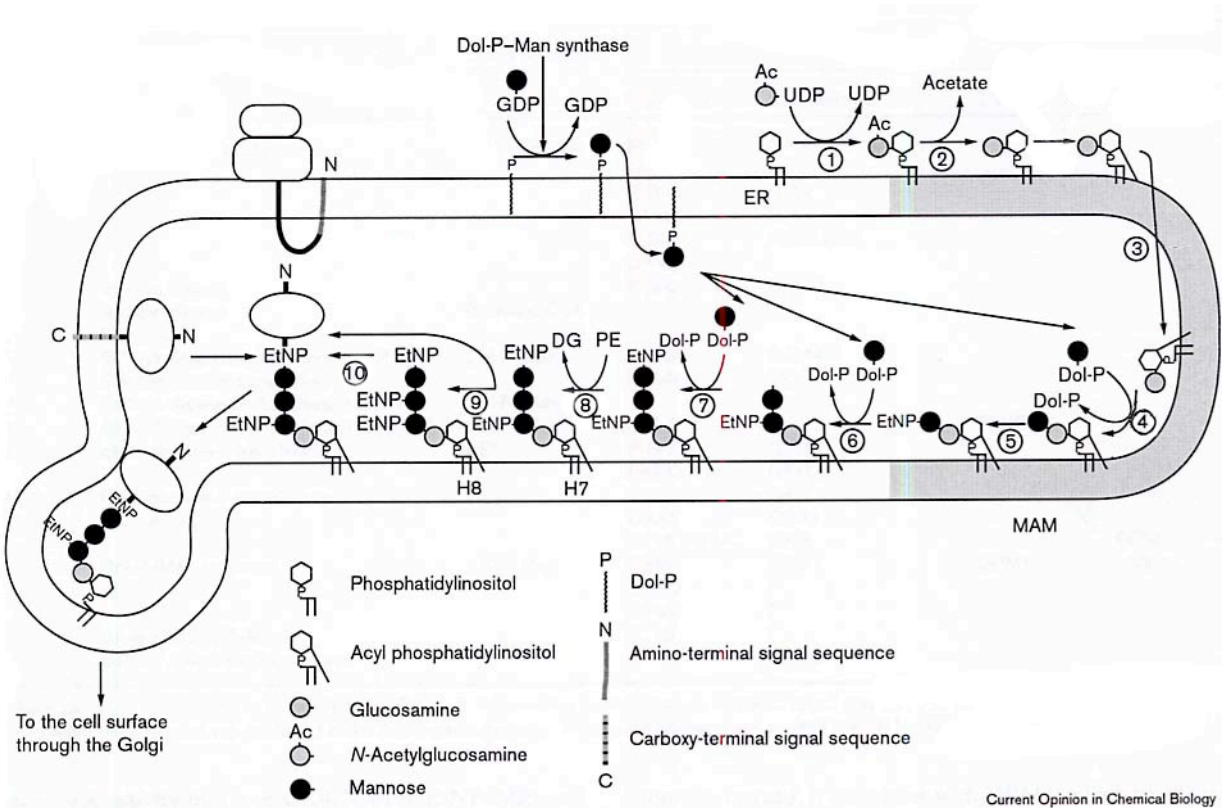


Figure 1.6: Biosynthesis of GPIs (modified from [187]). Biosynthesis of GPIs begins on the cytoplasmic side of the endoplasmic reticulum (ER). It starts with the addition of GlcNAc to phosphatidylinositol to form GlcNAc-PtdIns (1). GlcNAc-PtdIns is deacetylated (2) to GlcN-PtdIns that flips into the ER lumen (3). Three mannose residues from dolichol-phosphoryl-mannose (Dol-P-Man) [188] are then added (4-7) followed by the transfer of a ethanolaminephosphate (EtNP) from phosphatidylethanolamine (PE) (8) to form glycolipid A' (EtN-P-Man3-GlcN-Ins-P-diacylglycerol) [189]. In *T. brucei* the fatty acids on glycolipid A' are replaced by myristate to yield glycolipid A, the VSGp GPI anchor precursor. Once synthesized, the transfer of glycolipid A to VSG polypeptide is thought to occur in the lumen of the ER [160]. GPI-anchored VSG is targeted to the exoplasmic leaflet of the plasma membrane.

Figure 1.6


Dol-P–Man synthase
GDP
GDP
P
P
Ac
UDP
UDP
Ac
Acetate
ER
N
C
N
EtNP
EtNP
EtNP
DG
PE
EtNP
Dol-P
Dol-P
Dol-P
Dol-P
Dol-P
Dol-P
EtNP
EtNP
EtNP
EtNP
EtNP
EtNP
EtNP
EtNP
H8
H7
MAM
To the cell surface
through the Golgi
Phosphatidylinositol
Acyl phosphatidylinositol
Glucosamine
Ac
N-Acetylglucosamine
Mannose
P
Dol-P
N
Amino-terminal signal sequence
Carboxy-terminal signal sequence
C
Current Opinion in Chemical Biology

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CHAPTER 2

GPI-PHOSPHOLIPASE C OF *TRYPANOSOMA BRUCEI* IS A GLYCOSOME PROTEIN[1]

[1] Hardin, C.F., Mensa-Wilmot, K. To be submitted to *FEBS Journal*.

ABSTRACT

Bloodstream *Trypanosoma brucei* express a glycosyl phosphatidylinositol-specific phospholipase C (GPI-PLCp). This enzyme can cleave the GPI-anchor of variant surface glycoprotein (VSG), a surface molecule that is essential for the parasite's survival in the mammalian host. Interestingly, very little VSG is cleaved in non-differentiating cells under physiological conditions. The enzyme is a virulence factor that stimulates endocytosis of transferrin in bloodstream *T. brucei*. GPI-PLCp also contributes to stress-induced cleavage of intracellular GPIs. However, despite these interesting developments about the biological function of GPI-PLCp, the intracellular location of GPI-PLCp has not been completely characterized. Better knowledge of the intracellular location of GPI-PLCp is important for understanding the mechanism of the different functions attributed to the protein. Several attempts to elucidate the location of the enzyme have resulted in the partial localization of GPI-PLCp to the cytoplasmic leaflet of intracellular organelles. Herein we demonstrate using a combination of iodixanol-gradient centrifugation and immunofluorescence microscopy that GPI-PLCp associates with glycosomes.

INTRODUCTION

Trypanosoma brucei causes human African trypanosomiasis (HAT). In a mammalian host, the plasma membrane of the parasite is covered with approximately 10^7 molecules of a glycosyl phosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG) [1-3]. This coat is directly responsible for the parasite population's ability, through "antigenic variation," to sustain an infection despite the host's immune response [4]. *T. brucei* express GPI-specific phospholipase C (GPI-PLCp) that can solubilize VSG by cleaving the GPI-anchor [5-8]. Lysis of *T. brucei* induces release of VSG by GPI-PLCp [2, 5, 9]. However, in non-differentiating bloodstream parasites, there little or no release of VSG [10, 11].

GPI-PLCp is a membrane protein [5, 6, 8, 9, 12]. However, the membrane on which the enzyme resides has not been completely defined. GPI-PLCp influences parasitemia in mice and is considered a virulence factor [13, 14]. The biological functions of the enzyme are only now coming to light. GPI-PLCp stimulates endocytosis of transferrin in bloodstream *T. brucei,* indirectly contributing to iron uptake [15]. In stressed *T. brucei* (hypo-osmotic or mild alkaline conditions), GPI-PLCp cleaves intracellular GPIs [16]. In differentiating bloodstream *T. brucei*, GPI-PLCp contributes to the release of VSG [17, 18], although the physiological importance of this observation has been questioned [17]. How the enzyme participates in these processes is not entirely known. To further understand the mechanisms underlying the enzyme's functions in the parasite, it is important to determine the intracellular location of the protein.

Several techniques have been used to determine the location of GPI-PLCp in *T. brucei*. First, using differential sucrose and isopycnic Percoll centrifugation, GPI-PLCp was localized to the flagellar pocket [19]. However, some activity was observed to be associated with the Golgi. Second, using isopycnic sucrose-gradient centrifugation, GPI-PLCp appeared to co-sediment

with lysosomes, mitochondria, glycosomes and the flagellar pocket [7]. In the same study, immuno-electron microscopy studies suggested that GPI-PLCp might associate with the cytoplasmic side of intracellular vesicles instead of the lumen of the organelles [7]. Finally, in differentiating cells (short stumpies), biotinylation assays indicate that some GPI-PLCp can associate with the extracellular side of the plasma membrane [17, 20]. In summary, the field could benefit from any efforts to resolve the intracellular location of the enzyme. Herein, we use two approaches, density-gradient centrifugation and fluorescence microscopy to demonstrate that the bulk of GPI-PLCp is glycosomal in bloodstream *T. brucei*.

MATERIALS AND METHODS

Materials

Immobilon P membrane was purchased from Millipore (Bedford, MA). DE52 was from Whatman (Hillsboro, OR). 5-bromo-4-chloro-indolyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT) were from BioRad (Melville, NY). Goat anti-mouse and goat anti-rabbit IgG-alkaline phosphatase conjugate and goat anti-mouse IgG-conjugated Alexa Fluor-594 and goat anti-rabbit Alexa Fluor-488 were purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Complete, mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). Amicon ultra-15 centrifugal filter units and Beckman Coulter quick-seal ultra-clear 39 ml centrifuge tubes were from Fisher Scientific (Norcross, GA). OptiPrep density gradient medium was purchased from Grenier-Bio-One, Inc. (Longwood, FL). Serum Plus was from JRH Biosciences (Lenexa, KS). VectaShield mounting medium was purchased from Vector Laboratories (Burlingame, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Anti-glycosome antibody was a gift from Dr. Marylin Parsons (Seattle Biomedical Research Institute, Seattle, WA). Antibodies against trypanopain were a gift from Dr. James Bangs (University of Wisconsin, Madison, WI). Antibody against hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was a gift from Dr. Buddy Ullman (Oregon Health and Science University).

Cell Types / Strains

Monomorphic bloodstream *T. brucei* Lister 427 (MITat 1.5) was a gift from Dr. George Cross (Rockefeller University, New York, NY). Parasites were grown in rodents and harvested

by chromatography on DE52 [3]. The purified parasites were then used for density-gradient centrifugation. Culture-adapted bloodstream *T. brucei* 427 was a gift from C.C. Wang (University of California, San Francisco, CA, USA). *T. brucei* 427 (CA427) was cultured in HMI-9 medium [21] (supplemented with 10% fetal bovine serum and 10% Serum Plus) at 37 °C with 5% CO_2.

Cell Lysis and Density-Gradient Centrifugation

The following procedure was modified from Colasante et al, 2006 [22]. Bloodstream *T. brucei* 427 (10^{10}) were harvested from rats by cardiac puncture and purified by DE52 chromatography [3]. Cells were resuspended in 10 ml bicine buffered saline (BBS) plus glucose (10 mg/ml). The cell resuspension was pelleted (2000 x g, 4 °C, 10 min), washed in 50 ml of TEDS (25 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 250 mM sucrose) and then pelleted (2000 x g, 4 °C, 10 min) again. The pellet was resuspended in 5 ml TEDS with 15% glycerol and frozen in a dry ice/ethanol bath, stored at -80 °C for 30 min, and thawed in a 37 °C water bath with constant motion to prevent localized over-heating. After thawing, the cells were centrifuged (2000 x g, 4 °C, 10 min) and the pellet was resuspended in 2 ml homogenization buffer (HB) (5 mM MOPS, pH 7.2, 250 mM sucrose, 1 mM EDTA, 0.1% v/v ethanol, supplemented with 'complete EDTA-free' protease-inhibitor cocktail). The cells were added to two volumes of wet-weight (~ 10 g) silicon carbide (< 400 mesh) in a pre-chilled mortar, and ground three times for 10 s with 10 s rests in between grinding (to prevent over-heating). The cells were observed by light microscopy (40X objective) to confirm at least 90% lysis. The pestle was rinsed with 10 ml HB allowing the rinse to collect in the mortar. The slurry was mixed and added to a 50 ml centrifuge tube. The mortar was then rinsed with 10 ml HB to remove the remaining lysate and added to the centrifuge tube (from above). The mixture was

centrifuged (100 x g, 4 °C, 15 min) to pellet the silicon carbide. The supernatant was then centrifuged (1000 x g, 4 °C, 15 min) and the supernatant (~ 25 ml) was added to an Ultra-15 Centricon device (Amicon®) that was centrifuged (5000 x g, 4 °C, 15 min) to concentrate the solution. This last step was repeated until the supernatant was concentrated to 3 mls. Three ml, cleared lysate was layered onto a 36 ml iodixanol (OptiPrep) gradient (5-15%).

The OptiPrep gradient was prepared according to OptiPrep application sheet S9 (http://www.axis-shield-density-gradient-media.com/organelleindexes.htm) as follows: the OptiPrep medium was diluted by adding five volumes of OptiPrep to one volume diluent (30 mM MOPS, pH 7.2, 0.25 M sucrose, 6 mM EDTA, 0.6% ethanol) for a 50% OptiPrep working mixture. The working mixture was diluted in homogenization medium (HM) (5 mM MOPS, pH 7.2, 0.25 M sucrose, 1 mM EDTA, 0.1% ethanol) to produce solutions with different percentages (5, 7.5, 10, 12.5, 15 and 30%) of OptiPrep. Using a 10 ml syringe, a 20G1 (20 gauge, 25 mm) needle and plastic tubing connected to the needle, 3.5 ml of 30% OptiPrep was added first to a 39 ml quick-seal, ultra-clear centrifuge tube followed by 6.5 ml of each of the OptiPrep mixtures mentioned above (15, 12.5, 10, 7.5, and 5%, in this order). After pouring the gradient, the top was covered with parafilm and placed at 4°C for 12 h (for gradient equilibration).

The gradient containing the lysate was centrifuged (170,000 x g, 4 °C, 1 h) in a Beckman VTi-50 rotor (acceleration 9, deceleration 9). After centrifugation the gradient was mounted by ring stand and clamp. The top of the tube was pierced with a 20G1 syringe needle. The bottom of the tube was carefully pierced with a 25G7/8 (25 gauge, 177 mm) syringe needle, and 1 ml fractions (40 total) were collected by gravity. Fractions obtained were kept at 4 °C until use.

Proteins were precipitated with trichloroacetic acid (TCA) as follows: To 100 µl of each (gradient) fraction, 27 µl of sodium deoxycholate (0.01% w/v) was added, followed by 53 µl of

deionized water for a final concentration of 0.0015% sodium deoxycholate [23]. The mixture was incubated on ice for 30 min followed by addition of 20 µl TCA (100% w/v, 6.1 N) to yield a final concentration of 10% TCA. The mixture was vortexed, incubated on ice for 1 h and centrifuged (14,000 x g, 4 °C, 10 min). The pellet was gently washed with 500 µl ice-cold acetone and air dried for 10 min. Three microliters of 1 M Tris (pH 8.0) was added to each pellet to neutralize the residual acid, and 12 µl of 3X SDS sample buffer was added. Samples were heated for 5 min at 100 °C, proteins separated by SDS-PAGE (14%) [24], and transferred to Immobilon P membrane with a Trans-Blot semi-dry cell (BioRad) [25].

Immunoblotting

The Immobilon P membrane was blocked with PBS (pH 7.5) containing 5% (w/v) Carnation powdered milk, 1% (v/v) Tween 20 for 1 h at room temperature. The membranes were washed twice with PBS followed by incubation for 1 h with the following antibodies diluted in blocking buffer: anti-GPI-PLC RC300 (1:1000 dilution) [26]; anti-HGPRT (glycosomal) antibody (1:1000 dilution) [27] or anti-trypanopain (lysosomal) antibody (1:5000 dilution) [28]. The membranes were washed three times with PBS followed by 1 h incubation with relevant goat anti-mouse or goat anti-rabbit IgG-alkaline phosphatase (1:1000 in blocking buffer). The membranes were then washed three times with PBS followed by alkaline phosphatase reaction buffer (APRB) (75 mM Tris pH 9.5, 75 mM NaCl, 3.75 mM MgCl). The proteins were detected by color development with 5-bromo-4-chloro-indolyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT). Trypanopain and HGPRT were detected on the same membrane (concurrently) and GPI-PLCp was detected on a different membrane. Odd fraction numbers (of the 40 collected) were analyzed on three membranes: (i) Fractions 1-19, (ii) fractions 21-39, and (iii) fractions 11-29. Because fractions 1-39 were analyzed on two

membranes (i and ii) (Fig. 2.2B), the signals detected from blotting may not accurately represent the amount of protein in each fraction. To correct for this situation, fractions 11-29 (iii) were used as a reference and Adobe Photoshop was used to digitally adjust the signals of the fractions on first two membranes (i and ii) (Fig. 2.2B) to match the signals from the third membrane (iii) (Fig. 2.2A).

Fluorescence Microscopy

Late log phase cells (grown to no more than 10^6 cells/ml) were harvested ($2x10^6$ cells/ml), washed with phosphate buffered saline (PBS, pH 7.4) and fixed on ice in 400 µl of either PBS containing 0.1% formaldehyde for 5 min (for detection of trypanopain [29, 30]) or PBS containing 4% formaldehyde for 1 h (for detection of glycosomes [31]). The fixative was quenched with glycine (1 ml; 0.5 M in PBS). The cells were washed once with PBS and $2x10^6$ cells (in 200 µl) were settled on poly(L)-lysine coated coverslips for 45 min, and permeabilized with methanol (at -20 °C) for 3 min at -20 °C (anti-trypanopain) or with PBS containing 0.2% Triton X-100 for 25 min at room temperature (anti-HGPRT). Cells were washed twice with PBS and blocked with PBS containing 5% normal goat serum for 30 min at room temperature. Cells were double-labeled to detect either (i) GPI-PLCp and glycosomes or (ii) GPI-PLCp and lysosomes, as follows: To detect GPI-PLCp, monoclonal antibody 2A6.6 [32] (150 µl of a 1:200 dilution in blocking solution) was used. Glycosomes were detected using rabbit anti-glycosome antiserum [33] (150 µl of a 1:400 dilution in blocking solution). Lysosomes were detected using rabbit anti-trypanopain antibody [29] (150 µl of a 1:1600 dilution in blocking solution). Permeabilized cells were incubated with primary antibody for 1h at room temperature and washed as follows: once with PBS for 5 min, twice with high salt buffer (PBS containing 500 mM NaCl) for 2 min, and twice with PBS for 5 min. The cells were then incubated with Alexa

Fluor-594 goat anti-mouse IgG and Alexa Fluor-488 goat anti-rabbit IgG (150 µl of a 1:1000 dilution in PBS) for 1 h in the dark. Coverslips were rinsed twice with PBS before mounting on slides with 4 µl of VectaShield mounting medium containing DAPI (10 µM) (4′,6-diamidino-2-phenylindole). The DIC (differential interference contrast) and fluorescent images were captured with an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnap$_{HQ}$ CCD (charge-coupled device) camera driven by DeltaVision software (Applied Precision, Seattle, WA).

RESULTS

GPI-PLCp Localizes to Glycosomes by Immunofluorescence Analysis

In our first approach to determine the intracellular location of GPI-PLCp we used fluorescence microscopy. Permeabilized cells were double-labeled with antibodies to either (i) GPI-PLCp [32] and glycosomes with hypoxanthine guanine phosphoribosyl transferase (HGPRT) as a glycosome marker [31], or (ii) GPI-PLCp and lysosomes with trypanopain as a lysosome marker protein [29]. In these experiments, the GPI-PLCp distribution overlapped extensively with glycosomes (Fig. 2.1, *top panels*). There was no detectable overlap between GPI-PLCp and lysosomes (Fig. 2.1, *bottom panels*). From these data we conclude that the bulk of GPI-PLCp associates with glycosomes in bloodstream *T. brucei*.

GPI-PLCp Sediments with Glycosomes and Lysosomes on an Iodixanol Gradient

In a second approach, to complement the immunofluorescence studies, we used an iodixanol (OptiPrep) gradient to separate the glycosomes from other organelles. Iodixanol has been used to produce highly enriched peroxisome fractions from cell lysates [34]. Furthermore, iodixanol gradients are able to separate glycosomes from other organelles in *T. brucei* [22]. In our studies, we used 5-15% linear iodixanol-gradient to separate organelles after lysis of *T. brucei* with silicon carbide. Odd numbered fractions 1-39 were analyzed on two separate membranes containing fractions 1-21 on a first and fractions 23-39 a second. In an effort to correct discrepancies that may have occurred as a result of developing signals 1-19 and 21-39 on two separate membranes, fractions 11-29 (Fig 2.2A) were used as a reference to adjust the signal intensities of fractions 1-19 and 21-39 (Fig 2.2B).

The gradient separated glycosomes from lysosomes (Fig. 2.2B). Glycosomes were mostly found in the lighter part of the gradient with peak signal in fractions 3-15 (Fig. 2.2B),

however a significant amount was also found in denser areas of the gradient, fractions 35-39. Lysosomes were mostly in fractions 15-33 (Fig. 2.2B). However, they too were also detectable in 35-39. GPI-PLCp was found in fractions 5-19, co-sedimenting with both glycosomes and lysosomes (Fig. 2.2B). Second, a significant fraction of the protein was in fractions 29-39, again co-sedimenting with both glycosomes and lysosomes (fractions 35-39). The presence of all three proteins in fractions 35-39 suggests that they may associate with vesicles that sediment in a concentration of iodixanol denser than both glycosomes and lysosomes.

DISCUSSION

Knowledge of the intracellular location of GPI-PLCp is important for understanding biological functions in *T. brucei* [15-17]. Different investigators have reported apparently varying results [7, 19]. We used two approaches to independently determine the location of GPI-PLCp, iodixanol-density centrifugation and fluorescence microscopy. Fluorescence microscopy indicates that GPI-PLCp is predominantly a glycosome protein (Fig. 2.1). GPI-PLCp was not detectable on lysosomes.

The data from the subcellular fractionation studies was supportive but not independently conclusive, because GPI-PLCp co-sedimented with both glycosomes and lysosomes, although the organelles were reasonably resolved on the gradient. Subcellular fractionation alone was not able to determine the intracellular location of GPI-PLCp. Other fractionation attempts to determine the location of the enzyme produced similar results [7, 19]; GPI-PLCp associated with the flagellar pocket and Golgi complex when sucrose and isopycnic Percoll centrifugation were used [19]. With isopycnic sucrose-gradient centrifugation, GPI-PLCp co-sedimented with glycosomes, lysosomes, mitochondria, and the flagellar pocket [7].

Co-sedimentation of GPI-PLCp on glycosomes, lysosomes, Golgi, mitochondria and flagellar pocket may have several explanations. First, different organelles may have similar densities. Further, the same organelle at different stages of biogenesis (e.g. peroxisomes) can have different densities as a result of having different protein and lipid constituents [35-37]. The density of the lysosome may also vary depending on its constituents. Thus, GPI-PLCp may only associate with glycosomes *in vivo*, however, because a portion of glycosomes and lysosomes (or other organelles) may have similar densities, GPI-PLCp would co-sediment with other organelles as reported by other investigators. Second, lysis of *T. brucei* without prior fixation may cause

GPI-PLCp to be misdirected to other organelles such as the lysosome. Given this possibility, co-sedimentation of GPI-PLCp with other organelles may be an artifact generated by cell lysis, which is known to localize the enzyme to the plasma membrane [2].

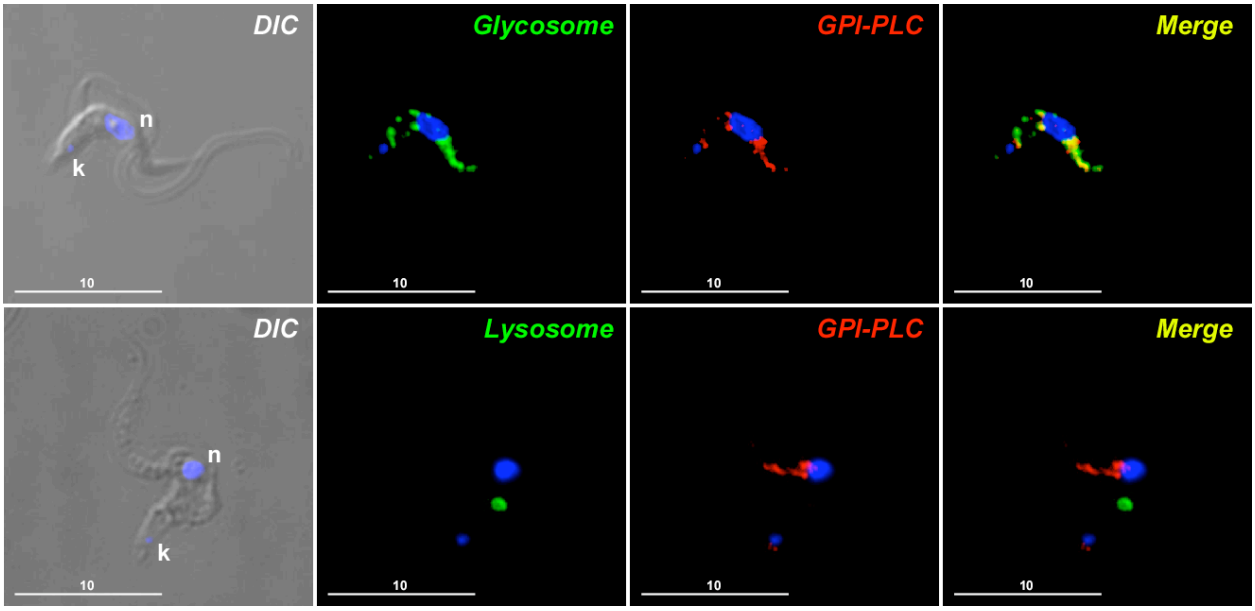
Detection of GPI-PLCp on glycosomes is significant for several reasons. First, this may explain why little or no VSG is released under physiological conditions in non-differentiating bloodstream *T. brucei*. In living cells, VSG may be released by cleavage of the GPI-anchor however, for this to occur GPI-PLCp and VSG must be in the same membrane [9]. VSG is located on the extracellular leaflet of the plasma membrane. Placement of GPI-PLC at the glycosome separates the enzyme from its substrates. Second, GPI-PLCp stimulates endocytosis of transferrin [38]. However, since GPI-PLCp is predominantly a glycosome protein, it does not localize with the endosomal machinery. GPI-PLCp cleaves phosphatidylinositol (PI) to yield diacylglycerol (DAG) [39]. Therefore, it is possible that GPI-PLCp stimulates endocytosis by cleaving PI or GPI at the glycosome [38].

The glycosome-targeting signal of GPI-PLCp remains to be identified. GPI-PLCp is a membrane protein [5, 6, 8, 9, 12]. Membrane proteins of glycosomes are expected to target to the organelle by a membrane peroxisomal targeting sequence (mPTS). The mPTS consists of two components adjacent to or in close proximity to one another: (i) a transmembrane domain and (ii) a PEX19-binding motif [31] that consists of approximately 11 hydrophobic or positively charged residues [31]. GPI-PLCp does not contain a transmembrane domain (from Kyte-Doolittle analysis of the primary structure [40]) and is therefore unlikely to have a mPTS. We hypothesize that the enzyme either (i) has a novel targeting sequence (yet to be identified) or (ii) initially associates with the endoplasmic reticulum membrane and is subsequently sorted to the glycosome. The latter hypothesis is based on recent findings that peroxisomes are derived from

specialized regions of the endoplasmic reticulum (ER) that then bud from the ER to form peroxisome precompartments and eventually, mature peroxisomes [41]. Several peroxisome membrane proteins are first directed to the ER before being sorted to the peroxisome [42-45] (reviewed in [41]). If GPI-PLCp is directed to the ER prior to localizing to the glycosome, it may explain why GPI-PLCp co-sediments with trypanopain on an iodixanol gradient. Trypanopain is presumed to be synthesized at the ER (due to the presence of a signal peptide, TrypDB [46]) prior to targeting to the lysosome. GPI-PLCp does not have a signal peptide and therefore may be targeted to the ER via a novel targeting sequence.

Figure 2.1: GPI-PLCp Co-localizes with Glycosomes in *T. brucei*. Bloodstream *T. brucei* (2 x 10^6) were fixed, permeabilized and incubated with mouse anti-GPI-PLC monoclonal 2A6.6 (GPI-PLCp, *red*) and rabbit anti-glycosome (glycosome) or anti-trypanopain (lysosome) (*green*) antibodies. Primary antibodies were detected with goat anti-mouse IgG Alexa Fluor 594 or goat anti-rabbit IgG Alexa Fluor 488. The cell nucleus (n) and kinetoplast (k) were detected with DAPI (*blue*). GPI-PLCp and glycosome or lysosome-labeled images were digitally overlaid using DeltaVision software (softWoRx, Applied Precision, Seattle, WA) (merge, *yellow*). The white bar represents 10 microns in length.

Figure 2.1


DIC
Glycosome
GPI-PLC
Merge
n
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DIC
Lysosome
GPI-PLC
Merge
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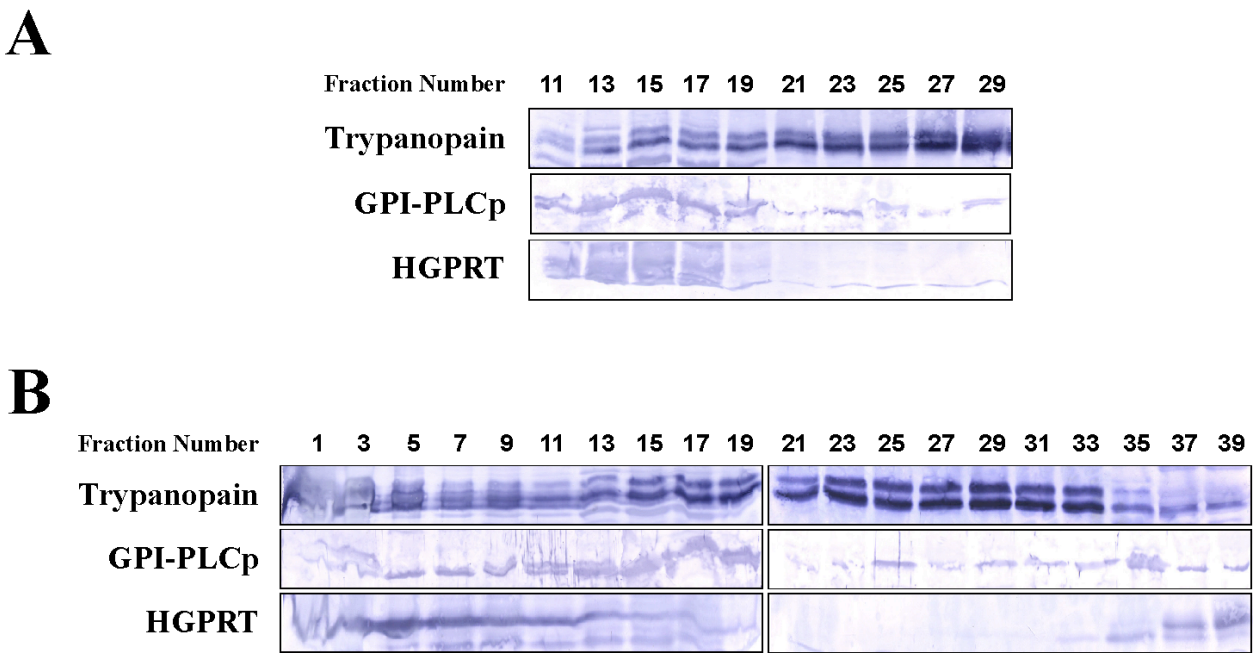
10

Figure 2.2: GPI-PLCp Co-sediments with Lysosomes and Glycosomes in an Iodixanol Gradient. A lysate from 10^{10} bloodstream *T. brucei* was layered on a 35 ml 5-15% iodixanol gradient that was centrifuged (170,000 x g, 4 °C, 1 h) and forty 1 ml fractions collected. Proteins in one hundred µl of each of the odd-numbered fractions were precipitated (TCA) and resolved by SDS-PAGE (14%). Proteins were transferred to Immobilon P membrane and western blot analysis was used to detect GPI-PLCp, glycosomes and lysosomes using RC300 (anti-GPI-PLCp) [26], anti-HGPRT [27], and anti-trypanopain [28] antibodies, respectively. The trypanopain, HGPRT and GPI-PLCp signals in fractions 11-29 **(A)** were used to adjust the respective signals in fractions 1-19 and 21-39 **(B)** in an effort to correct for variation in different gels when the same quantities of protein were detected.

Figure 2.2


A
Fraction Number 11 13 15 17 19 21 23 25 27 29
Trypanopain
GPI-PLCp
HGPRT
B
Fraction Number 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Trypanopain
GPI-PLCp
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CHAPTER 3

CHEMICAL VALIDATION OF PROTEIN TYROSINE KINASE AS A TARGET FOR ANTI-TRYPANOSOME DRUG DISCOVERY[1]

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ABSTRACT

Tyrosine phosphorylation of proteins plays important roles in diverse biological processes in response to internal and external stimuli. The role of tyrosine kinases in the pathophysiology of cancer has led to the development of drugs that inhibit these kinases. Although tyrosine kinase activity is present and important for viability of *Trypanosoma brucei*, pathways for tyrosine kinase signaling in the trypanosome have not been extensively studied. *Trypanosoma brucei* causes human African trypanosomiasis (HAT), an emerging disease for which new drugs are needed. In this study we demonstrate that three drugs that inhibit specific tyrosine kinases kill cultured *T. brucei*. AEE788, a pyrrolopyrimidine that inhibits epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)-like kinases, is a potent anti-trypanosomal compound. AEE788 induces a morphological transformation of the parasite, reduces tyrosine phosphorylation of proteins, blocks endocytosis of transferrin, and triggers cell death. To further understand the pathways and processes associated with tyrosine kinase signaling in bloodstream *T. brucei*, phosphotyrosine affinity chromatography and LC-MS/MS were used to characterize the phosphotyrosine proteome of *T. brucei*. We identified 134 proteins involved in 14 biological pathways and processes including signal transduction, glucose metabolism, endocytosis and protein folding and stability. Elongation factor 1-γ and an ATP-dependent DEAD/H RNA helicase were confirmed to have phosphorylated tyrosine residues. Together these data contribute to our understanding of the role(s) of tyrosine phosphorylation in *T. brucei*. Further, our studies validate PTKs as a target for anti-trypanosome drug discovery.

INTRODUCTION

Tyrosine phosphorylation by protein tyrosine kinases (PTKs) plays an important role in regulation of several cellular processes including differentiation, proliferation, survival, apoptosis, transcription, endocytosis, cell shape and migration [1, 2]. Given their role in normal homeostasis, malfunction of PTKs is implicated in several human disorders such as leukemia, psoriasis and cancer [2, 3]. The importance of PTKs in cancer has made them the focus of an enormous drug discovery effort that is largely focused on small molecule ATP-mimics that bind to the ATP-binding region of the kinases. Epidermal growth factor receptor (EGFR) activation is associated with most human solid tumors. Therefore, EGFR has become a major target for discovery of anti-cancer drugs. Of these, many are based on the anilinoquinazoline scaffold (reviewed in [3]).

Trypanosoma brucei causes human African trypanosomiasis (HAT). Only four drugs are used to treat the disease. Unfortunately, these drugs are generally toxic (sometimes lethal) and very difficult to administer (due to the need for parenteral administration in a hospital) [4, 5]. There is a severe need for newer, safer, orally administered drugs [4].

Although the *T. brucei* genome lacks conventional protein tyrosine kinases [6], several lines of evidence indicate that tyrosine kinase signaling pathways are present in *T. brucei*. First, over 50 Tyr-phosphorylated proteins have recently been identified in the phosphoproteome of bloodstream *T. brucei*, including mitogen-activated protein (MAP) kinases, glycogen synthase 3 kinase (GSK3) and dual-specificity tyrosine phosphorylation regulated kinases (DYRKs) [7]. Second, *T. brucei* cell extracts phosphorylate poly($Glu^{80}Tyr^{20}$) [8] a synthetic polymeric substrate for some PTKs. The tyrosine kinase inhibitor genistein [9] reduced phosphorylation of poly($Glu^{80}Tyr^{20}$) approximately 7-fold and inhibited replication of the parasite 10-fold. Third,

proteins that contain phosphotyrosine (pTyr) have been detected in the parasite by western analysis using pTyr-specific antibodies [10-14]. Interferon-γ (IFN-γ) is an activator of PTKs in mammalian cells [15-17]. When added to *T. brucei*, IFN-γ increases tyrosine phosphorylation of *T. brucei* proteins [18]. Furthermore, tyrphostin A47, a PTK inhibitor, reduces IFN-γ-induced tyrosine phosphorylation in the parasite. [18-20]. Finally, tyrphostin A47 inhibits endocytosis of transferrin (Tf) in *T. brucei* (S. Subramanya, PhD Thesis, University of Georgia). In the trypanosome, transferrin is essential for the uptake of iron [21-23]. Taken together, these data suggest that PTK activity is present and may be essential for viability of *T. brucei*.

Given the development of anti-cancer drugs that inhibit tyrosine kinases (reviewed in [24], and the apparent importance of tyrosine kinase activity in *T. brucei* we wanted hypothesize that some of the anti-cancer drugs could be used as "lead compounds" for anti-trypanosome drug discovery. Herein we demonstrate that Canertinib and the pyrrolopyrimidines [3], PKI166 and AEE788, kill *T. brucei*. These data validate PTKs as targets for drug discovery in *T. brucei* and introduce 4-anilinoquinazolines and pyrrolopyrimidines as novel scaffolds for anti-trypanosome drug discovery. We also demonstrate the use of anti-phosphotyrosine affinity chromatography and LC-MS/MS to characterize the phosphotyrosine (pTyr) proteome of bloodstream *T. brucei*. We report the identification of 2 pTyr-proteins and 134 putative pTyr-proteins involved in 14 biological pathways and processes. Together these data contribute to our understanding of some of the biological pathways or processes associated with tyrosine kinase signaling in bloodstream *T. brucei*.

MATERIALS AND METHODS

Materials

Immobilon P membrane was purchased from Millipore (Bedford, MA). Sodium dodecyl sulfate (SDS), 5-bromo-4-chloro-indoyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT) were from BioRad (Melville, NY). Leupeptin and aprotinin were purchased from Roche (Indianapolis, IN). Formaldehyde, hydrogen peroxide, dimethyl sulfoxide (DMSO) and Triton X-100 were purchased from Fisher Scientific (Pittsburgh, PA). GelCode blue stain reagent was from Pierce (Rockford, IL). DAPI (4',6-diamidino-2-phenylindole), goat anti-mouse and goat anti-rabbit IgG-alkaline phosphatase conjugate and transferrin from human serum Alexa Fluor 488 conjugates were purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Serum Plus was from JRH Biosciences (Lenexa, KS). VectaShield mounting medium was purchased from Vector Laboratories (Burlingame, CA). 12G10 (anti-tubulin antibody) was purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). P-Tyr-100 (anti-phosphotyrosine mouse monoclonal antibody) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). PY20-AC was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Antibody against *T. brucei* BiP was a gift from Dr. James Bangs (University of Wisconsin, Madison, WI). NVP-AEE788 and PKI166 were a gifts from Novartis Pharmaceutical Corporation (Vienna, Austria). CI-1033 (Canertinib) was a gift from Pfizer Inc (New York, NY).

Cell Culture

Bloodstream *T. brucei* 427 was obtained from C.C. Wang (University of California, San Francisco, CA) and cultured in HMI-9 medium [25] (supplemented with 10% fetal bovine serum and 10% Serum Plus) at 37 °C, 5% CO_2.

Bioinformatics Analysis of Tyrosine Kinases in T. brucei

A genome-wide search for the EGFR kinase domain in the *T. brucei* proteome (TrypDB, [26]was performed by BLAST analysis [27]. The probability (P(N)) of relatedness between the trypanosome protein and the EGFR kinase domain was obtained from BLAST analysis. Next, PFAM [28] was used to confirm tyrosine kinase domains (PFAM 07714) in the proteins discovered. The 12 best candidates (those with PTK (protein tyrosine kinase) E-values less than 10^{-24} [29, 30]) were listed.

Drug Treatment of T. brucei

T. brucei was cultured to a density of 10^6 cells/ml and then diluted to 10^4 cells/ml in HMI-9 medium. Five hundred µl of cells (5 x 10^3 cells) were added to each well of a 24-well plate. AG1478, AEE788 and PKI166 stock solutions were prepared in dimethyl sulfoxide (DMSO), and Canertinib was dissolved in deionized water. To cells in which no inhibitor or drug was added (0 µM), the drug vehicles (DMSO or deionized water) were added as a control. To treat cells with drugs 2.5 µl of a 200-fold concentrated stock was added to each well to yield the stated final concentrations in each figure. Cells were cultured for 24 or 48 h and cell density was determined with a hemocytometer. The IG_{50} is determined as the concentration of inhibitor (or drug) that reduces growth of *T. brucei* by 50% during culture. The concentration range needed to cover IG_{50} was determined empirically for each inhibitor.

Transient Drug Treatment of T. brucei

Late log phase cells (grown to no more than 10^6 cells/ml) were harvested and 2 x 10^6 cells/ml in HMI-9 media were aliquoted into 1.5 ml tubes. Three µl of DMSO or AEE788 (10 mM) (30 µM final concentration) was then added to the tubes. The cells were incubated with caps open for 90 min at 37 °C, 5% CO_2 and mixed by pipetting every 30 min. The cells were centrifuged (1500 x g, 4 °C, 10 min), washed twice with HMI-9 media and diluted in 5 ml HMI-9 media to concentrations of 10^5 or 10^4 cells/ml. Cells were cultured for 24 or 48 h, respectively and cell density was determined with a hemocytometer.

Drug Treatment and SDS-PAGE

Cells were harvested and treated with AEE788 as described above. The cells were centrifuged (1500 x g, 4 °C, 10 min) and the supernatant was removed. The cell pellet was resuspended in 15 µl of 3X SDS-PAGE sample buffer and heated for 5 min at 100 °C. Proteins were separated by SDS-PAGE (14%) [31] and transferred to Immobilon P with a Trans-Blot semi-dry cell (Bio-Rad) [32].

For detection of BiP or tubulin (see Immunoblotting section) one cell pellet from above was resuspended in 100 µl of 3X SDS-sample buffer. Proteins in ten µl of sample (2 x 10^5 cell equivalents) were loaded into each well of an SDS-polyacrylamide mini gel (14%), separated by electrophoresis [31] and transferred to Immobilon P with a Trans-Blot semi-dry cell (Bio-Rad) [32].

Immunoblotting

The detection of phosphotyrosine (pTyr) using P-Tyr-100 antibody was carried out by modification of the manufacture's protocol. An Immobilon P membrane was washed with TBS (Tris buffered saline, pH 7.6) for 5 min at room temperature and then blocked in TBS containing

0.1% Tween-20 (TBS/T) and 5% (w/v) Carnation powdered milk for 1 h at room temperature. The membrane was washed three times for 5 min each with TBS/T. The primary antibody P-Tyr-100 (1:1000) in TBS/T with 5% bovine serum albumin (BSA) was incubated with the membrane overnight at 4°C with gentle shaking. The membrane was washed three times for 5 min with 5 ml of TBS/T at room temperature. Secondary antibody, goat anti-mouse IgG-alkaline phosphatase conjugate (1:1000 dilution in blocking buffer (from above)) was added for 1 h at room temperature with gentle shaking. The membrane was washed three times with TBS/T (as previously described) and twice with alkaline phosphatase reaction buffer (APRB, 75 mM Tris pH 9.5, 75 mM NaCl, 3.75 mM MgCl) for 10 min. The membrane was then incubated in 10 ml APRB supplemented with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 1% v/v) and p-nitro blue tetrazolium chloride (NBT, 1% v/v). The signal was developed until a satisfactory intensity was acheived (i.e. with a good signal-to-noise ratio) and stopped by washing the membrane three times with deionized water within 10 min.

To detect BiP, tubulin and GPI-PLCp, immunoblotting was carried out as follows: Immobilion P membrane was blocked with PBS (pH 7.4) containing 5% (w/v) Carnation powdered milk, 1% (v/v) Tween 20 for 1 h at room temperature with gentle shaking. GPI-PLCp was detected using 2A6.6 monoclonal antibody (1:1000) [33], BiP was detected using anti-BiP antibody (1:5000) [34], and tubulin was detected with 12G10 antibody (1:5000) (all antibodies were diluted in blocking buffer). After incubation (1 h at room temperature with gentle shaking) the membranes were washed three times with PBS followed by incubation with goat anti-mouse or goat anti-rabbit IgG-alkaline phosphatase conjugate antibodies diluted (1:1000) in blocking buffer for 1 h at room temperature with gentle shaking. The membranes were then washed three times with PBS and the protein signals were developed as described above.

Transferrin Endocytosis Assays

Bloodstream *T. brucei* were harvested as described above, and 2 x 10^6 cells were resuspended in 1 ml of serum-free HMI-9 medium. The cells were treated with either 2 µl DMSO, 2 µl of 5 mM AEE788 (10 µM, final concentration) or 2 µl of 10 mM AEE788 (20 µM, final concentration) for 30 min at 37 °C, 5% CO_2. The cells were mixed by pipetting every 10 minutes. After this initial incubation, 5 µl of human transferrin AlexaFluor 488 conjugate (5 µg/µl) was added to each tube and the cells were incubated for 20 min at 37 °C, 5% CO_2. The cells were pelleted (1500 x g, 5 min, 4°C) and washed 3 times with ice-cold PBS pH 7.4 containing 30 µM sodium azide, and fixed in 300 µl 1% formaldehyde (in PBS) on ice for 5 min. The formaldehyde was quenched with 700 µl of 100 mM glycine (in PBS) for 5 min at room temperature. The cells were washed twice with PBS and resuspended in 300 µl PBS.

The relative fluorescent intensity of 50,000 events was measured using a CyAn ADP Analyzer (Beckman Coulter, Hialeah, FL; excitation 488 nm, emission 530 – 540 nm). Data analysis (with FlowJo software (Tree Star, Inc. Ashland, Oregon)) was performed on whole cells (~ 45,000 events) by selecting gates to exclude extracellular debris.

Differential Interference Contrast Microscopy

T. brucei bloodstream cells were harvested as described earlier and 2 x 10^6 cells/ml were aliquoted into 1.5 ml tubes. The cells were treated with either 3 µl of DMSO or 3 µl of 10 mM AEE788 (final concentration of 30 µM). The cells were allowed to incubate at 37 °C, 5% CO_2 for 90 min after which they were pelleted (as described above) and washed once with PBS (pH 7.4) containing 10 mg/ml glucose. The cells were fixed in 300 µl 4% formaldehyde on ice for 1 h, and quenched with glycine (700 µl, 100 mM in PBS) for 5 min at room temperature. The cells were washed once with PBS (pH 7.4), resuspended in 200 µl PBS, and allowed to settle on

poly(L)-lysine-coated coverslips for 45 min. The coverslips were rinsed twice with PBS and mounted on slides with 4 µl of VectaShield mounting medium.

DIC (differential interference contrast) images were captured using an Olympus IX-71 inverted fluorescence microscope with a Photometrix $CoolSnap_{HQ}$ CCD (charged-coupling device) camera driven by DeltaVision software (Applied Precision, Seattle, WA).

Affinity-Enrichment of Tyr-phosphorylated Proteins

Bloodstream *T. brucei* late log phase cells (1.35×10^8, grown to a density of no more than 10^6 cells/ml) were pelleted (1500 x g, 5 min), and washed once with 10 ml BBS/G (buffered saline plus glucose, 50 mM bicine, 50 mM NaCl, 5 mM KCl, 1% glucose, pH 7.4) containing sodium vanadate (1 mM), and then centrifuged again as described above. The supernatant was removed without dislodging the pellet, and the cells were lysed with 250 µl of lysis buffer (LB) (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2% Triton X-100, 1 mM EGTA, 1 mM PMSF, 2 µg/mL aprotinin, 5 µg/mL leupeptin, 37 µg/mL TLCK, 2 µM FMK024 and 1 mM sodium vanadate). The lysate was incubated in an ice-water bath and occasionally mixed by pipetting (3 times in 20 min). Lysis was verified by phase contrast microscopy. Two hundred and fifty µl of LB (without Triton X-100) was then added to the lysate to reduce the concentration of Triton X-100 from 2% to 1%. The lysate was centrifuged (14,000 x g, 10 min, 4 °C) and the supernatant was retrieved into a 1.5 ml tube. The supernatant was pre-cleared by adding a 50 µl slurry (15 µl packed) of Protein G agarose, equilibrated with LB containing 1% Triton X-100, and incubated for 1 h at 4 °C. The slurry was then centrifuged (5000 x g, 25 s) and the supernatant was removed. Fifty µl (15 µl packed bed) PY20-agarose was added and the slurry was rotated for 3 h at 4 °C. Agarose was recovered by centrifugation (5000 x g, 25 s), and the supernatant removed. The column was washed three times with 15 µl LB containing 1% Triton X-100. Proteins bound

to the PY20-agarose column were eluted with three washes of 15 µl of PBS containing 100 mM phenylphosphate. To 40 µl of the eluate, 13 µl of 3X SDS-sample buffer was added and the sample was heated for 5 min at 100 °C. The proteins were separated by SDS-PAGE (10% non-stacking), and the gel was stained with GelCode blue overnight at 4 °C. The gel was destained the next day for 2 h in deionized water and then analyzed by LC-MS/MS (see below).

In some experiments bloodstream *T. brucei* were treated with pervanadate prior to lysis as follows: cells (1.35 x 10^8, grown to a density of no more than 10^6 cells/ml) were divided equally into two 50 ml culture flasks (30 ml in each flask). To each flask, 300 µl of freshly prepared 100 mM pervanadate (1 mM final concentration) was added and then incubated for 1 h at 37 °C, 5% CO_2. Pervanadate (100 mM) was prepared by mixing 500 µl of 200 mM sodium vanadate with 500 µl of 200 mM H_2O_2 and allowing the mixture to incubate in the dark for 15 min at room temperature [35]). After treatment the cells were washed, lysed and the Triton X-100 concentration reduced as described previously. The lysate was centrifuged (14,000 x g, 10 min, 4 °C), the supernatant was retrieved and added to a 1.5 ml tube. Fifty µl (15 µl packed) of PY20-agarose or Protein G-agarose (as a control to identify non-specific binding proteins) was added to equivalent amounts of lysate and rotated for 3 h at 4 °C. The beads were washed three times with 15 µl LB 1% Triton X-100, pelleted, resuspended in 35 µl of SDS-sample buffer and heated for 5 min at 100 °C. The proteins were resolved and gels prepared for LC-MS/MS as described above.

In-Gel Trypsin Digest

SDS-polyacrylamide gel lanes containing proteins stained with GelCode blue were cut into 5 pieces. Each of the 5 pieces were futher cut into 2 x 2 mm pieces, dehydrated with acetonitrile and reswollen in 40 mM ammonium bicarbonate [36]. This step was repeated, the

gel pieces were reduced with DTT (10 mM) for 1 h at 56 °C, and then carboxyamidomethylated with iodoacetamide (55 mM) in the dark for 45 min. The gel pieces were dehydrated and reswollen (as previously described) twice and dried in a Speed Vac for 5 min. Dried gel pieces were then slowly reswollen in ammonium bicarbonate (40 mM) with 20 ng/µl trypsin (Promega) on ice for 45 min and then incubated overnight at 37 °C. The peptides were extracted three times for 20 min with 5% formic acid, 50% acetonitrile and dried down in a Speed Vac [36].

Analysis of Peptides by LC-MS/MS

Tryptic peptides were identified by LC-MS/MS [37]. Briefly, dried peptides (from above) were resuspended with 39 µl (for 1 of the 5 pieces of gel from above) of mobile phase A (0.1% formic acid (FA) in water) and 1 µl of mobile phase B (80% acetonitrile and 0.1% FA in water). Resuspended peptides were filtered with 0.2 µm filters (Nanosep, PALL, East Hills, NY) and samples were loaded offline onto a nanospray tapered capillary column (75 µm by 8.5 cm, PicoFrit, New Objective, Woburn, MA) self-packed with C18 RP (reverse phase) resin (5 µM, Waters, Milford, MA) in a nitrogen pressure bomb. Peptides were separated with a linear gradient (160 min) of increasing mobile phase B using a flow rate of 200 nl/min after split. MS/MS analysis was performed on a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray ion source. A full mass spectrum (350-2000 *m*/z) was collected followed by eight collision induced dissociation (CID) MS/MS spectra (normalized collision energy of 34% with a 2.2 *m*/z isolation width and dynamic exclusion set at 2). The resulting data was searched against *T. brucei/T. cruzi/Leishmania* database (released on July 26th, 2006) obtained from the National Center for Biotechnology Information (NCBI) using TurboSequest (Bio-Works 3.3.2, Thermo Electron, San Jose, CA). DTA (SEQUEST common file format) files were generated for spectra with a threshold of 15

ions and a TIC (total ion current) of 2 x 10^3. Zero stack algorithm (ZSA), correct ion, combion and ionquest were all applied over a range of MH^+ 600-4000. The SEAQUEST parameters were set to allow 1.4 Da of precursor ion mass tolerance and 0.5 Da of fragment ion tolerance with monoisotopic mass. Only strict tryptic peptides were allowed with up to three missed internal cleavage sites. Dynamic mass increases of 15.99, 57.02 and 79.97 Da were allowed to search for peptides containing oxidized methionine, alkylated cysteine and phosphorylated tyrosine, respectively. The results of the SEAQUEST search were filtered by ProteoIQ (BioInquire, Athens, GA) with a false discover rate (FDR) of 5% [37] against an inverted database.

RESULTS

T. brucei Contains Protein Kinases with EGFR-Like Kinase Domains

To determine if 4-anilinoquinazoline drugs that inhibit human EGFR could be used against *T. brucei* we used bioinformatics analysis to search the *T. brucei* genome for protein kinases with EGFR-like domains. First, a genome-wide search for EGFR kinase domains was performed, by 'BLASTing' the EGFR-kinase domain against the parasite proteome. Next, PFAM [28] was used to confirm the tyrosine kinase domains in the proteins discovered (not shown). The systematic IDs of the 12 best candidates (PTK E-value < 10^{-24} [29, 30]) are listed along with the residues that define the kinase domain (Table 3.1). The probability (P(N)) of homology between the trypanosome protein (obtained from BLAST) and the EGFR kinase domain are shown (Table 3.1). This analysis revealed several *T. brucei* protein tyrosine kinases that may have EGFR-like kinase domains (e.g., Tb10.406.0580, Tb927.8.5730, Tb927.7.3580, and Tb10.70.5760). These data suggest that *T. brucei* has a group of proteins that may be inhibited by EGFR-specific inhibitors.

4-Anilinoquinazolines Kill T. brucei

In a pharmacological approach to determine if bloodstream *T. brucei* contains PTKs that may be inhibited by EGFR-kinase specific drugs, cells were treated with the 4-anilinoquinazoline AG1478 [38]. Replication of the parasite was reduced 95% by 5 μM AG1478 while 7.5 μM of the inhibitor killed all *T. brucei* (Fig. 3.1A). Canertinib is a 4-anilinoquinazoline developed to treat some cancers [39, 40]. Because it is an analog of AG1478, we hypothesized that it too would kill *T. brucei*. Canertinib killed *T. brucei* (no cells were detected on exposure to 5 μM of the drug), and the IG_{50} (drug concentration that reduces growth

of the parasite by 50%) was determined to be approximately 3 μM (Fig. 3.1B). These data along with the bioinformatics analysis suggest that 4-anilinoquinazoline drugs may be effective against *T. brucei*.

The Pyrrolopyrimidines PKI166 and AEE788 Kill T. brucei.

PKI166 and AEE788 both contain a pyrrolopyrimidine scaffold (Fig. 3.2A and B). PKI166 is an EGFR-kinase inhibitor [41] whereas AEE788 inhibits both EGFR and VEGFR (vascular endothelial growth factor receptor) [42, 43]. To determine whether drugs containing the pyrrolopyrimidine scaffold could kill *T. brucei,* cells were treated with PKI166 or AE788. Both PKI166 and AEE788 killed *T. brucei*. The IG_{50} for PKI166 is between 3-5 μM (Fig. 3.2A) and for AEE788 it was 600 nM (Fig. 3.2B). Thus, AEE788 is approximately 5-fold more potent than PKI166. Because AEE788 is the more potent lead drug against *T. brucei*, we used it for the remainder of this study.

AEE788 Transforms the Shape of T. brucei

While determining if AEE788 could kill *T. brucei* we serendipitously noticed that cells exposed to AEE788 were rounded up. To investigate this result, we treated cells with AEE788 and analyzed them by DIC microscopy. AEE788 changed the shape of *T. brucei* within 45 min of cell exposure (*not shown*), whereas cells treated with DMSO (drug vehicle) maintained the normal shape (Fig. 3.3). After 90 min treatment with AEE788, approximately 90% of the cells acquired a round shape (Fig. 3.3). At this time point, the length of the cell bodies and distance between kinetoplast and nucleus, of AEE788 treated cells, were reduced by at least 2-fold when compared to cells treated with DMSO; the cells were no longer motile and the flagellum (when detectable) was beating in only 25% of the round cells (*not shown*).

Transient Exposure of T. brucei to AEE788 Triggers Death

To determine if the morphological change caused by AEE788 correlated with viability of *T. brucei*, we treated bloodstream cells with AEE788 for 90 min, removed the medium containing the drug, and cultured the treated cells in fresh media for 24 or 48 hours. Cells were not detected 24 or 48 hours after AEE788 treatment (Fig. 3.4A and B, respectively), whereas cells treated with DMSO grow normally (Fig. 3.4A and B). These data indicate that transient exposure of *T. brucei* to AEE788 triggers cell death.

AEE788 Reduces Tyrosine Phosphorylation of Proteins in T. brucei

Antibodies can be used to detect tyrosine-phosphorylated proteins [13, 44-46]. Because AEE788 inhibits EGFR and VEGFR kinases, we hypothesized that the compound would reduce the amount of phosphotyrosine (pTyr) detected on *T. brucei* proteins. To test this prediction, we treated *T. brucei* with AEE788 and probed for Tyr phosphorylation by western blotting with anti-pTyr-antibody P-Tyr-100 [47, 48].

AEE788 reduces Tyr phosphorylation of most *T. brucei* proteins when compared to control cells treated with DMSO (vehicle for AEE788) (Fig. 3.5). However, the pTyr signal on one protein increased after AEE788 treatment (Fig. 3.5). To determine if the reduction (or increase) of Tyr phosphorylation was specific to pTyr proteins we monitored the amounts of tubulin, BiP and GPI-PLCp. After AEE788 treatment, the signal intensity of these proteins on western blots does not change (Fig. 3.5). These data suggest that the loss (or gain) of pTyr signal on proteins (Fig. 3.5) arises from inhibition of PTK activity in *T. brucei*.

Identification of Tyrosine Phosphorylated Proteins in Bloodstream T. brucei

Tyrosine kinase signaling is essential for viability of bloodstream *T. brucei*. To further understand the cellular processes associated with tyrosine kinase signaling it is important to

identify Tyr-phosphorylated proteins in bloodstream *T. brucei*. Recently, over 50 Tyr-phosphorylated proteins were identified in the phosphoproteome of bloodstream *T. brucei* [7]. In this study serine, threonine and tyrosine phosphopeptides of a cytosolic protein fraction were enriched by strong cation exchange and titanium-dioxide chromatography prior to MS. However, it has been shown that due to the presence of low levels of Tyr-phosphorylated proteins in cells, enrichment of tyrosine phosphorylated proteins is essential [49] to more fully catalog Tyr-phosphorylated proteins in a cell. Therefore it is possible that some of the Tyr-phosphorylated proteins were not identified in the study. Because of our interest in the phosphotyrosine proteome of bloodstream *T. brucei*, we concentrated Tyr-phosphorylated proteins with phosphotyrosine-affinity chromatography and identified them by LC-MS/MS [50-52]. Prior to pTyr-enrichment, protein G-agarose was used to pre-clear proteins that non-specifically bind to the agarose beads. Proteins adsorbed to the PY20-agarose column were eluted with either phenylphosphate (100 mM), a pTyr mimic that can be used to desorb pTyr-containing proteins from a pTyr antibody [35], or phenylethanol (as a control). Proteins that were specifically eluted from the PY20-agarose column were obtained by "subtracting" proteins that were eluted with phenylethanol. Using this approach, eight proteins were identified as putative Tyr-phosphorylated proteins (Table 3.2) and two proteins, elongation factor 1-γ (Tb11.01.4660) and an ATP-dependent DEAD/H RNA helicase (Tb927.4.2630) (Table 3.2), were confirmed to contain phosphorylated tyrosine.

Because the number of putative pTyr-proteins identified using the previous approach was low compared to other studies [35, 53-55], we used a second approach to identify Tyr-phosphorylated proteins in bloodstream *T. brucei*. In this approach, *T. brucei* were treated with pervanadate (a potent phosphatase inhibitor [35, 56, 57]) for 1 hour prior to affinity

chromatography with PY20-agarose or protein G-agarose (as a control). Beads with bound proteins were eluted with SDS-sample buffer, resolved by SDS-PAGE and analyzed by LC-MS/MS. From these experiments, we identified over 140 proteins bound to the protein G-agarose (not shown) and over 190 proteins bound to the PY20-agarose (not shown). Approximately 67 proteins were bound to both columns. Of these 67 proteins, twelve were enriched by at least 5-fold with the PY20-agarose column (Table 3.3). To determine which proteins specifically bind PY20-agarose, proteins that bound to protein G-agarose (control) were "subtracted" from proteins bound to PY20-agarose. Using this method, we identified 127 proteins that associated with PY20-agarose (Table 3.4).

Discovery of Protein Kinases in Bloodstream T. brucei

Most PTKs have an activation loop, a 20-35 amino acid long, flexible region that links the N and C lobes of the kinase domain [58]. Activation loops are delineated by an N-terminal [D,N]F[G,R], and a C-terminal [A,P][P,E][E,M], motif. The most common method for kinase regulation is phosphorylation of the activation loop. An unphosphorylated activation loop sterically hinders substrate binding. Phosphorylation of activation loop threonine or tyrosine residues displaces the loop, and allows substrate binding to the PTK (reviewed in [58]). In *Saccharomyces cerevisiae*, tyrosine phosphorylation of the activation loop is rare, however one site that it frequently occurs is in the activation loop of kinases belonging to the CMGC (cyclin-dependent kinases (CDK), mitogen-activated protein kinases (MAPK), glycogen synthase kinases (GSK) and CDK-like kinases) group. In this group of kinases, the phosphorylatable tyrosine residue is generally found 8 residues N-terminal of the APE tripeptide [59]. To determine if any of the kinases identified in the *T. brucei* pTyr proteome (Table 3.4, N) contain phosphorylatable tyrosine residues within the activation loop we first identified active kinases by

searching for conserved catalytic residues [60, 61] (Fig. 3.6A). We then searched for activation loops (Fig. 3.6B) within the enzymatically active kinases. Sequence alignments of the activation loops of the kinases revealed four kinases with phosphorylatable tyrosine residues namely, Tb10.61.3140, Tb10.389.1730, Tb927.6.1780, Tb09.160.4770. The data indicate that bloodstream *T. brucei* contains protein kinases that may be regulated by tyrosine phosphorylation.

AEE788 Inhibits Endocytosis of Transferrin in Bloodstream T. brucei

Clathrin-mediated endocytosis is essential for viability of *T. brucei* [22]. In general, endocytosis maintains cell volume, protects the cell from antibody-mediated killing [62, 63] and is used to take up essential nutrients (e.g. iron) from the host bloodstream (reviewed in [64]). The pTyr proteome of *T. brucei* includes proteins involved in clathrin-mediated endocytosis (e.g. clathrin heavy chain (Tb10.70.0830), clathrin coat assembly protein (Tb11.02.2410) and epsin (Tb11.0890)) (Table 3.4, E). These data suggest that Tyr phosphorylation of proteins may be important for regulation of clathrin-mediated endocytosis in *T. brucei*.

To determine if Tyr phosphorylation affects clathrin-mediated endocytosis, uptake of transferrin (Tf) endocytosis was tested in cells, and the effect of AEE788 on the process was evaluated. AEE788 inhibited Tf endocytosis by bloodstream *T. brucei*. Accumulation of fluorescent Tf was reduced 2-fold by 50 nM AEE788 and 10-fold by 100 nM (Fig. 3.7B). These data suggest that clathrin-mediated endocytosis is regulated by tyrosine kinase signaling in bloodstream *T. brucei*.

DISCUSSION

Chemical Validation of PTKs as Drug Targets in T. brucei

Protein tyrosine kinases are important regulators of numerous processes in eukaryotes [1, 2]. In trypanosomes the pathways regulated by these enzymes are not known. Moreover, the phosphotyrosine proteome of bloodstream *T. brucei* has not been completely characterized. Our interest in both of these issues stems from the extraordinary advances in cancer biology toward new drugs that act on PTKs (reviewed in [3]).

Constitutive activation of EGFR (due to mutations, over expression, deletions, viral infection, and/or failure in down-regulation (reviewed in [65]) is associated with the most common human solid tumors [2, 66]. Drugs that inhibit this kinase are currently used to treat some cancers (reviewed in [24]). Bioinformatics suggest that *T. brucei* contains kinases with enzyme domains similar to EGFR (TbEGFR-like kinase) (Table 3.1). Support of this prediction came from pharmacological studies. AG1478, a 4-anilinoquinazoline and potent inhibitor of EGFR kinase [67, 68], killed *T. brucei* with an IG_{50} of 7 μM (Fig 1A). Canertinib, also a 4-anilinoquinazoline [40], kills *T. brucei* with IG_{50} of 3 μM (Fig. 3.1B).

Several classes of molecules, based on different chemical scaffolds have been developed to inhibit EGFR kinase [69]. Ultimately, the scaffold that exhibits the highest potency against *T. brucei* would be the best scaffold for lead drug discovery. PKI166 [41, 70] and AEE788 [42, 43, 71-73]) belong to a class of EGFR kinase inhibitors that use a pyrrolopyrimidine scaffold. [3]. Both PKI166 and AEE788 kill *T. brucei* with an IG_{50} between 3 and 5 μM, and approximately 600 nM, respectively (Fig. 3.2). AEE788 is 5 times more potent than PKI166. These data establish 'proof of principle' that pyrrolopyrimidine analogs exhibit different potencies against *T. brucei*. Further, the pyrrolopyrimidine scaffold may be good for the discovery of new drugs

against *T. brucei*. Studies of other derivatives of pyrrolopyrimidines may reveal compounds that are more potent against *T. brucei* than they are against human cells. Such compounds would be the best candidates to develop into drugs against *T. brucei.* The structure activity relationships (SAR) of pyrrolopyrimidines against EGFR kinase indicate a preference for bulky substituents at 5-and/or 6-position of the pyrrole ring (similar to the SAR of 4-anilinoquinoazolines) and for halogens at the 3-position of the anilino moiety (reviewed in [69]). Pyrrolopyrimidines with these types of substituents should be tested to assess their effectiveness against bloodstream *T. brucei*.

AEE788 Changes Morphology of T. brucei and Transient Exposure to the Drug Triggers Cell Death

While conducting the experiments above we serendipitously observed that AEE788 caused a change in *T. brucei* morphology; cells round up within 45 minutes of exposure to the compound (*not shown*). After 90 minutes of exposure the majority of the cell population (> 90%) were transformed into round cells. (Fig. 3.3) Interestingly, normal cell morphology was not restored upon removal of the drug and the cells died eventually (Fig. 3.4).

In *T. brucei* a subpellicular microtubule cytoskeleton underlying the plasma membrane contributes to cell shape [74] and plays a role in cell division [75]. Because AEE788 inhibits Tyr phosphorylation (Fig. 3.5) and causes a change in cell morphology in *T. brucei*, it is possible that proteins involved in the organization or dynamics of the cytoskeleton in *T. brucei* could be regulated by tyrosine phosphorylation. This idea is supported by the identification of several proteins involved in cell morphology (i.e. α- and β-tubulin, Table 3.4, C) as possibly being Tyr-phosphorylated or associated with proteins that contain pTyr residues. Tubulin is Tyr-phosphorylated by tyrosine kinases in other cells [76-80], however the function of the modification has not been completely characterized. *In vitro* experiments indicate that Tyr

phosphorylation of tubulin on the C-terminal tyrosine inhibits polymerization of microtubules [81] further, in Jurkat T-cells Tyr-phosphorylated tubulin is restricted to tubulin that is not polymerized and that is found exclusively in cytosolic fractions [82]. It has therefore been postulated that tyrosine phosphorylation of tubulin may play a role in regulating the pool of tubulin monomers available for polymerization [78]. If tubulin polymerization is regulated by tyrosine kinase signaling in *T. brucei*, TbPTK inhibitors may kill the parasite by disrupting cytoskeleton dynamics.

AEE788 Inhibits Tyrosine Phosphorylation in T. brucei

AEE788 was confirmed as an inhibitor of most of the protein Tyr phosphorylation in *T. brucei* (Fig. 3.5). Although AEE788 reduced detection of pTyr on trypanosome proteins, the drug had no effect on polypeptide levels of tubulin, GPI-PLCp or BiP in cells (Fig. 3.5). This data suggests that the drug specificity inhibits pTyr synthesis in *T. brucei.*

Tyrosine phosphorylation of one protein increased after AEE788 treatment (Fig. 3.5). This observation may be explained by data regarding the regulation of Src tyrosine kinase. Src is inhibited by tyrosine phosphorylation promoted by the tyrosine kinase Csk [83, 84] (reviewed in [85]). Tyrosine phosphorylation of Src leads to intramolecular domain interactions that inhibit the kinase. If a similar regulatory mechanism existed in *T. brucei*, it could explain why some protein Tyr phosphorylation increases after AEE788 treatment.

The Putative Tyrosine Phosphoproteome of Bloodstream T. brucei

One of our long-term goals is to understand how drugs against TbEGFR-like kinases might kill the parasite. This objective may be advanced by knowledge of the repertoire of tyrosine-phosphorylated proteins in *T. brucei* (i.e. pTyr proteome). The phosphoproteome of bloodstream *T. brucei* has been discovered [7] however, in this study the investigators did not

enrich Tyr-phosphorylated proteins prior to LC-MS/MS. Because the abundance of pTyr proteins is relatively low in cells, the pTyr proteome may not have been completely described. Therefore, we initiated a study of the pTyr proteome of bloodstream *T. brucei*. To accomplish this task, we used anti-phosphotyrosine (PY20-agarose) affinity chromatography and LC-MS/MS, as has been done with other cells [54, 55, 86], to characterize the pTyr proteome. In one approach, we used phenylphosphate to elute proteins bound to PY20-agarose. Using this elution method, we identified two tyrosine-phosphorylated proteins and eight proteins that may associate with Tyr-phosphorylated proteins (Table 3.2). Elongation factor 1-γ (Tb11.01.4660) and ATP-dependent DEAD/H RNA helicase (Tb927.4.2630) were confirmed to have phosphorylated tyrosine residues. Neither protein has been studied extensively in *T. brucei*, but in other organisms EF1-γ and ATP-dependent DEAD/H RNA helicase are important for protein synthesis [87, 88]. In cancer cells Tyr-phosphorylation of p68, a DEAD RNA helicase is required growth factor-stimulated cell proliferation [89]. The role of Tyr-phosphorylation on EF1-γ and ATP-dependent DEAD/H RNA helicases in bloodstream *T. brucei* has yet to be determined, however if they are regulated Tyr phosphorylation PTK inhibitors might kill the parasite by interfering with protein synthesis or proliferation.

Tyrosine phosphorylation is regulated by tyrosine kinases and tyrosine phosphatases. Under physiological conditions, the fraction of Tyr-phosphorylated proteins is small [54, 90]. In a second approach to characterize the pTyr proteome we aimed to increase sensitivity of detection by treating *T. brucei* with pervanadate to inhibit phosphatases of pTyr [56, 91]. Further, we tried to identify all proteins bound to the protein PY20-agarose by eluting bound proteins with SDS-sample buffer instead of phenylphosphate. Using this approach we identified over 127 Tyr-phosphorylated proteins or proteins that form complexes with Tyr-phosphorylated

proteins (Table 3.4). The putative Tyr-phosphorylated proteins are associated with biological processes such as signal transduction, glucose metabolism, protein folding, proteolysis and endocytosis (Table 3.4) among others. Sixty-seven proteins were associated with both protein G-agarose and PY20-agarose. Of these 12 were found to be enriched at least 5-fold by PY20-agarose (Table 3.3). This data suggest that these proteins may have Tyr-phosphorylated proteins. Because we did not identify Tyr-phosphorylated peptides in our second approach to characterize the pTyr proteome of bloodstream *T. brucei*, it is necessary to do so in order to confidently report the pTyr proteome of bloodstream *T. brucei*. Further, because some Tyr-phosphorylated proteins may bind to protein G-agarose, it may be necessary to search for pTyr in proteins bound to this column as well.

The enrichment of Tyr-phosphorylated proteins prior to LC-MS/MS identified over 50% more putative Tyr-phosphorylated proteins than those identified in the bloodstream *T. brucei* phosphoproteome [7]. Again, we must confirm the presence of pTyr in order to better compare these data. Nonetheless, of the 134 total proteins we identified, six proteins (Tb927.8.3680, Tb927.8.7400, Tb10.61.3140, Tb10.406.0020, Tb927.6.1780, and Tb927.1.4310) were also identified and confirmed to contain pTyr in an independent experiment characterizing the bloodstream *T. brucei* phosphoproteome [7].

Tyrosine Phosphorylation and Signal Transduction in T. brucei

We identified 11 proteins kinases in the pTyr proteome of bloodstream *T. brucei* (Table 3.4, M). Seven of the proteins have conserved catalytic residues and an activation loop (Fig. 3.6). These data indicate that the enzymes are active in bloodstream *T. brucei* and may be regulated by Tyr-phosphorylation. Alternatively, the enzymes might associate with other Tyr-phosphorylated proteins.

Many protein kinases are regulated by phosphorylation of an activation loop [58]. However, regulation of some kinases by tyrosine phosphorylation outside of the activation loop has also been described [59, 92, 93]. In *S. cerevisiae*, mitogen-activated protein kinases (MAPK) and glycogen synthase kinases (GSK) are phosphorylated on tyrosine in their activation loop 8-residues N-terminal to an APE tripeptide motif [59]. MAPKs are phosphorylated by MAPK kinases (MAPKK/STE7), dual-specificity kinases that phosphorylate both Thr and Tyr [59, 94]. The GSK family of kinases autophosphorylate on a Tyr in their activation loops [95, 96]. Four of the protein kinases we identified (MAPKs Tb927.6.1780 and Tb10.389.1730, GSK3 Tb10.61.3140 and NAK Tb09.160.4770) have a phosphorylatable tyrosine in their activation loops (Fig. 3.6B). Recently, these Tyr residues in MAPK (Tb927.6.1780) and GSK3 (Tb10.61.3140) were confirmed to be phosphorylated [7]. These data support the hypothesis that these kinases are regulated by Tyr phosphorylation in *T. brucei*. Furthermore, GSK3 has recently been characterized in *T. brucei* and is required for cell survival [97]. If AEE788, or other TbPTK inhibitors inhibit tyrosine phosphorylation of GSK3 in bloodstream *T. brucei* it could explain why the drug kills the parasite.

It is possible that the other active kinases identified (CAMKK Tb11.02.4860, NRKA Tb927.8.6930 and STE11 Tb927.2.2720) are regulated by Tyr phosphorylation outside of the activation loop. NRKA has been characterized in *T. brucei* and is predicted to have two Tyr phosphorylation sites outside of the activation loop [98]. However, because our studies do not confirm the presence of pTyr in any of the kinases it will be important to obtain direct evidence of Tyr-phosphorylation of these kinases, in future work.

Tyrosine Phosphorylation and Glucose Metabolism in T. brucei

T. brucei depends completely on glycolysis for ATP generation [99]. Our studies have identified nine glycolytic proteins that may be Tyr-phosphorylated (Table 3.4, B). This data suggests that Tyr phosphorylation may play a role in regulation of glucose metabolism in *T. brucei*. In mammalian cells exposed to growth factors or over-expressing the PTK Src, three glycolytic enzymes, namely, enolase, phosphoglycerate mutase and lactate dehydrogenase are tyrosine-phosphorylated [100]. Further the degree of tyrosine phosphorylation is correlated with the rate of glycolysis [101]. In addition, LDH-A, enolase, GAPDH, PGM, and PFK are phosphorylated on tyrosine residues by EGFR in transformed fibroblast cells [102]. Tyrosine phosphorylation of glycolytic proteins (Table 3.4, B) in *T. brucei* remains to be confirmed. The precedent in human cells suggests that the *T. brucei* enzymes may be Tyr-phosphorylated. If tyrosine phosphorylation regulates these enzymes in *T. brucei*, inhibitors of TbPTKs might kill the parasite by interfering with energy metabolism.

Tyrosine Phosphorylation and Protein Chaperones in T. brucei

The identification of protein chaperones by phosphotyrosine affinity chromatography and LC-MS/MS suggests that these proteins contain pTyr-residues (or associate with proteins that do). Tyrosine phosphorylation of protein chaperones has been detected in other cells [103-108] however, the function of the modification is not entirely known. In Cos-1 cells, tyrosine phosphorylation of Hsp72, a molecule closely related to Hsp70, regulates the nuclear accumulation of the protein [109]. The role of protein chaperone tyrosine phosphorylation remains to be defined in *T. brucei*, however if the modification affects localization of these proteins in the cell, TbPTK inhibitors could cause the proteins to be misdirected, possibly interfering with folding or stability of proteins.

Evidence for Tyrosine Kinase Regulation of Endocytosis of Transferrin in T. brucei

In our analysis, clathrin, clathrin coat assembly protein, and epsin were identified as putative tyrosine-phosphorylated proteins. These proteins participate in clathrin-mediated endocytosis which is essential in *T. brucei* [22]. In mammalian cells PTKs facilitate endocytosis by phosphorylating clathrin, clathrin coat assembly proteins (i.e. AP-2 and AP180) and others [110-114]. Inhibition of Src tyrosine kinase attenuates endocytosis [115-118] (reviewed in [114]). Further, the tyrosine kinase inhibitor genistein attenuates the internalization of EGFR, B-cell antigen receptor and asialoglycoprotein receptor [119-121] and partially inhibits endocytosis of the transferrin (Tf) receptor [122].

Tyrphostin A47 blocks endocytosis of Tf in *T. brucei* (S. Subramanya, PhD Thesis, University of Georgia). Given this, we wanted to determine if AEE788 could also inhibit Tf endocytosis. We found that AEE788 reduces Tf endocytosis between 50 and 90 (Fig. 3.7B). These data along with identification of clathrin and epsin as having pTyr suggest that tyrosine phosphorylation may be involved in regulating Tf endocytosis in *T. brucei*. Iron, which is acquired by the Tf receptor, is required for *T. brucei* growth [23, 123]. Inhibition of Tf uptake by AEE788 may result in iron starvation and kill the parasite cells.

Conclusions

In this study, through the use of the PTK inhibitors AG1748, Canertinib, PKI166 and AEE788 we provide evidence that tyrosine kinase activity is a valid target for drug discovery in *T. brucei*. The pyrrolopyrimidine chemical scaffold was the most effective scaffold discovered. We have identified numerous proteins in bloodstream *T. brucei* that are tyrosine phosphorylated. Some of these proteins were confirmed to be substrates for tyrosine kinases. Finally, we have identified processes that may be influenced by tyrosine phosphorylation in *T. brucei* such as

signal transduction, glucose metabolism, protein folding and endocytosis. Together, our findings strongly suggest the presence of active and essential tyrosine kinase signaling pathways in bloodstream *T. brucei.*

Figure 3.1: The 4-Anilinoquinazolines AG1478 and Canertinib, Kill *T. brucei*. Indicated concentrations of **(A)** AG1478 or **(B)** Canertinib (x-axis) were added to bloodstream *T. brucei* (5×10^3 cells/500 µl) and cells were cultured for 48 h. Control cells (0 µM) received DMSO or water, the solvent for the drugs. After 48 h, cell density was determined by hemocytometer. Mean ± standard deviation of duplicate samples are plotted.

Figure 3.1

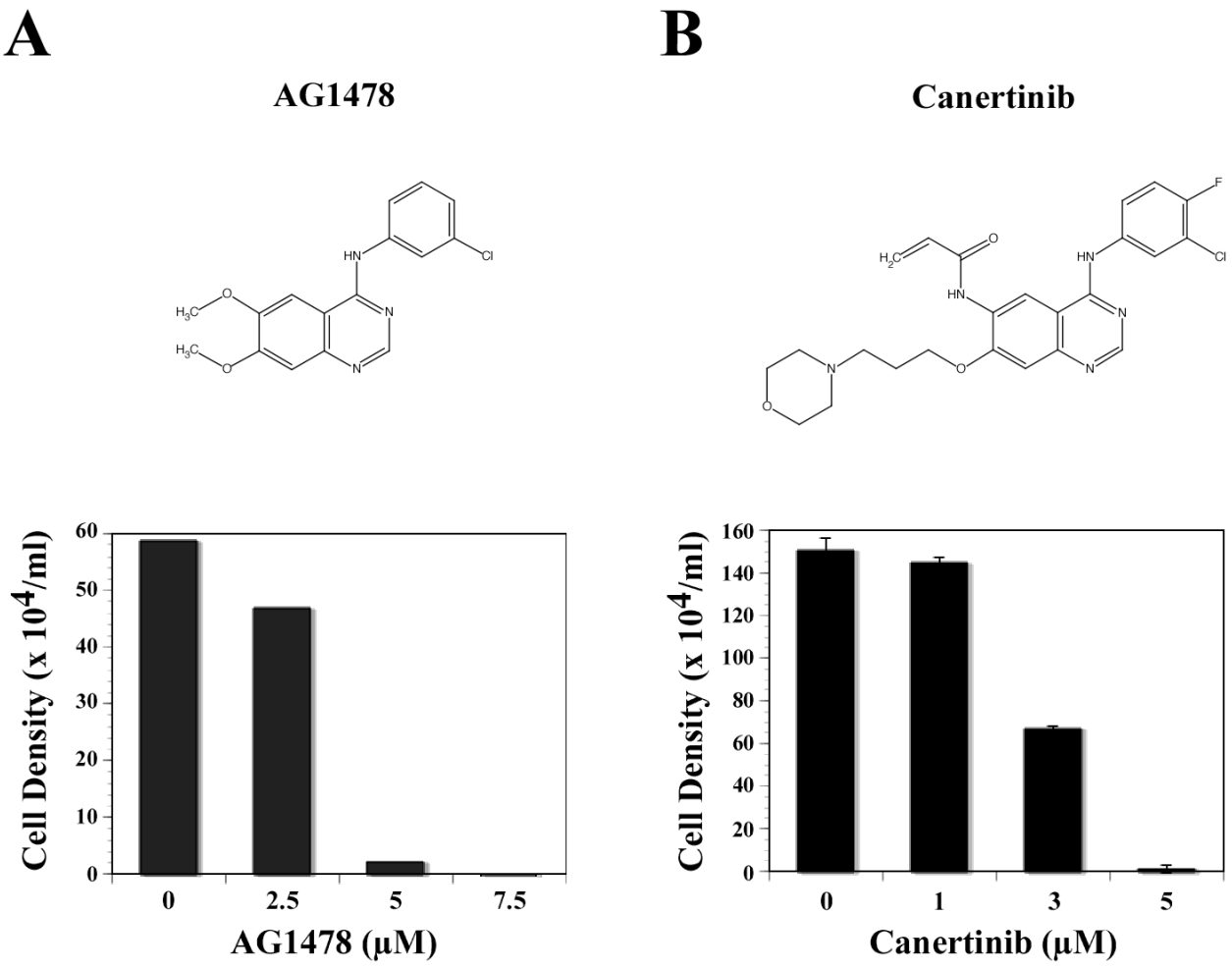

A
AG1478
B
Canertinib
Cell Density (x 10^4/ml)
AG1478 (μM)
0
2.5
5
7.5
Cell Density (x 10^4/ml)
Canertinib (μM)
0
1
3
5

Figure 3.2: The Pyrrolopyrimidines PKI166 and AEE788 Kill *T. brucei*. Indicated concentrations of **(A)** PKI166 or **(B)** AEE788 were added to bloodstream *T. brucei* (5×10^3 cells/500 µl) and the cells were cultured for 40 h. Control cells (0 µM) received DMSO, the solvent for the drugs. After 40 h, cell density was determined by hemocytometer. At 15 µM, PKI166 killed all the *T. brucei* cells (not shown). Mean ± standard deviation of duplicate samples are plotted.

Figure 3.2

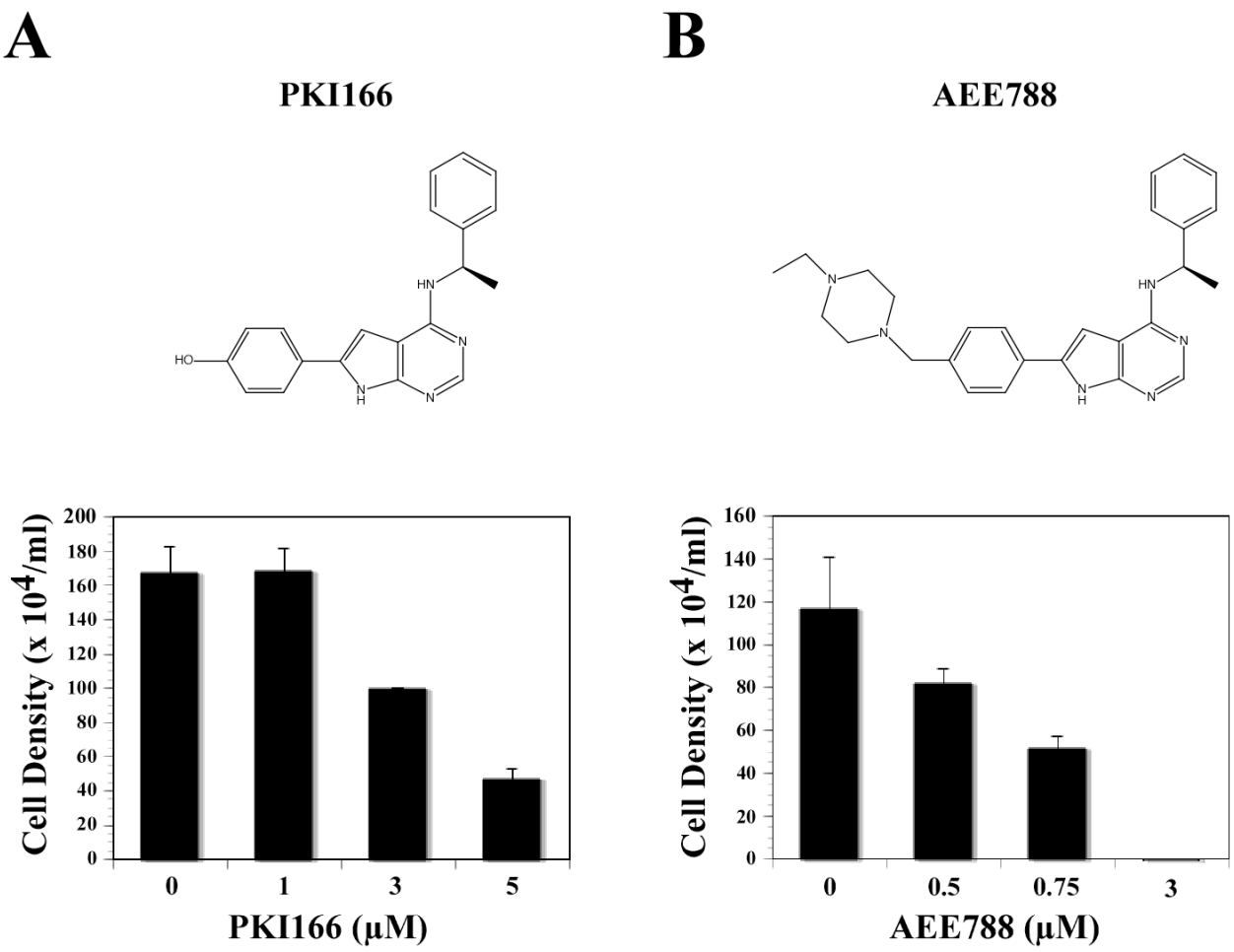

A
PKI166
HO
HN
N
N
H
N
Cell Density (x 10^4/ml)
200
180
160
140
120
100
80
60
40
20
0
0
1
3
5
PKI166 (μM)
B
AEE788
N
N
HN
N
N
H
N
Cell Density (x 10^4/ml)
160
140
120
100
80
60
40
20
0
0
0.5
0.75
3
AEE788 (μM)

Table 3.1: Identification of Putative Tyrosine Kinases in the *T. brucei* Genome. A genome-wide search for the EGFR kinase domain in the parasite proteome was performed by BLAST analysis. Next, PFAM was used to confirm protein kinase domains in the proteins discovered (not shown). The systematic ID of the 12 best candidates (those with PK (protein kinase) E-values less than 10^{-24}) is listed along with the residues that define the kinase domain. The probability of relatedness (P(N)) between the trypanosome protein (obtained from BLAST analysis) and the EGFR kinase domain is presented.

Table 3.1

Systematic ID	PK (E-value)	Relatedness to Kinase Domain (P(N))
		EGFR
Tb927.7.3580	49-201 (3.6×10^{-27})	3.4×10^{-14}
Tb927.8.5730	20-271 (5.0×10^{-29})	1.2×10^{-12}
Tb10.406.0580	342-604 (4.4×10^{-41})	1.7×10^{-11}
Tb927.5.2820	29-294 (2.6×10^{-32})	2.7×10^{-11}
Tb11.02.2050	28-278 (1.3×10^{-27})	6.3×10^{-11}
Tb10.70.5760	14-288 (1.8×10^{-25})	1.8×10^{-11}
Tb10.61.2330	4-253 (4.3×10^{-35})	2.4×10^{-10}
Tb927.3.3190	20-278 (1.8×10^{-26})	6.7×10^{-10}
Tb10.70.7860	4-258 (2.9×10^{-29})	6.8×10^{-9}
Tb927.7.6680	608-881 (6.1×10^{-28})	1.4×10^{-8}
Tb927.6.2030	240-496 (1.5×10^{-29})	4.2×10^{-9}
Tb11.46.0003	233-476 (3.9×10^{-25})	1.4×10^{-8}

Figure 3.3: AEE788 Changes Morphology of *T. brucei*. Bloodstream *T. brucei* (2×10^6 cells/ml) were treated with DMSO (-AEE788) or AEE788 (+AEE788, 30 μM) for 90 min. The cells were fixed, mounted on coverslips containing DAPI, and visualized by DIC and fluorescence microscopy. The nucleus (n) and kinetoplast (k) are labeled accordingly. The yellow bar is 6.7 microns in length.

Figure 3.3

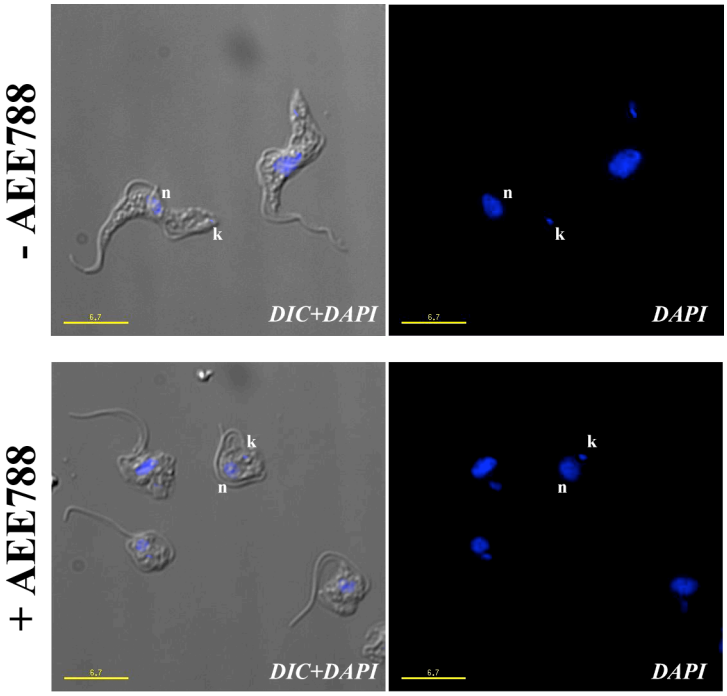


Figure 3.4: Transient Exposure of *T. brucei* to AEE788 Triggers Death. Bloodstream *T. brucei* (2 x 10^6 cells/ml) were treated with DMSO (0 μM AEE788) or AEE788 (30 μM) for 90 min. **(A)** Cells were washed, diluted (10^5 cells/ml) and cultured for 24 h or **(B)** diluted (10^4 cells/ml) and cultured for 48 h. Cells density was determined by hemocytometer. An (*) indicates that no cells were detected. Mean ± standard deviation of duplicate samples are plotted.

Figure 3.4

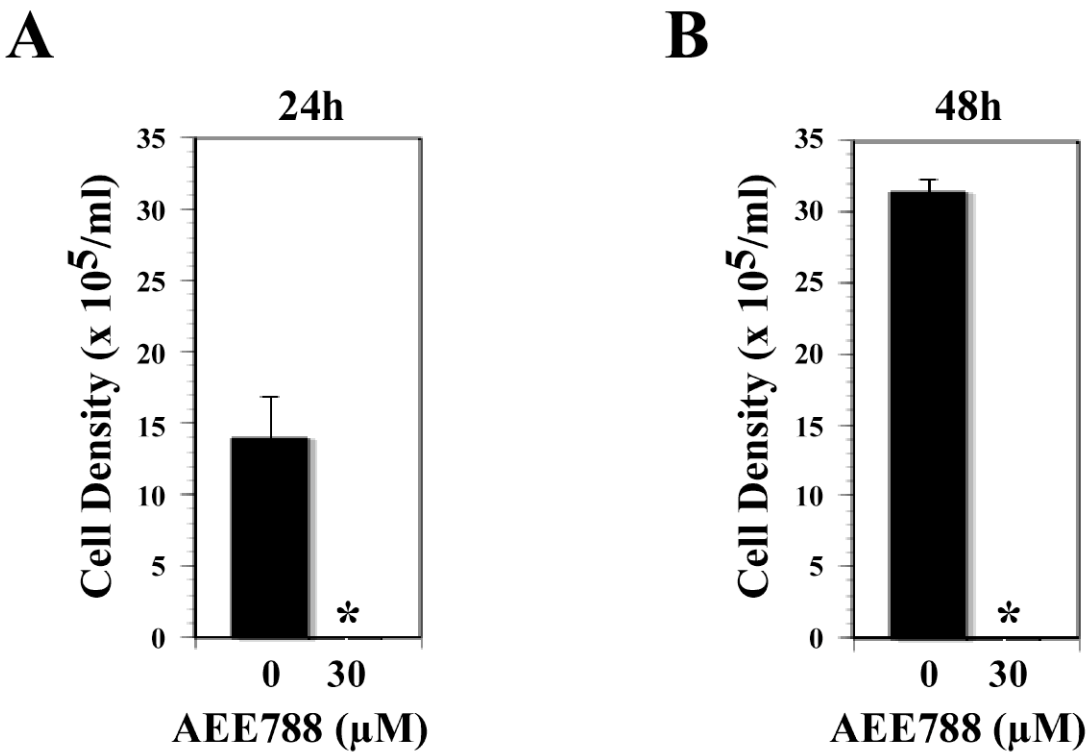

A
24h
Cell Density (x 10^5/ml)
35
30
25
20
15
10
5
0
*
0
30
AEE788 (μM)
B
48h
Cell Density (x 10^5/ml)
35
30
25
20
15
10
5
0
*
0
30
AEE788 (μM)

Figure 3.5: AEE788 Reduces Tyrosine Phosphorylation of Proteins in *T. brucei.*

Bloodstream *T. brucei* (2 x 10^6 cells/ml) were treated with DMSO (-) or AEE788 (+, 30 μM). Cells were lysed and the proteins resolved by SDS-PAGE. The proteins were transferred to Immobilion P membrane and Western blot analysis was used to detect phosphotyrosine (anti-pTyr), tubulin, BiP or GPI-PLCp. An (∗) indicates decreased phosphotyrosine (pTyr) in the band after AEE788 treatment; (■) indicates increased pTyr in the band.

Figure 3.5

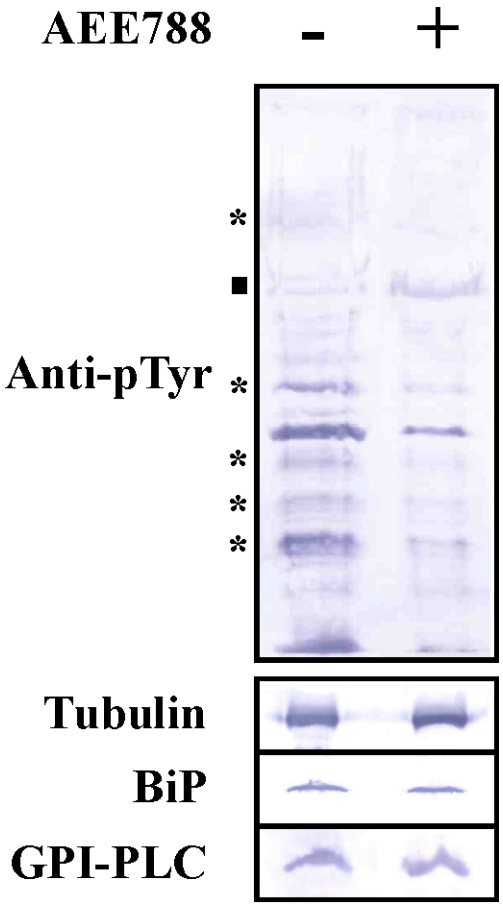


Table 3.2: Proteins Eluted with Phenylphosphate After Binding to Anti-phosphotyrosine Antibody. Bloodstream *T. brucei* (1.35 x 10^8 cells) were lysed, precleared with protein G-agarose and precipitated with PY20-agarose. Bound proteins were eluted with phenyl ethanol (control) or phenylphosphate, resolved by SDS-PAGE, digested with trypsin and analyzed by LC-MS/MS. Peptide sequences were searched against *T. brucei/T. cruzi/Leishmania* database from the NCBI. BLAST analysis against the *T. brucei* database (TrypDB) was used to determine the systematic ID of each protein. To determine which proteins were specifically eluted with phenylphosphate, proteins identified by phenyl ethanol elution were subtracted from those identified by phenylphosphate elution. Proteins specifically eluted with phenylphosphate were 'BLASTed' against the *T. brucei* database (TrypDB) to determine the systematic ID of each protein. The description and molecular weight of each protein was obtained from TrypDB. For *Proteins unique to Trypansomatidae* (F), domain information was obtained from PFAM, InterPro or SMART. (None detected) indicates that no domains have been identified in the protein. The superscript (†) indicates proteins whose phosphopeptides identified by LC-MS/MS.

Table 3.2

	Systematic Id	Description	MW (kDa)	Total Peptides	Total Spectra
(A)	**Carbohydrate Metabolism**				
	Tb927.7.7480	*Trans*-sialidase	90.2	3	2
(B)	**Endocytosis**				
	Tb10.70.0830	Clathrin heavy chain	190.4	2	2
(C)	**Protein Degradation**				
	Tb11.47.0035	Calpain-like cysteine peptidase	520.7	3	3
	Tb11.01.2050	Zinc carboxypeptidase	131.1	2	5
(D)	**Protein Synthesis**				
	Tb11.01.4660	Elongation factor 1-γ[†]	46.3	2	2
(E)	**RNA Metabolism**				
	Tb927.4.2630	ATP-dependent DEAD/H RNA helicase[†]	97.2	2	2
(F)	**Proteins Unique to Trypanosomatidae**				
		Domain(s) Present			
	Tb927.7.3330	Helix-loop-helix DNA binding, t-SNARE, prefoldin domain	227.7	2	2
	Tb09.160.0520	Prefoldin domain	182	2	2
	Tb10.70.7440	None detected	71	1	1
	Tb11.02.3670	None detected	63.9	3	5

Table 3.3: Non-Specifically Associated Bloodstream *T. brucei* Proteins Enriched by Anti-Phosphotyrosine Affinity Chromatography. Bloodstream *T. brucei* (1.35 x 10^8 cells) were treated with pervanadate, lysed and precipitated with either protein G-agarose (control) or PY20-agarose. Bound proteins were eluted with SDS-sample buffer, resolved by SDS-PAGE, digested with trypsin and analyzed by LC-MS/MS. Peptide sequences were searched against *T. brucei/T. cruzi/Leishmania* database from the NCBI. Identified proteins that bound to both protein G-agarose and PY20 (67 total) were analyzed and proteins that were enriched at least 5-fold (by comparing the number of total peptides identified from Protein G and PY20) were 'BLASTed' against the *T. brucei* database (TrypDB) to determine the systematic ID of each protein. The description and molecular weight of each protein was obtained from TrypDB. For *Proteins unique to Trypansomatidae* (F) the domain information was obtained from TrypDB.

Table 3.3

	Systematic Id	Description	MW (kDa)	Total Peptides	
				Protein G	PY20
(A)	**DNA/RNA Metabolism**				
	Tb927.8.1510	ATP-dependent DEAD/H RNA helicase	62.4	5	40
	Tb10.61.2130	ATP-dependent DEAD/H RNA helicase	71.3	5	34
	Tb09.211.2150	Polyadenylate-binding protein 1	62.1	4	22
	Tb927.2.340	Retrotransposon hot spot protein	97.8	2	22
	Tb11.02.3670	Retrotransposon hot spot protein 6a	63.9	3	16
	Tb927.1.120	Retrotransposon hot spot protein 41	98	2	24
(B)	**Endocytosis, Organelle Biogenesis and Acidification**				
	Tb10.70.1190	Valosin-containing protein homolog	85.8	4	22
(C)	**Lipid Metabolism**				
	Tb09.211.3550	Glycerol kinase	56.3	9	111
	Tb927.3.3580	Lipophosphoglycan biosynthetic protein	87.7	2	17
(D)	**Protein Chaperones**				
	Tb927.7.710	70kDa heat shock protein	70.2	1	14
(E)	**Protein Synthesis**				
	Tb927.5.1090	Threonyl-tRNA synthetase	90.9	2	13
(F)	**Proteins Unique to Trypanosomatidae**				
		Domain(s) Present			
	Tb927.6.4770	PIN domain-like superfamily	83.3	2	11

Table 3.4: Bloodstream *T. brucei* Proteins Enriched by Anti-phosphotyrosine Affinity Chromatography. Bloodstream *T. brucei* (1.35×10^8 cells) were treated with pervanadate, lysed and precipitated with either protein G-agarose (control) or PY20-agarose. Bound proteins were eluted with SDS-sample buffer, resolved by SDS-PAGE, digested with trypsin and analyzed by LC-MS/MS. Peptide sequences were searched against *T. brucei/T. cruzi/Leishmania* database from the NCBI. To determine which proteins were specifically bound to PY20-agarose, identified proteins bound to protein-G agarose were subtracted from proteins bound to PY20-agarose. PY20-specific proteins were 'BLASTed' against the *T. brucei* database (TrypDB) to determine the systematic ID of each protein. The description and molecular weight of each protein was obtained from TrypDB. For *Proteins unique to Trypansomatidae* (N) the domain information was obtained from PFAM, InterPro or SMART. (None detected) indicates that no domains have yet been identified in the protein.

Table 3.4

	Systematic Id	Description	MW (kDa)	Total Peptides	Total Spectra
(A)	**Amino Acid Metabolism**				
	Tb09.160.4560	Arginine kinase	44.7	5	14.1
	Tb927.2.4610	Branched-chain amino acid aminotransferase	40.4	41	11.6
	Tb927.6.2790	L-threonine 3-dehydrogenase	36.9	2	10.6
	Tb927.7.7040	Methylthioadenosine phosphorylase	33.4	1	1.6
(B)	**Carbohydrate/Glucose Metabolism**				
	Tb10.6k15.2620	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	60.6	14	29.4
	Tb11.01.3040	Cytosolic malate dehydrogenase	35.1	8	17.7
	Tb10.70.4740	Enolase	46.5	23	54
	Tb10.70.1370	Fructose-bisphosphate aldolase	41	114	70.8
	Tb927.1.3830	Glucose-6-phosphate isomerase	67.6	35	58.2
	Tb927.6.4280	Glyceraldehyde 3-phosphate dehydrogenase	43.8	28	14.6
	Tb10.6k15.2040	Hexose transporter	56.4	5	9
	Tb927.1.700	Phosphoglycerate kinase	47.2	17	37.3
	Tb10.61.2680	Pyruvate kinase	94.9	22	45
	Tb927.7.7480	*Trans*-sialidase	90.2	2	4.1
(C)	**Cell Morphology and Motility**				
	Tb927.1.2380	Alpha tubulin	49.7	34	49.7
	Tb927.1.2370	Beta tubulin	49.7	29	46.4
	Tb11.02.0030	Dynein heavy chain	484.9	2	2
	Tb927.4.3740	Flagellar attachment zone protein	192.5	4	10
	Tb927.3.5490	Flagellar transport protein	77.6	1	5.9
	Tb11.55.0006	Intraflagellar transport protein IFT88	89.7	8	9
	Tb927.6.1770	Kinesin	69.4	3	8.3
	Tb10.6k15.2330	T-complex protein 1, θ-subunit	58	12	18
(D)	**DNA/RNA Metabolism**				
	Tb10.406.0020	Argonaute-like protein 1	97.9	20	51
	Tb09.211.3510	ATP-dependent DEAD/H RNA helicase	82.7	5	6
	Tb927.6.5110	Damage-specific DNA binding protein	138.2	5	9
	Tb927.4.3810	DNA-directed RNA polymerase II, subunit 2	134.5	5	12
	Tb10.70.5840	Major vault protein	95	3	4
	Tb10.70.6450	NOT1 transcription regulatory protein	259.1	5	5
	Tb09.211.0930	Polyadenylate binding protein 1	63	3	9.0
	Tb10.26.0140	Pumilio RNA-binding protein	93	4	7
	Tb927.1.120	Retrotransposon hot spot protein	98	22	45
	Tb10.70.2470	Ribonuclease II	53.9	1	1.5
	Tb11.01.5570	RNA binding protein	28.7	6	16.5
	Tb927.8.7400	RNA polymerase IIA, largest subunit	196.7	3	5

	Systematic Id	Description	MW (kDa)	Total Peptides	Total Spectra
	Tb927.7.1370	Spliced leader RNA PSE-promoter transcription factor	68.8	2	0.5
	Tb927.3.2820	TFIIF-stimulated CTD phosphatase	62.3	5	8.0
	Tb927.2.2240	tRNA exportin	134.8	1	1
	Tb927.8.3750	U3 snoRNP component	54.3	1	5.9
(E)	**Endocytosis, Organelle Biogenesis and Acidification**				
	Tb10.61.0840	ABC Transporter	67.5	2	2.4
	Tb927.5.3400	Calcium-translocating P-type ATPase	110.3	5	8
	Tb10.70.0830	Clathrin heavy chain	190.6	4	12
	Tb11.02.2410	Clathrin coat assembly protein (ANTH domain family)	59.4	2	8
	Tb11.01.3740	Coatomer (γ-subunit)	97.5	1	2
	Tb11.0890	Epsin	53.9	8	17.5
	Tb927.8.3660	Sec23	106.6	1	2
	Tb11.02.4040	Sec31	131.1	1	1
	Tb10.389.1170	P-type-H^+-ATPase	100.6	3	3
	Tb927.4.1080	V-type-ATPase, (A subunit)	67.7	3	7.9
(F)	**Lipid Metabolism**				
	Tb927.8.7100	Acetyl-CoA carboxylase	242.9	6	5
	Tb10.70.4200	Acyl-CoA synthetase	78.9	14	29.6
	Tb927.8.3530	Glycerol 3-phosphate dehydrogenase	37.8	18	35.6
	Tb927.4.1920	GPI transamidase component GPI16	75.8	3	5.4
(G)	**Nucleotide Metabolism**				
	Tb927.5.3800	Carbomyl-phosphate synthetase	204	7	10
(H)	**Plasma Membrane Proteins**				
	Tb927.2.3280	Invariant surface glycoprotein	48.2	4	11.2
(I)	**Protein Degradation**				
	Tb11.02.0070	Aminopeptidase	60.5	6	8.5
	Tb927.2.5980	ATP-dependent Clp protease	97	10	14
	Tb10.70.7090	Serine carboxypeptidase III precursor	51.5	2	5.3
(J)	**Protein Modification**				
	Tb11.01.1680	Polyubiquitin	76.6	6	30
(K)	**Protein Chaperones**				
	Tb11.01.3110	70kDa heat shock protein	75.3	12	56.0
	Tb927.8.7410	Calreticulin	45	10	18.1
	Tb927.6.4090	Chaperonin HSP60	58.3	1	3.2
	Tb11.02.5500	Glucose-regulated protein 78	71.4	31	26
(L)	**Protein Synthesis**				
	Tb09.244.2630	40S ribosomal protein S6	28.4	2	6.4
	Tb927.3.5050	60S ribosomal protein L4	41.8	10	22.7
	Tb10.389.0630	Branched-chain aminoacyl-tRNA synthetase	90.9	11	16.7
	Tb927.6.1880	Aspartyl-tRNA synthetase	62.9	11	23.7

	Systematic Id	Description	MW (kDa)	Total Peptides	Total Spectra
	Tb09.160.3270	Eukaryotic initiation factor 4A	45.3	24	24.1
	Tb10.6k15.1220	Isoleucyl-tRNA synthetase	130.6	3	4
	Tb11.02.1210	Leucyl-tRNA synthetase	121.8	4	4
	Tb927.8.1600	Lysyl-tRNA synthetase	66.7	7	16.7
	Tb10.70.6470	Methionyl-tRNA synthetase	86.9	7	9
	Tb927.4.3570	Translation elongation factor 1-β	28.4	2	7.3
	Tb927.6.4480	Valyl-tRNA synthetase	110.9	7	9
(M)	**Signal Transduction**				
	Tb11.02.4860	Calmodulin-activated protein kinase kinase (CAMKK)	75.8	1	1.5
	Tb10.61.3140	Glycogen synthase kinase 3 (GSK3)	40.3	12	13.5
	Tb11.01.3180	Guanine nucleotide-binding protein	34.6	6	16.9
	Tb927.6.1780	Mitogen-activated protein kinase (MAPK)	46.1	4	11.9
	Tb10.389.1730	Mitogen-activated protein kinase	47.3	25	50.9
	Tb09.160.4770	NF-κB-activating protein kinase (NAK)	76.4	2	3
	Tb927.8.6930	NIMA-related kinase A (NRKA)	47.9	1	2.6
	Tb09.211.4890	Protein kinase CK2 alpha (casein-kinase 2)	41	11	21.8
	Tb11.01.2590	Protein kinase CK2 beta	35.5	4	8.8
	Tb927.6.4970	Serine/Arginine protein kinase	82.6	8	16
	Tb927.2.2720	STE11/Mitogen-activated protein kinase kinase kinase (MAPKKK)	162.1	5	7
(N)	**Proteins Unique to Trypanosomatidae**				
		Domain(s) Present			
	Tb10.6k15.0640	C2 domain	132.5	5	9
	Tb927.8.5580	DysF domain, galactose-binding like	526.4	3	5
	Tb10.70.5020	Zinc finger (C2H2-type) domain	84.6	3	4
	Tb09.211.1070	Zinc finger (CCCH-type) domain, Phosphopantetheine attachment site	114.7	2	5
	Tb09.211.1390	Hsp70 domain	79.8	7	9
	Tb09.211.1620	SET, post-SET zinc binding region	160.6	7	8
	Tb10.61.2260	WD40-repeat domain	186.6	4	5
	Tb11.01.3290	Tetratricopeptide-like helical, CS domain	68.9	3	11.8
	Tb11.01.5680	Nucleotide-binding domain	51.6	10	19.3
	Tb11.01.5690	Nucleotide-binding domain, RNA recognition motif	54.7	27	35.2
	Tb11.01.5780	Tudor-Stapholoccal nuclease domain, RNA-induced silencing complex	100.4	12	23
	Tb11.01.6200	P-loop containing nucleoside triphosphate hydrolase domain	70.3	4	9.6
	Tb11.01.6550	Ribosomal protein L29 conserved site	363.3	5	5
	Tb11.01.7010	HEAT-repeat domain	117.8	6	13
	Tb11.02.0690	LisH dimerization motif, Quinoprotein amine dehydrogenase β chain-like domain	184.7	5	10
	Tb11.02.0810	T-complex protein 10 (C-terminal)	107.8	3	5
	Tb11.46.0009	Zinc finger (CCCH-type) domain, DNA/RNA helicase (C-terminal)	58.4	6	11.1

Systematic Id	Domain(s) Present	MW (kDa)	Total Peptides	Total Spectra
Tb927.1.4310	PDZ/DHR/GLGF domain	183.7	8	13
Tb927.3.3560	Zinc finger (RING/FYVE/PHD-type), U-box domain	69	15	23.8
Tb927.3.4070	Major facility superfamily 1 domain	64.3	2	5
Tb927.4.2080	C2 domain, lipid-binding region	104.8	5	6
Tb927.5.3330	Ribosomal protein S2 domain	521.7	5	1.8
Tb927.6.2210	Armadillo/beta-catenin-like repeat	123.8	3	4
Tb927.6.4440	NTF2-like superfamily domain	37.7	5	20.4
Tb927.8.3680	BRCT domain	69.5	20	38.1
Tb927.8.4540	Ataxin-2 domain (N-terminal)	59.5	15	18.9
Tb09.160.1150	None detected	15.1	1	1.7
Tb927.8.3840	None detected	80	5	7
Tb927.8.1470	None detected	62.3	6	8.8
Tb927.6.4100	None detected	94.3	11	26
Tb927.6.1910	None detected	56.6	11	15
Tb927.4.710	None detected	30.4	2	2
Tb927.3.1590	None detected	72.6	75	111.2
Tb927.1.3940	None detected	101.7	3	3
Tb11.02.4300	None detected	48.8	25	39.4
Tb11.03.0530	None detected	31.2	9	28.2
Tb10.70.7880	None detected	65.6	4	4
Tb10.70.6480	None detected	134.8	14	5
Tb10.61.3130	None detected	74.6	7	16.5
Tb10.61.2850	None detected	50.4	5	9.7
Tb09.160.0400	None detected	71.8	13	24.1
Tb09.211.4200	None detected	38.5	2	2
Tb927.7.3700	None detected	33.7	1	1

Figure 3.6: Sequence Alignments of Catalytic and Activation Loops of Some Protein Kinases Detected on an Anti-phosphotyrosine Affinity Column. (A) Alignment of the catalytic loops. Catalytic residues are in bold lettering. **(B)** Alignments of "activation loops". The conserved DFG and APE (or tolerated variants), at the N- and C-termini of the activation loop are in bold lettering. Phosphorylatable tyrosine (Y) in the loop is highlighted in yellow. Residues in lowercase or numbers in parentheses indicate additional residues between each segment. CamKK, calcium-calmodulin dependent protein kinase kinase; GSK3, glycogen synthase kinase 3; MapK, mitogen-activated kinase; NAK, NF-κB-activating protein kinase; NRKA, NIMA-related kinase A; Ste11, MapK kinase kinase.

Figure 3.6

A

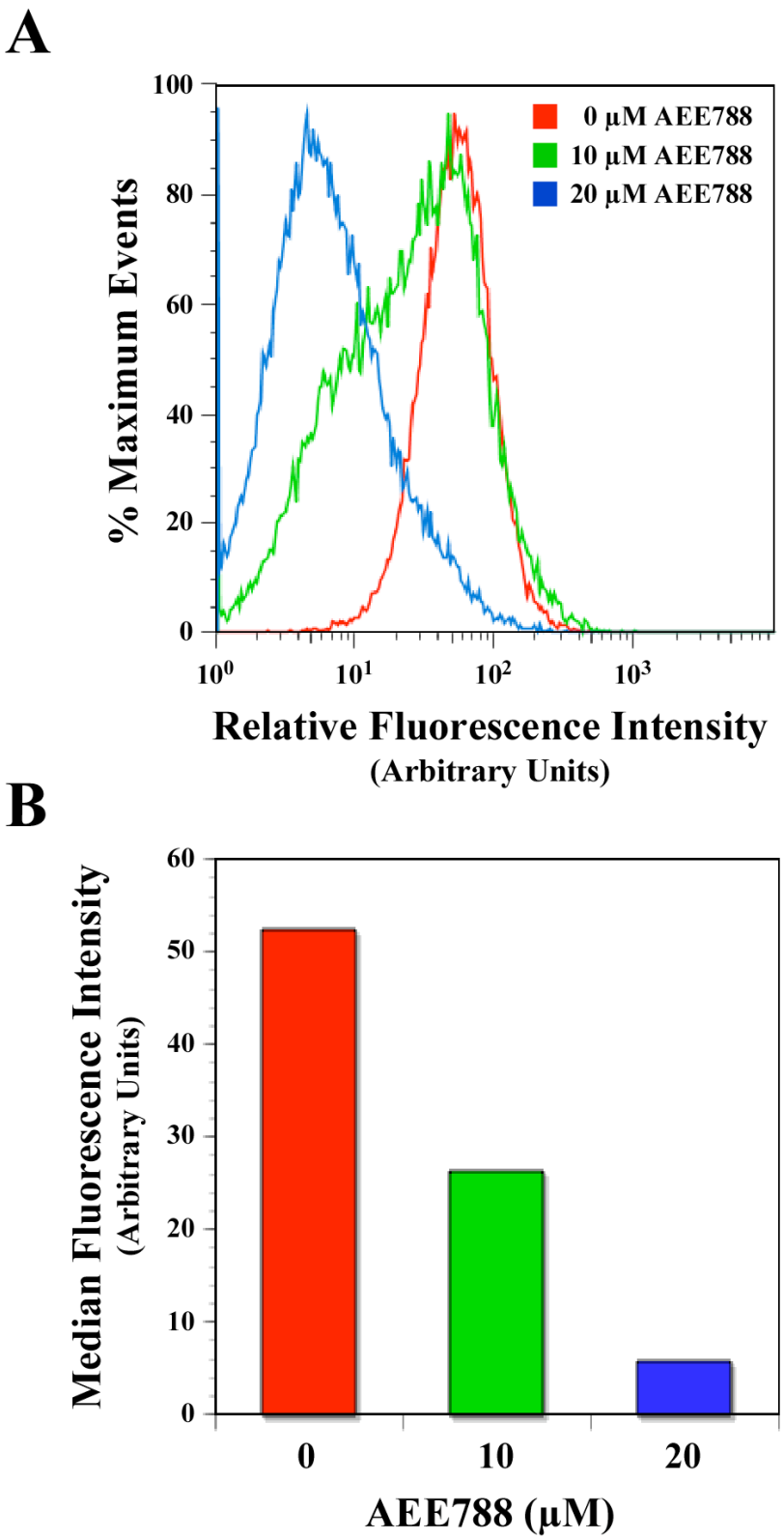
		Catalytic Loop
Tb11.02.4860-CAMKK	305	KIA**HRD**IKPE**N**ILVSK
Tb10.61.3140-GSK3	146	NIC**HRD**IKPH**N**VLVDE
Tb10.389.1730-MAPK	144	GVI**HRD**ITPA**N**ILVNK
Tb927.6.1780-MAPK	151	DII**HRD**LKPA**N**ILTDS
Tb09.160.4770-NAK	146	PVA**HRN**INPS**N**VLIHS
Tb927.8.6930-NRKA	142	KML**HRD**IKSA**N**VLLTS
Tb927.2.2720-STE11	1240	HIT**HRD**LKPE**N**ILFRD

B

		Activation Loop
Tb11.02.4860-CAMKK	326	LA**DFG**VAE...VFDVSARE(29)TKGTILFI**APE**IW
Tb10.61.3140-GSK3	169	LC**DFG**SAK...RLAADEFNva..**Y**ICSRYYR**APE**LI
Tb10.389.1730-MAPK	165	IC**DFG**LSKeesDQGEHMTD....**Y**VTMRWYR**APE**LV
Tb927.6.1780-MAPK	172	VC**DFG**LAR...GVGVNVTS....**Y**VVTRWYR**PPE**LL
Tb09.160.4770-NAK	171	LY**NFR**SAM...TEAYHCEN(12)**Y**TTAGYRA**PEM**LD
Tb927.8.6930-NRKA	162	LG**DFG**FSH...TVSGVVASt...FCGTPYYL**APE**LW
Tb927.2.2720-STE11	1260	VS**DFG**TAV...QRDSGLKN....TRGTLAYM**APE**VL

Figure 3.7: AEE788 Inhibits Endocytosis of Transferrin in *T. brucei*. (A) Bloodstream *T. brucei* (2 x 10^6 cells/ml) were treated with DMSO (0 μM AEE788) or AEE788 (10 or 20 μM) for 30 min in serum-free HMI-9 medium. Transferrin-Alexa Fluor 488 (25 μg) was added, the cells were incubated for 30 min and the relative fluorescence measured using flow cytometry. **(B)** Median fluorescence intensity (per 45,000 cells) is plotted for each treatment described in panel A. A representative result from duplicate studies is shown.

Figure 3.7


A
100
80
60
40
20
0
% Maximum Events
0 µM AEE788
10 µM AEE788
20 µM AEE788
10^0
10^1
10^2
10^3
Relative Fluorescence Intensity
(Arbitrary Units)
B
60
50
40
30
20
10
0
Median Fluorescence Intensity
(Arbitrary Units)
0
10
20
AEE788 (µM)

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CHAPTER 4

DISCUSSION

The extracellular surface of *Trypanosoma brucei's* plasma membrane is covered with glycosyl phosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG) [1]. This coat is essential for the parasite's survival in the mammalian host [2, 3]. Bloodstream *T. brucei* express a GPI-specific phospholipase C (GPI-PLCp) [4-6] that can cleave the GPI-anchor [6, 7]. Interestingly, in non-differentiating bloodstream parasites, very little VSG is released [8, 9].

GPI-PLCp is a membrane protein [5-7, 10]. However, prior to this study, the subcellular location of GPI-PLCp was not completely resolved [11, 12]. Recently, several functions have been attributed to GPI-PLCp in non-differentiating bloodstream cells. The enzyme stimulates endocytosis of transferrin [13], and contributes to stress-induced cleavage of intracellular GPIs [14]. However, how the enzyme participates in these processes is not known. To further understand the biological function of GPI-PLCp in *T. brucei*, it is valuable to determine the intracellular location of the protein.

In this work we used fluorescence microscopy and density-gradient centrifugation to determine that GPI-PLCp is a glycosomal protein. Sequestration of GPI-PLCp to the glycosome, away from the plasma membrane, may explain why little or no VSG is released under physiological conditions in non-differentiating *T. brucei*.

The glycosome targeting signal of GPI-PLCp remains to be identified. Membrane proteins of glycosomes are expected to target to the organelle by membrane peroxisomal targeting sequences (mPTS) composed of a transmembrane domain and a PEX19-binding motif [15]. However, it is unlikely that GPI-PLCp contains a mPTS due to the lack of a transmembrane domain. Therefore we hypothesize that the enzyme either (i) has a novel targeting sequence (yet to be identified), or (ii) associates with the endoplasmic reticulum membrane initially and is subsequently sorted to the glycosome. The latter hypothesis is based on recent findings that peroxisome membranes are derived from the ER (reviewed in [16]) and some peroxisomal membrane proteins are first directed to the ER before being sorted to the peroxisome [17, 18]. Further work should be done to determine how GPI-PLCp is targeted to glycosomes.

Tyrosine kinase activity is present in *T. brucei* [26-30] and is important for cell viability [26]. We have found that some drugs, based on the 4-anlinoquinazoline and pyrrolopyridmine scaffolds, that inhibit specific tyrosine kinases kill cultured *T. brucei*. Furthermore, within 90 min of exposure, the pyrrolopyrimidine AEE788 reduces tyrosine phosphorylation *in vivo*, reduces endocytosis of transferrin, causes a morphological change in the parasite, and triggers cell death. These studies validate PTK activity as a valid target for drug discovery and establish the pyrrolopyrimidine scaffold as a good lead for drug discovery against *T. brucei*. Studies of other derivatives of pyrrolopyrimidines may reveal compounds that are more potent against *T. brucei* than they are against human cells. Such compounds would be the best candidates for lead drug development against the parasite.

In *T. brucei*, little is known about PTKs (none have been directly characterized biochemically), their substrates, or pathways that are regulated by the enzymes. To understand how inhibition of these PTKs might kill the parasite it is necessary to determine which proteins contain phosphotyrosine (pTyr). Using anti-pTyr affinity chromatography and LC-MS/MS, we identified 134 proteins involved in several biological pathways as putative Tyr-phosphorylated polypeptides. With the identification of these proteins we now have the tools to begin asking very specific questions regarding the role of these proteins in tyrosine kinase signaling pathways.

Finally, the tyrosine kinases inhibited by AEE788 have not been identified. The identification of these kinases would provide a better understanding of how the drugs affect *T. brucei*. Moreover, because tyrosine kinases have not been characterized in the parasite, identifying these tyrosine kinases would also contribute to understanding the biological function of these enzymes in *T. brucei*.

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