THE EFFECT OF TYROSINE KINASE INHIBITORS IN *TRYPANOSOMA BRUCEI*

by

ARIEL BIANCA LANE

(Under the direction of Kojo Mensa-Wilmot)

ABSTRACT

Human African trypanosomiasis (HAT) is caused by the protozoan parasite *Trypanosoma brucei*. This disease affects millions of people throughout Africa. The drugs used against this disease are toxic, have shown signs of resistance, and are difficult to administer, prompting the need for safer more effective drugs.

There is evidence of both tyrosine kinase activity and tyrosine phosphorylated proteins in *T. brucei*. Bioinformatic analysis suggests that tyrosine kinase-like domains are also present in *T. brucei*. Protein tyrosine kinases have become a target to fight some cancers; tyrosine kinases inhibitors (TKI) are currently being used in some treatments. Lastly, tyrphostin A47, a general tyrosine kinase inhibitor has been shown to inhibit growth of *T. brucei*.

To elucidate how TKIs kill *T. brucei*, we used 2 TKIs lapatinib and SU14813, with different targets/pathways in mammalian cells, and measured their function in inhibition of growth, cell morphology, protein phosphorylation, and endocytosis. Lapatinib and SU14813 were used in cell growth inhibition experiments and shown to both have IG_{50} in the 1-2 micromolar range. To establish whether or not a cell shape change was evident, we used differential interference contrast (DIC) along with light scatter plots from flow cytometry and found that cells treated with lapatinib, but not SU14813 demonstrated a rounded up phenotype compared to control cells. Effect in tyrosine phosphorylation was revealed through western blot

analysis using PTyr100, an antibody that recognizes phosphorylated tyrosines. Both TKIs exhibited decrease in tyrosine phosphorylated proteins, with lapatinib having a stronger effect. Receptor-mediated endocytosis was tested on cells treated with the TKIs and only lapatinib seemed to exhibit a block in uptake of transferrin. These data confirm PTKs as drug discovery targets in *T. brucei* and gives some clues as to the mechanisms by which they cause cell death.

INDEX WORDS: *Trypanosoma brucei,* Human African Trypanosomiasis, Protein tyrosine kinase, Tyrosine kinase inhibitors, tyrosine phosphorylation, lapatinib, SU14813

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1 *Trypanosomes*

1.1 Human African Trypanosomiasis

Trypanosoma brucei is an obligate parasite that causes African trypanosomiasis or sleeping sickness in humans and nagana in animals. The estimated number of human sleeping sickness cases is currently between 50,000 and 70,000, with the prevalence of disease differing from one country to another (WHO 2006). Human African trypanosomiasis (HAT) is caused by two subspecies of *T. brucei*: *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. The former represents the majority of the cases reported and causes a chronic and often asymptomatic state; people are often infected for years without any signs. *T. b. rhodesiense* causes an acute infection and symptoms often develop within the first few weeks or months. Accordingly, the disease progression of *T. b. gambiense* is slower than *T. b.rhodesiense*. Screening the population in Africa for sleeping sickness requires many scarce resources, resulting in many undiagnosed individuals, particularly infected with *T. b. gambiense*, spreading disease unknowingly and eventually dying. In both the acute and chronic disease, the trypanosomes first multiply in the subcutaneous tissues, blood and lymph, eventually crossing the blood brain barrier into the central nervous system. Thus, there are two stages of African trypanosomiasis: the haemolymphatic and neurological stage. The first stage is characterized by fevers, headaches and joint pains. The second stage, when the parasites have entered into the central nervous system, includes confusion, poor coordination and disturbance of sleep cycle (HAT reviews: (Smith, Pepin et al. 1998; Fevre, Wissmann et al. 2008; Welburn, Maudlin et al. 2009).

1.2 Human African Trypanosomiasis Chemotherapy

Treatment of African sleeping sickness is dependent on the stage of the disease. Drugs used during the haemolymphatic stage are less toxic and are relatively effective. The earlier the identification of infection, the greater the chance of survival. This is complicated by the often long asymptomatic, first stage of infection from *T. b. gambiense*. Pentamidine is often used during the first stage for individuals infected with *T. b. gambiense* and suramin for individuals infected with *T. b. rhodesiense* (Denise and Barrett 2001). Melarsoprol is one drug used for the second stage; a combination of Nifurtimox and Eflornithine (DFMO) is an alternative to Melarsoprol, but only works with infections caused by *T. b. gambiense (Baral).* Adverse side effect as well as increase in resistance to some drugs exacerbates the necessity to create new drugs for use against HAT(reviewed in (Denise and Barrett 2001)).

1.3 ***Trypanosoma brucei Life Cycle***

African trypanosomiasis is transmitted through the tsetse fly which acquires its infection from a human or animal already harboring the parasites. Once an infected teste fly takes a bite of a mammalian host it injects metacylic trypomastigotes into skin tissue; the parasites pass into the bloodstream and transform into bloodstream trypomastigotes. They continue replicating through binary fission and travel to the blood and lymph. An uninfected tsetse fly takes a blood meal and is infected with bloodstream trypomastigotes, which transform into procyclic trypomastigotes in the fly's midgut. The parasites continue to multiply, eventually leaving the midgut and transforming into epimastigotes. The epimastigotes reach the salivary gland and continue to replicate; once the now infected tsetse fly takes another bite of a mammalian host the cycle continues (summarized by Vickerman 1985; CDC 2006)(Figure 1).

2 Antigenic Variation

An important aspect of *T. brucei* cell structure is its plasma membrane being covered by variant surface glycoproteins (VSG) in the bloodstream form. Approximately 10^7 molecules of VSG cover the trypanosome's surface, permitting host immune evasion enabling chronic infection. The VSG coat shields the innate human immune system from coming into contact

with the plasma membrane, which displays surface antigens. In addition, the VSG coat frequently switches the variants expressed on the surface; different VSGs forces the immune system to continuously respond and create a new immune response to different VSGs. Variant surface glycoprotein switching often is called antigenic variation (Vanhamme, Lecordier et al. 2001; Stockdale, Swiderski et al. 2008). Because it takes several days for an immune response to begin, VSG coat switching prolongs the life of the infecting parasite population. Thousands of VSG genes are located in *T. brucei*'s genome, facilitating *T. brucei*'s survival and the inability of the production of a vaccine (Baral ; Luscher, de Koning et al. 2007).

3 Endocytosis

T. brucei relies on the process of endocytosis from its host for most of its nutrients. Endocytosis is the process in which cells internalize materials from their surroundings by formation of membrane vesicles at the plasma membrane. Endocytosis is very important in eukaryotes in general, regulating many processes including cell adhesion, cell polarity, cell signaling and drug delivery. In addition, endocytosis aids the parasite in clearing host antibodies from its membrane, thus prolonging survival (Morgan, Hall et al. 2002). There are three main types of endocytosis: phagocytosis, pinocytosis and receptor-mediated (Figure 2). Pinocytosis and phagocytosis are non-receptor mediated, but only pinocytosis is nonspecific for what particles they engulf. Phagocytosis is defined as the internalization of solid particles and is involved in acquiring nutrients and removing pathogens. Pinocytosis (fluid-phase endocytosis) is the transport of small particles into the cell via small vesicles (reviewed in (Mellman 1996; Mukherjee, Ghosh et al. 1997). Receptor-mediated endocytosis is specific for the particles that are engulfed. Bloodstream *T. brucei* performs both fluid-phase and receptor-mediated endocytosis, with a higher rate of endocytosed material in the latter (Coppens, Opperdoes et al. 1987). In addition, bloodstream form *T. brucei* have a higher rate of endocytosis compared to its procyclic counterpart (Morgan, Hall et al. 2002).

An important growth factor for *T. brucei* is transferrin. Iron is essential for the vitality of *T. brucei*; the iron chelator deferoxamine inhibits the growth of bloodstream forms of *T. brucei* 10 times more compared to mammalian cells (Coppens, Opperdoes et al. 1987; Steverding 1998; Breidbach, Scory et al. 2002). The transferrin receptor is made up of two subunits: ESAG6, which is anchored to the membrane by a glycosylphosphatidylinositol (GPI) and ESAG7, a peripheral membrane protein. The proteins of ESAG6 and ESAG7 form a GPI-transferrin binding protein (TFBP) complex. Within the endocytosis pathway, the transferrin along with the TFBP are separated in the lysosome, where the TFBP complex is recycled back to the surface and the transferrin degraded, making iron available for use to the parasite; this process of uptake of transferrin in *T. brucei* is novel compared to iron retrieval in mammalian cells; transferrin is degraded after uptake in trypanosomes, but is recycled to the cell surface in mammalian cells (Steverding, Stierhof et al. 1995).

In *T. brucei* the vesicles are engulfed into the flagellar pocket (FP), an invagination of the membrane. These clathrin coated pits define an endocytosis pathway and are essential in *T. brucei* (Allen, Goulding et al. 2003). The polarized cell of *T. brucei* is required to maintain the process of endocytosis and exocytosis. After a ligand binds to its receptor clathrin assembly occurs followed by membrane invagination. The ligand and receptor are then engulfed by these clathrin-coated pits and eventually uncoat and fuse with other vesicles to create the early endosome (Morgan, Hall et al. 2002). The endocytic pathway is characterized by different compartments that are responsible for either recycling the molecules back to the surface (early and recycling endosomes) or degrading them (late endosomes and lysosomes). The route begins at the early endosome, which is the site of cargo sorting in the endocytic pathway. Here receptors and cargo proteins are separated due to the acidic pH of the endosome and receptors recycled back to the surface. The early endosome fuses with another compartment termed the late endosome; its contents eventually travel to the last stop in the endocytic pathway: the lysosome. The lysosome contains lysosomal hydrolases and thus here all of its contents are

degraded. (Review of Endocytosis Bloom and Puszkin 1981; Besterman and Low 1983; Mellman 1996; Morgan, Hall et al. 2002; Morgan, Hall et al. 2002).

3.1 Protein Components of the Endocytic Pathway

Clathrin is an essential protein in all eukaryotes. In bloodstream *T. brucei*, reduced expression of clathrin by RNAi results in an oversized flagellar pocket, accumulation of the endocytotic markers conconavilin A, a lectin protein used to characterize glycoproteins and FM4-64, a dye that embeds into vesicles upon endocytosis stimulation and cessation of continued movement through the trans-Golgi network (TGN) (Allen, Goulding et al. 2003). Another protein found to be important in *T. brucei* endocytosis is actin (Garcia-Salcedo, Perez-Morga et al. 2004). Although found in both procyclic and bloodstream, actin seems to play more of an essential role in endocytosis in the bloodstream form; RNAi of actin resulted in an arrest in cell division, termination of vesicular traffic, loss of endocytic activity and cell death (Garcia-Salcedo, Perez-Morga et al. 2004). Actin also plays an important role in mammalian cells; depletion of actin monomers with the use of the drug latrunculin A inhibited receptor-mediated endocytosis (Lamaze, Fujimoto et al. 1997). Both clathrin and actin have paramount responsibilities in *T. brucei* and may prove to regulate endocytosis and any signal transduction pathways associated with endocytosis.

4 Protein Kinases

Protein kinases are enzymes that transfer phosphates from ATP to other proteins modifying them, eventually affecting several cell signaling pathways. Phosphoproteins are involved in essential cell processes such as cell replication, cell death and movement (Graves and Krebs 1999). There are three basic types of kinases, based on their target of phosphorylation: serine/threonine kinases, tyrosine kinases (PTKs) and dual specificity kinases. The basic structure of all three contain a conserved catalytic core, consisting of the glycine rich N-terminal region and an aspartic acid in the central most region, shown to be important for catalytic activity (Hanks and Quinn 1991).

4.1 Protein Tyrosine Kinases

PTKs make up the largest group of protein kinases in humans (Manning, Whyte et al. 2002). Overall, all protein kinases have a high sequence similarity, which coincides to their secondary structures. The main difference in the structure of PTKs compared to other kinases has to do with the specificity for tyrosine (Cowan-Jacob 2006). With the comparison of the crystal structures cAMP-dependent kinase and insulin receptor kinase, the similarities and differences are more deeply discovered. Two regions that differ in PTKs compared to serine-threonine kinases are the large lobe of the activation loop and the differing positioning of the phosphate acceptor (Taylor, Radzio-Andzelm et al. 1995). These variations are used in the design of inhibitors specific for serine-threonine or tyrosine kinases (reviewed by Fabbro, Ruetz et al. 2002; Johnson 2009).

Based on their cellular location, tyrosine kinases can be divided into two subgroups. Receptor tyrosine kinases consist of an extracellular ligand-binding domain, transmembrane domain and an intracellular kinase domain. Non-receptor tyrosine kinases or NRTKs are located in the cytoplasm of the cell. Examples of mammalian receptor tyrosine kinases include vascular endothelial growth factor receptor (VEGFR), endothelial growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). There are not as many NRTKs as RTKs; examples of NRTKs include the Jak and Src families (Neet and Hunter 1996). Substrates of both PTK subgroups have the ability to bind proteins containing SH2 (phosphotyrosine binding domains) (Hubbard and Till 2000). These particular proteins recognize and bind phosphorylated tyrosine residues initiating a series of signal transduction events that lead to several different cellular effects (Schlessinger 2000; Hubbard and Miller 2007).

Signal transduction is an important aspect of cellular communication (Figure 3). In terms of RTKs, a ligand recognizes a receptor in the extracellular domain and binds, initiating dimerization of the receptors, which leads to intrinsic tyrosine phosphorylation activity in the intracellular domain. Once tyrosine phosphorylation occurs, proteins containing SH2 and/or

PTB domains recognize and bind to phosporylated tyrosines eventually leading to several downstream cellular events, including cellular differentiation, death and mobility (Schlessinger 2000). NRTKs not only contain a tyrosine binding domain, but SH2 and SH3 domains which interact with other proteins, a lipid binding domain (Pleckstrin homology domain (PH)) as well as other domains (Hubbard and Till 2000).

4.2 Bioinformatic Analysis of T. brucei Tyrosine Kinases

Despite evidence of tyrosine phosphorylated proteins and tyrosine kinase activity no tyrosine kinase or tyrosine-like groups have been discovered in *T. brucei*. Atypical tyrosine kinases like Wee1 and dual-specificity kinases like DYRKs are predicted to be in its kinome (Parsons, Worthey et al. 2005). Although conventional receptor tyrosine kinases or cytosolic tyrosine kinases are not found in *T. brucei*, a bioinformatic search of the *T. brucei* genome revealed 156 protein kinases (Naula, Parsons et al. 2005). Analysis of phosphopeptides and phosphorylation sites revealed 44 protein kinases that could be classified in conventional eukaryotic protein kinase groups (Nett, Martin et al. 2009). A study on the bloodstream form of *T. brucei* determined that most of the tyrosine kinases belong to the STE and CMGC groups, which regulate cell differentiation and/or stress response (Nett, 2009). Common accessory Pfam domains, such as SH2 and the PTB found in other eukaryotic protein kinases, remain to be identified in *T. brucei* and other members of the trypanosomatids (Parsons, Worthey et al. 2005). Immunofluorescence using anti-phosphotyrosine antibodies demonstrate a high degree of localization with the cytoskeletal structures, the basal body and flagellum. In addition, most *T. brucei* protein kinases lack major sequence similarity with other well known eukaryotic kinases. This observation can be exploited and inhibitors/drugs can be targeted to these particular kinases. In an analysis of human protein kinases and the relationship between their sequence and inhibitor structure and function, a general rule of the probability of inhibitors affecting protein kinases of different organisms was identified. If there is >60% sequence similarity among the protein kinases then it is more likely for inhibitors to affect both proteins

(Vieth, Higgs et al. 2004). As aforementioned since there is a lack of sequence agreement between human and *T. brucei* protein kinases, inhibitors designed specifically for *T. brucei* kinases may have a specific effect on this organism. Protein kinase inhibitors, more specifically tyrosine kinase inhibitors (TKIs), have recently become popular drug candidates for many different cancers and are currently being used in the clinic (Hartmann, Haap et al. 2009). Most EGFR inhibitors are from the class known as 4-anilinoquinazolines and pyrrolopyrimidines, defined by structural motifs (Levitzki and Mishani 2006).

4.3 Evidence of Protein Tyrosine Kinases in T. brucei

156 catalytically active eukaryotic protein kinases have been identified in *T. brucei* (Parsons, 2005). Although there are no receptor tyrosine kinases or receptor tyrosine-like kinases located in *T. brucei*, tyrosine phosphorylation does occur as aforementioned. The dual-specificity kinases (DYRK family and Wee) most likely are involved in tyrosine phosphorylation in *T. brucei*. The CMGC group is the largest group within the DYRKs and includes the cyclin-dependent (CDKs) and mitogen-activated protein (MAP) cascades. Several cell division cycle 2-related protein kinases as well as MAP kinases were found to be phosphorylated on tyrosine (Nett, 2009).

Extensive phosphorylation in *T. brucei* has been demonstrated; specifically serine/tyrosine phosphorylation of the protein pp44/pp46 occurs upon differentiation from slender bloodforms to procyclic (Parsons, Ledbetter et al. 1994). Tyrosine phosphorylated proteins have also been identified in the bloodstream form of *T. brucei;* 13 phosphorylated tyrosine residues on protein kinases have been identified by mass spectrometry (Nett, 2009). Kinase activity has also been confirmed in *T. brucei* through the use of the tyrosine kinase inhibitor genistein, which prevented parasite replication (Wheeler-Alm and Shapiro 1992). *T. brucei* releases a trypanosome lymphocyte-triggering factor (TLTF) that has been shown to decrease its response to interferon gamma when treated with the tyrosine kinase inhibitor

genistein (Bakhiet, Olsson et al. 1990). This suppression suggests that a tyrosine kinase may be involved in the communication of CD8 T-cells.

5 Cell Structure and Tyrosine Phosphorylation

The cell structure of *T. brucei* is characteristic of most eukaryotes having the same key organelles, a nucleus, mitochondria, Golgi, etc. Similarly a complex array of microtubules form the cytoskeleton and are responsible for the cell shape of *T. brucei* (Angelopoulos 1970). A round cell shape has previously been observed with the treatment of the TKI AEE788 (Hardin 2009). Tubulin has been observed to be tyrosine phosphorylated in human T lymphocytes and fetal rat brain nerve cells (Maness and Matten 1990; Marie-Cardine, Kirchgessner et al. 1995). Tyrosine phosphorylated tubulin has even been shown to prevent incorporation into microtubules (Wandosell, Serrano et al. 1987). The protein calpain-like cysteine peptidase, important to *T. brucei* cell shape, was discovered during immunoprecipitation with the anti-phosphotyrosine antibody PY20 (Hardin 2009). Specifically, the calpain-like protein Cap5.5V is required for cell morphogenesis in bloodstream *T. brucei*. Decreased expression of this protein by RNAi results in defective organization of subpellicular microtubule, the foundation of *T. brucei* cell structure (Olego-Fernandez, Vaughan et al. 2009). Thus, it is possible a pathway exist involving tyrosine phosphorylation and the cytoskeleton of *T. brucei* and the regulation of this begets the overall cell shape.

6 Tyrosine Kinase Inhibitors

Mutations in common protein kinases are associated with the cause for many cancers, due to its upregulation of expression. These oncogenes result in constant activation, which may eventually lead to uncontrolled cell division and subsequently cancer. Protein kinase inhibitors are small molecules that inhibit the phosphorylation of a specific protein residue. Similar to protein kinases they are specific for the amino acid they inhibit; tyrosine kinase inhibitors (TKIs) inhibit the phosphorylation of a tyrosine residue on a protein. Within the last 10 years, TKIs have become extremely important in the treatment of cancer. TKIs can be divided into 4

groups: ATP competitive, substrate specific, activation inhibitors and irreversible inhibitors. Most TKIs fall into the ATP competitive category due to the specific differences of the ATP binding pocket among different PTKs (Hubbard and Till 2000).

Tyrosine phosphorylation decreases in *T. brucei* when treated with the epidermal growth factor (EGFR) inhibitor AEE788 (Hardin 2009). Two other TKIs of interest are lapatinib and SU14813 (Figure 4). Both inhibit tyrosine kinases by mimicking ATP and subsequently binding to the ATP pocket, preventing self-phosphorylation and downstream signal transduction cascades. Lapatinib, from the class of EGFR inhibitors known as the 4-anilinoquinazolines, is a dual small molecule inhibitor of the epidermal growth factor receptor (EGFR) family, specifically ErbB1 (EGFR/Her1) and ErbB2 (Her2/neu) tyrosine kinases. Eukaryotic signaling pathways affected by EGFR include MAPK-Erk1/2 and PI3K-Akt, which involve cell proliferation and cell survival respectively (Pines, Kostler et al. ; Xia, Mullin et al. 2002) . Lapatinib has been studied in the clinic and has triggered apoptosis in breast cancer cell lines, which overexpress ErbB2 (Xia, Gerard et al. 2005). Lapatinib inhibits the activation of proliferation and survival pathways in both ErbB2 and EGFR-dependent tumors (Xia, Mullin et al. 2002). In ErbB2-overexpressing breast cancer cells lapatinib (1 micromolar) inhibits the MAPK-erk1/2 and PI3K-Akt signaling pathways triggering the cells to undergo apoptosis (Xia, Gerard et al. 2005). SU14813 is a broad-spectrum small molecule inhibitor of VEGFR, PDGFR, stem cell factor receptor (KIT) and fms-like tyrosine kinase 3 (FLT3). Similar to EGFR these RTKs also play a role in cell growth and survival. SU14813 inhibits VEGFR-2, FLT3-ITD and PDGFR-beta phosphorylation and biological activity in vivo (human xenograft tumors). SU14813 has been shown not to strongly inhibit epidermal growth factor-dependent DNA synthesis in cells overexpressing EGFR, confirming its specific target (Patyna, Laird et al. 2006).

A genome-wide search for EGFR kinase domains was performed using the program BLAST (Basic Local Alignment Search Tool) by comparing the different kinase domains against the parasite proteome (Altschul, Gish et al. 1990). The program PFAM was used to verify the

tyrosine kinase domains in the newly discovered proteins (Finn, Tate et al. 2008). The systematic identification of the 12 best candidates with a PTK E-value < 10^{-24} are listed along with the residues that define the kinase domain and the probability (P(N)) of homology between the trypanosome protein and the the various kinase domains are shown (Pearson 1996) (Table 1). This table shows PTKs in *T. brucei* that may have EGFR-like, c-Abl-like, src-like or VEGFR-like domains and is more evidence of a tyrosine signaling pathway in *T. brucei*. These findings are validated with the use of the 4-anilinoquinazolines and EGFR inhibitors, AG1478, which killed *T. brucei* with an IG_{50} of 7 μM; Canertinib kills *T. brucei* with an IG_{50} of 3 μM (Hardin 2009).

Familiarity with *T. brucei*'s kinome identifies the connection with the identified tyrosine kinases, being mostly of the STE/CMGC group and the use of lapatinib, which has shown inhibition of the MAPK signaling cascades (see above). On the other hand PDGFR is also associated with the MAPK pathway; the precursor to SU14813, Sunitinib has been observed to have a greater affect on inhibiting growth in leukemia cells when MEK/ERK signaling is prevented, suggesting a communication between the pathways (Nishioka, Ikezoe et al. 2008). It would be of interest to observe the differences and similarities between lapatinib, being a specific targeted RTK inhibitor and SU14813 a multiple target RTK inhibitor.

Figure 1.1: Life cycle of *Trypanosoma brucei* (CDC 2006). Human African trypanosomiasis is transmitted via the tsetse fly. The infected tsetse fly injects the trypanosome into human or animal skin tissue. During a blood meal on the mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into skin tissue. **1**: The parasites enter the lymphatic system and pass into the bloodstream. **2**: Inside the host, they transform into bloodstream trypomastigotes, **3**: are carried to other sites throughout the body and continue the replication by binary fission. The entire life cycle of African Trypanosomes is represented by extracellular stages. **4,5**: The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. **6**: In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, **7**: leave the midgut, and transform into epimastigotes. **8**: The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission. The cycle in the fly takes approximately 3 weeks. Humans are the main reservoir for *T. b. gambiense*, but this species can also be found in animals. Wild game animals are the main reservoir of *T. b. rhodesiense*. Eventually, the parasite enters the central nervous system of the host.

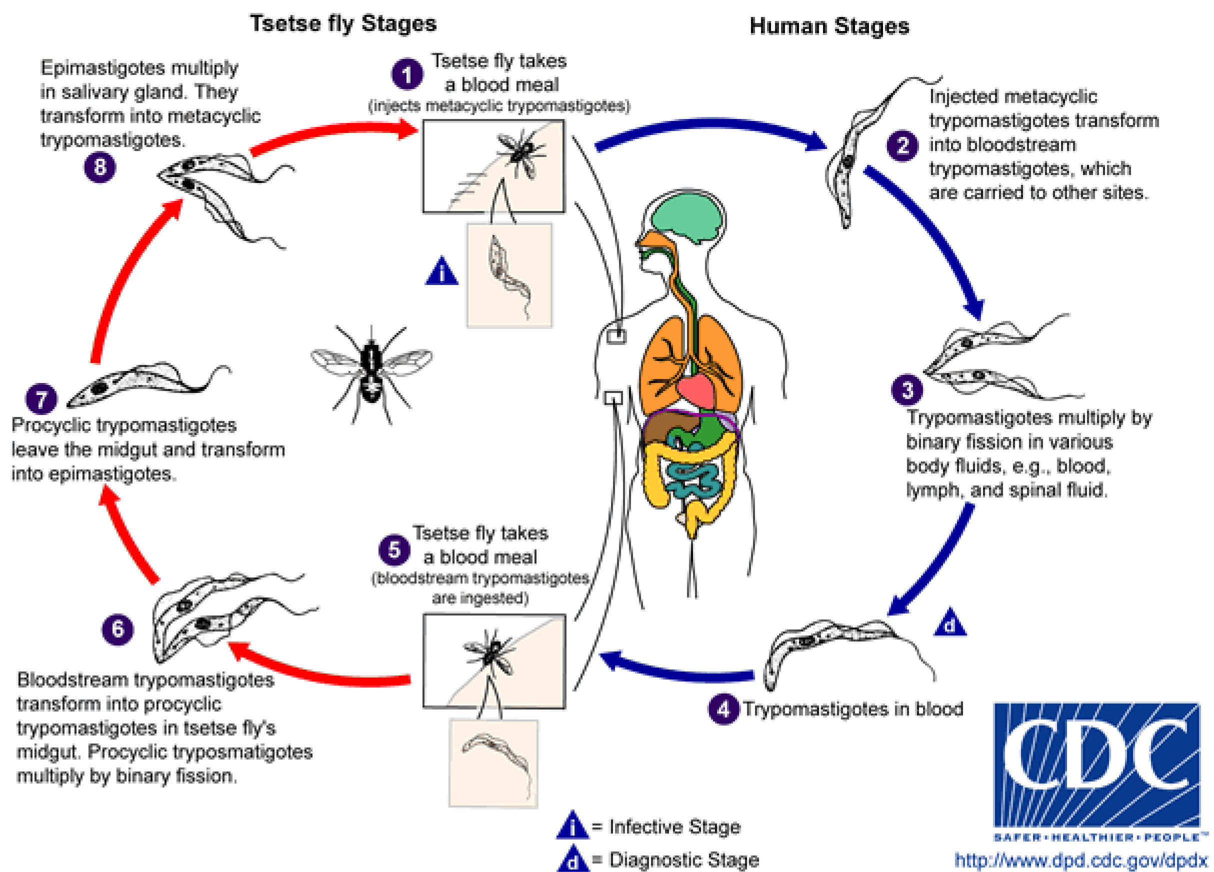


Figure 1.1

Figure 1.2: Eukaryotic Endosomal System (Clyker.com). Three forms of endocytosis are shown: Phagocytosis, Fluid-phase and Receptor-mediated. All forms initiate from the membrane and travel to the first portion of the pathway, the early endosome. Phagocytosis is characterized by the engulfing of large particles; pinocytosis or fluid-phase endocytosis involves small particles and/or fluid entering the cell. Receptor-mediated endocytosis uses a receptor to transport specific molecules into the cell. Specifically clathrin-coated vesicles assist in this process and clathrin-mediated endocytosis is essential in T. brucei (Allen, 2003).

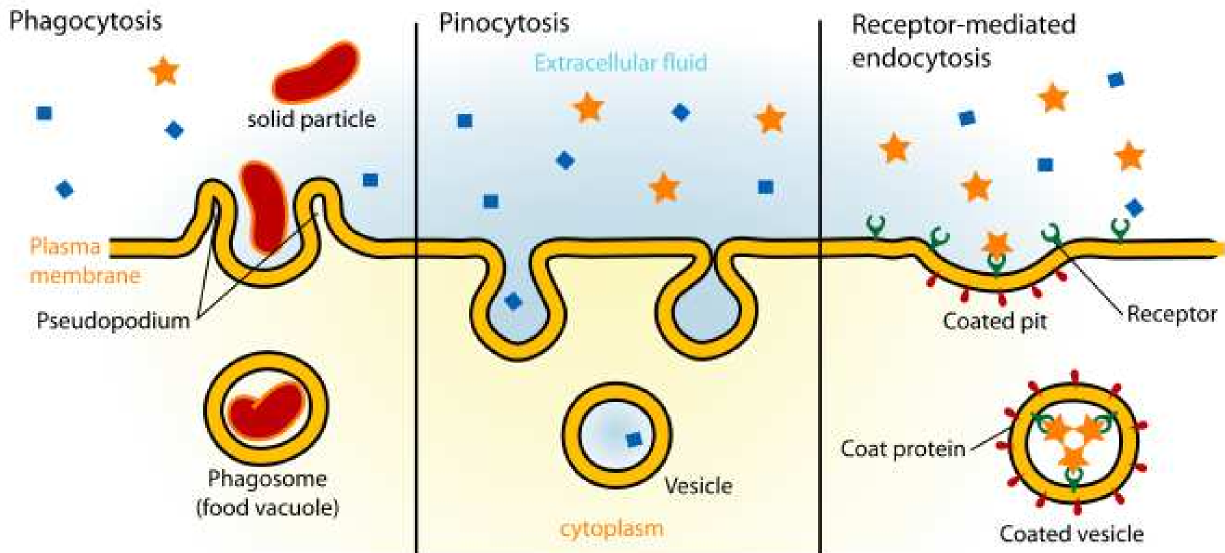


Figure 1.2

Figure 1.3: Receptor-tyrosine Kinase Schematic Nature Reviews Molecular Cell Biology 5, 464-471 (June 2004). **(A)** RTKS are in an inactive state when no ligand is present; they can be monomeric (one receptor) or dimeric (2 individual receptors). **(B)** To activate, a ligand (pink) binds each of the monomeric receptors (blue) prompting dimerization of both. Dimerization of the receptors initiates phosphorylation in the intracellular kinase domain (red). **(C)** Phosphorylation of the tyrosine domain creates available phosphotyrosines (black circles that act as binding sites for proteins with Src Homology 2 (SH2) and phosphotyrosine (PTB) binding sites; these specific proteins bind to the domain and eventually initiate several respective signal transduction pathways producing a fully active kinase domain (green). The TKIs Lapatinib and SU14813 both act on the intracellular tyrosine kinase domain, preventing phosphorylation by ATP by binding to the domain.

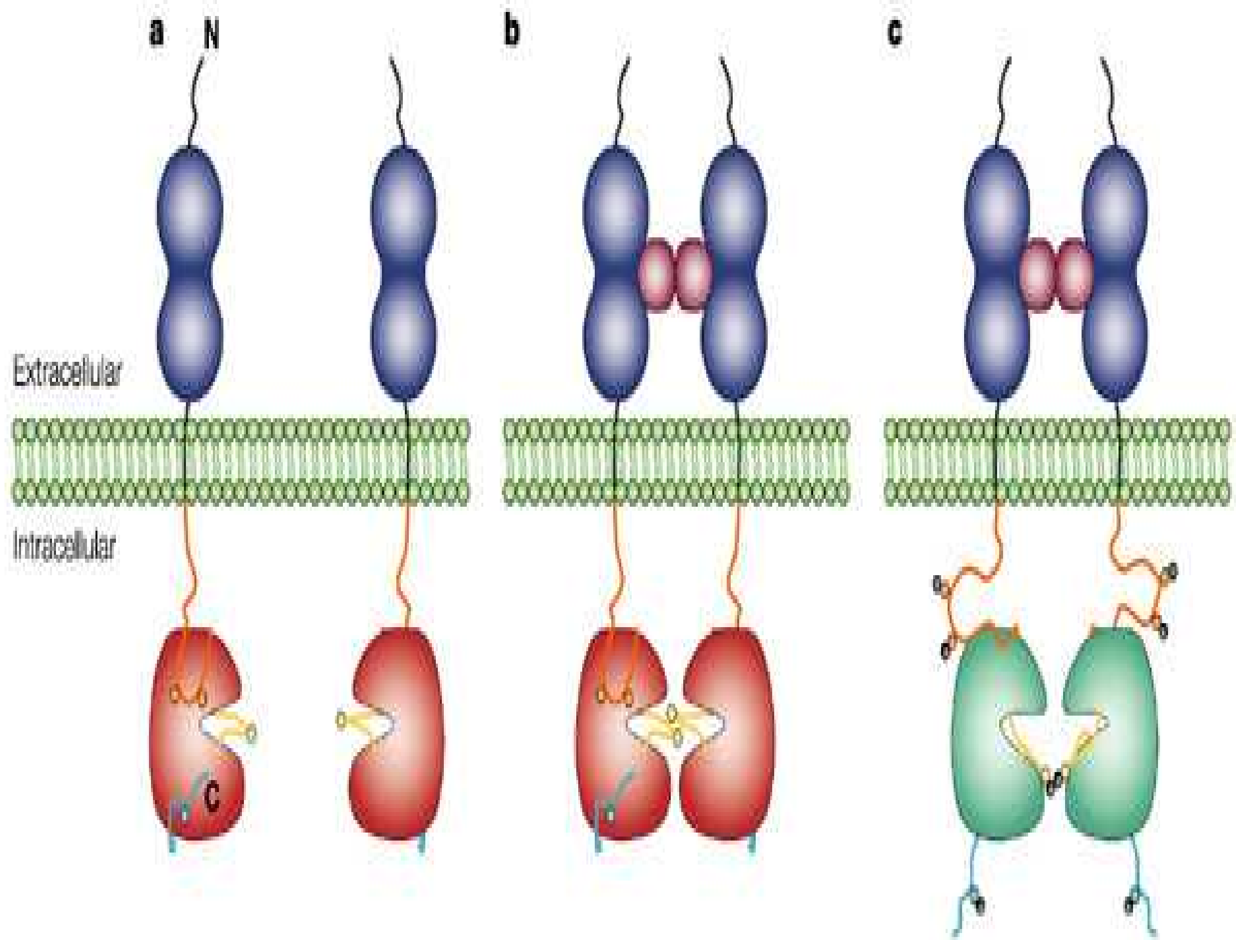
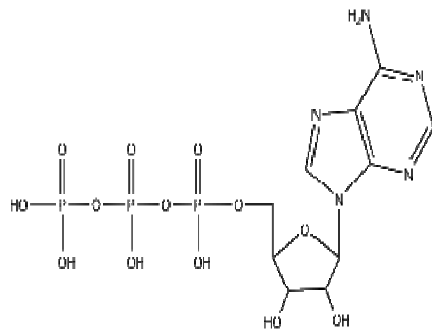


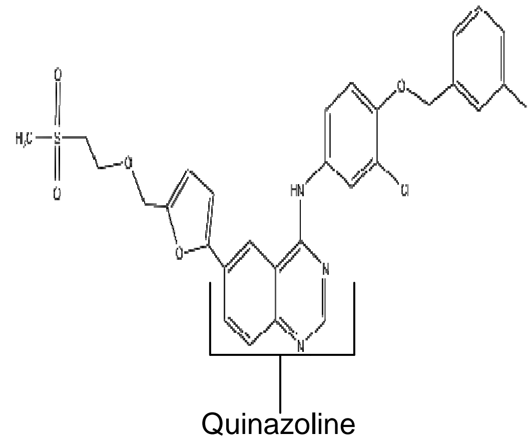
Figure 1.3

Figure 1.4: Tyrosine Kinase Inhibitor Structures. Structural figures of **(A)** ATP and **(B)** Lapatinib (trademarked Tykerb) (*N*-[3-chloro-4-[(3-fluorophenyl)methoxy]phenyl]-6-[5-[(2-methylsulfonylethylamino)methyl]-2-furyl]quinazolin-4-amine) and **(C)** SU14813 5-[(Z)-(5-fluoro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)methyl]-*N*-[(2*S*)-2-hydroxy-3-morpholin-4-ylpropyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide maleate); Lapatinib and SU14813 exhibit the same mechanism of inhibition: preventing ATP from binding to the intracellular tyrosine kinase domain, hindering subsequent phosphorylation of the kinase domain and other proteins; thus impeding downstream signaling events from happening. ATP is shown as a comparison to show the structure similarity, as both inhibitors are categorized as ATP competitive inhibitors or ATP mimics. Lapatinib a quinazoline is part of one of the largest groups of inhibitors defined based on their structure. SU14813 has a pyrrole group which is part of a pyrrolopyrimidine structure, another group of TKIs.

A. ATP



B. Lapatinib



C.SU14813

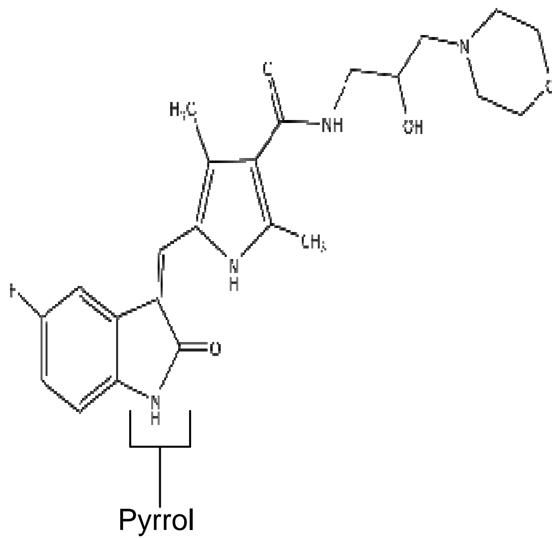


Figure 1.4

Table 1: Putative Tyrosine Kinases in the *T. brucei* Genome. A genome-wide search for the EGFR, c-Abl, src and VEGFR kinase domains was performed by the program BLAST with the kinase domains and the parasite proteome. PFAM was used to confirm the presence of protein kinase domains in the specific proteins. The systematic ID of the 12 best candidates (those with PTK E-values < 10^{-24}) is listed in addition to the residues that define the kinase domain (numbers beside E-values). The probability (P(N)) between the trypanosome protein and the kinase domains are located in the third column.

Systematic ID	PTK (E-value)*1	Homology to Specific Kinase Domain (P(N))			
		EGFR*2	c-Abl*3	c-Src*4	VEGFR*5
Tb10.406.0580	342-604 (4.4×10^{-41})	**1.7×10^{-11}**	5.0×10^{-9}	**6.0×10^{-22}**	**2.1×10^{-18}**
Tb10.61.2330	4-253 (4.3×10^{-35})	2.4×10^{-10}	**2.8×10^{-14}**	**1.1×10^{-20}**	**1.8×10^{-18}**
Tb927.5.2820	29-294 (2.6×10^{-32})	**2.7×10^{-11}**	**1.2×10^{-13}**	5.7×10^{-19}	**3.8×10^{-19}**
Tb927.6.2030	240-496 (1.5×10^{-29})	4.2×10^{-9}	4.2×10^{-9}	**3.8×10^{-21}**	1.8×10^{-15}
Tb10.70.7860	4-258 (2.9×10^{-29})	6.8×10^{-9}	1.3×10^{-10}	5.4×10^{-18}	7.4×10^{-16}
Tb927.8.5730	20-271 (5.0×10^{-29})	**1.2×10^{-12}**	3.7×10^{-10}	5.0×10^{-17}	**7.3×10^{-17}**
Tb927.7.6680	608-881 (6.1×10^{-28})	1.4×10^{-8}	1.5×10^{-6}	**2.4×10^{-20}**	1.7×10^{-8}
Tb11.02.2050	28-278 (1.3×10^{-27})	6.3×10^{-11}	**9.7×10^{-13}**	2.4×10^{-19}	1.4×10^{-15}
Tb927.7.3580	49-201 (3.6×10^{-27})	**3.4×10^{-14}**	**5.7×10^{-11}**	2.7×10^{-18}	**8.6×10^{-17}**
Tb927.3.3190	20-278 (1.8×10^{-26})	6.7×10^{-10}	**1.3×10^{-13}**	3.8×10^{-17}	5.1×10^{-15}
Tb10.70.5760	14-288 (1.8×10^{-25})	**1.8×10^{-11}**	2.4×10^{-10}	1.7×10^{-14}	1.5×10^{-7}
Tb11.46.0003	233-476 (3.9×10^{-25})	1.4×10^{-8}	4.8×10^{-8}	**1.7×10^{-19}**	2.5×10^{-14}

Table 1.1

Materials and Methods

Materials

Immobilon P (PVDF) membrane was purchased from Millipore (Bedford, MA). Sodium dodecyl sulfate (SDS), 5-bromo-4-chloro-inodyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT) were from BioRad (Melville, NY). Formaldehyde and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). GelCode blue stain reagent was from Pierce (Rockford, IL). 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). P-Tyr-100 (anti-phosphotyrosine mouse monoclonal antibody) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). 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). Lapatinib (lapatinib ditosylate) was a gift from GlaxoSmithKline (GSK) and SU14813 was a gift from Pfizer.

Cell Culture

Bloodstream *T. brucei* 427 was obtained from C.C. Wang (University of California, San Francisco, CA) and cultured in HMI-9 medium (Hirumi and Hirumi 1991) supplemented with 10% fetal bovine serum and 10% Serum Plus) at 37ºC, 5% CO_2.

Inhibitor Treatment of *T. brucei*

T. brucei was cultured to a density of 10^5 cells/ml and then diluted to 10^4 cells/ml in HMI-9 medium. 5×10^3 cells (500 µl) were added to each well of a 24-well plate. Lapatinib and SU14813 10mM stock solutions were prepared in DMSO. To cells where no inhibitor was added, DMSO was added as a control. To treat cells with inhibitors 2.5 µl of a 200-fold concentrated stock was added to each well to yield the stated final concentrations in each experiment. Cells were cultured for 48 hours and cell density was determined with a

hemacytometer. The IG_{50} is determined as the concentration of inhibitor that reduces growth of *T. brucei* by 50% during culture. The concentration range needed to cover IG_{50} was determined from previous experiments for each inhibitor.

Transferrin Endocytosis Assay

Bloodstream *T. brucei* were harvested as aforementioned and 10^4 cells were resuspended in 1 ml of HMI-9 medium for each sample. The cells were treated with 0.25 or 1 microliter of DMSO, .25 or 1 microliter of 1 mM Lapatinib (final concentration of 0.25 or 1 μM), or 0.25 or 1 microliter of 1mM SU14813 (final concentration 0.25 or 1 μM) for 30 minutes at 37ºC, 5% CO_2. The cells were then centrifuged at 2000 x g for 5 minutes and washed 2 times in serum-free HMI-9 media with the final concentration of DMSO or drug corresponding to each sample in the cold room. 1 ml of serum-free HMI-9 was added to each sample along with 5 μl of human transferrin AlexaFluor 488 conjugate (5 μg/μl) and the final concentration of DMSO or drug corresponding to each sample. The samples were incubated for 15 minutes at 37ºC, 5% CO_2. The cells were pelleted (2000 x g, 5 minutes), washed 3 times with ice-cold PBS pH 7.4 containing 1% glucose and fixed in 300 μl of 1% formaldehyde in PBS on ice for 5 minutes in the cold room. 600 μl of ice-cold 100mM glycine in PBS was added to quench the formaldehyde and the samples incubated for 5 minutes on ice. The cells were then washed twice with ice-cold PBS and resuspended in 300 μl PBS. This protocol was designed by Sarah Thomas.

The relative fluorescence intensity of 2,000 events at a Side Scatter threshold of 2% 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 approximately 2000 events by selecting gates to exclude extracellular debris.

Inhibitor Treatment and SDS-PAGE

10^7 cells were harvested and treated with 1.5 μl DMSO, 0.5 μl from a 10mM stock (final concentration 5 μM), 1 μl from a 10mM stock (final concentration 10 μM) and 1.5 μl from a

10mM stock (final concentration of 15 μM) of Lapatinib or SU14813 for 1 hour at 37ºC, 5% CO_2. The cells were centrifuged (2000 x g, 5 min) and the supernatant removed. The cell pellet was resuspended in 10 μl of 2X SDS-PAGE sample buffer and heated for 5 minutes at 100º C. Proteins were separated by SDS-PAGE (10% or 14%) (Sambrook 2001) and transferred to a PVDF membrane with a Trans-Blot semi-dry cell (Bio-Rad) (Armah and Mensa-Wilmot 1999).

Hsp70 was used as the control antibody; for its detection 2 μl from the aforementioned samples was diluted into 8 μl 2x SDS-PAGE sample buffer (2×10^5) and cells were loaded into each well of an SDS-PAGE gel (10%) and the protocol continued (see above).

Immunoblotting

The detection of phosphotyrosine (pTyr) using the P-Tyr-100 antibody was performed according to manufacturer's instructions, with a few modifications. After transfer the PVDF membrane was washed with TBS (Tris buffered saline, pH 7.6) for 5 minutes at room temperature and then blocked in TBS containing 0.1% Tween-20 (TBS/T) and 5% (w/v) Carnation powdered milk for 1 hour at room temperature. The membrane was washed three times for 5 minutes each with TBS/T. The primary antibody P-Tyr-100 (1:250) in TBS/T with 5% bovine serum albumin (BSA) was incubated with membrane overnight at 4ºC with gentle shaking. The membrane was washed three times for 5 minutes each with 5 ml of TBS/T at room temperature. Secondary antibody, goat anti-mouse IgG-alkaline phosphatase conjugate (1:1000 dilution in blocking buffer as aforementioned) was added for 1 hour at room temperature with gentle shaking. The membrane was then washed three times for 5 minutes each with TBS-T and incubated twice with alkaline phosphatase reaction buffer (APRB, 75mM Tris pH 9.5, 75mM NaCl, 3.75mM 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 achieved (least amount of background as possible) and stopped by washing the membrane with deionized water for 10 minutes, changing the water at least once. Detection of Hsp70 was

performed as aforementioned, with the following adaptations: blocked with PBS (pH 7.4) containing 5% (w/v) Carnation non-fat dry milk, 0.1% (v/v) Tween 20 for 1 hour at room temperature with gentle shaking. BiP was detected using anti-Bip antibody (1:5000) diluted in blocking buffer. The membrane was then washed three times for 5 minutes each with PBS, 0.1% Tween 20. The membrane was incubated with goat anti-rabbit IgG –alkaline phosphatase conjugate antibody diluted (1:1000) in blocking buffer for 1 hour at room temperature with gentle shaking. The membrane was washed three times for 5 minutes each with PBS, 0.1% Tween 20 and detection performed as described above.

Cell Shape Analysis

T. brucei bloodstream cells were harvested as aforementioned and 10^7 cells/ml were treated with either 1 µl DMSO or 1 µl of 10mM Lapatinib or SU14813 (final concentrations of 10 µM). The cells were incubated at 37ºC, 5% CO_2 for 15 minutes, 30 minutes, and 1 hour intervals after which they were pelleted (as aforementioned) and washed three times with PBS containing 10mg/ml glucose. The cells were then fixed in 300 µl of 1% formaldehyde on ice for 5 minutes and quenched with 600 µl of 100mM glycine in PBS for 5 minutes at room temperature. Cells were pelleted, washed twice in PBS and resuspended in 300 µl of sterile PBS.

The relative fluorescent intensity of 50,000 events at a forward scatter threshold of 37% 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 approximately 50,000 events by selecting gates to exclude extracellular debris.

Differential Interference Contrast Microscopy

T. brucei bloodstream cells were harvested as aforementioned and 10^7 cells/ml were aliquoted into 1.5 ml tubes. The cells were treated with either 1 µl of DMSO, 1 µl of 10 mM Lapatinib or SU14813 (final concentrations of 10 µM). The cells incubated at 37ºC, 5% CO_2;

200 μl of sample was taken out at 15 minutes, 30 minutes and 1 hour intervals after which they were pelleted (as aforementioned) and washed once with PBS (pH 7.4) containing 10mg/ml glucose. The cells were fixed in 300 μl of 4% formaldehyde at room temperature for 1 hour and quenched with 700 μl of 100mM glycine in PBS for 5 minutes at room temperature. Poly(L)-lysine-coated coverslips were made by adding 150μls of a diluted (1/10) in DiH_2O poly(L)-lysine solution to a sheet of parafilm and laying coverslip on top. The coverslip and poly(L)-lysine was incubated for at least 1 hour. After incubation the slips were removed and allowed to dry wet side up. The cells were washed once with PBS (pH 7.4), resuspended in 40 μl PBS and allowed to settle on poly(L)-lysine-coated coverslips for 45 minutes. Coverslips were rinsed twice with PBS and mounted on slides with 4 μl of VectaShield mounting medium and DAPI.

DIC (differential interference contrast) images were captured using a Zeiss Axioskop microscope (Carl Zeiss Microimaging, Thornwood, NY) using a cool charge-coupled device Retiga Exi Fast 1394 camera (Qimaging, Burnaby, BC, Canada) and IPLab Spectrum software (Scanalytics, Billerica,MA).

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CHAPTER 2

LAPATINIB AND SU14813 ACTIVITY IN TRYPANOSOMA BRUCEI

Human African trypanosomiasis is caused by the protozoan parasite *Trypanasoma brucei*. It causes over 30,000 deaths per year and is endemic in many African countries (Stuart, Brun et al. 2008). Throughout its life cycle *T. brucei* undergoes many changes, including phenotypically and metabolically in order to survive in the insect or human (Parsons, Valentine et al. 1991; Morgan, Allen et al. 2001; Garcia-Salcedo, Perez-Morga et al. 2004; Hall, Smith et al. 2005). The signaling pathways involved in these changes remain to be identified. Clues as to the specific proteins and/or pathways associated with a particular cellular event can be manipulated and used as drug targets to aid in curing HAT.

Pentamidine and suramin are used before the parasite invades the central nervous system. Melarsoprol and eflornithine (only effective in *T. b. gambiense*) are used in the late stage of HAT. All four drugs have several side effects including blindness and *T. brucei* is likely to develop resistance against them (Kennedy 2008). In addition they must be administered intravenously; the toxicity of the drugs alone exacerbates the idea of prolonged treatment of the patients. Clearly there is an immediate need for safer, more effective drugs.

Protein phosphorylation by tyrosine kinases control many cellular processes including differentiation, survival, endocytosis, growth and cell shape (Abrami, Kunz et al. ; Matten, Aubry et al. 1990; Ji, Winnier et al. 1997; Mani, Venkatasubrahmanyam et al. 2009). Albeit there is a conventional existence of PTKs in many cell types their aberrant functions lead to many disorders including cancer and consequently have become good drug targets (Pines, Kostler et al. ; Wu, Tseng et al. ; Bennasroune, Gardin et al. 2004). Over the past decade drugs that

mimic ATP also known as small molecule tyrosine kinase inhibitors have been significantly used in many forms of cancer. These inhibitors work by binding to the ATP binding pocket of the intracellular kinase domain of tyrosine kinases preventing protein phosphorylation and any subsequent signal transduction cascades from occurring.

There is no receptor-tyrosine or tyrosine-like kinases in the *T. brucei* genome. Despite this there is evidence of tyrosine phosphorylation of proteins and tyrosine activity (Wheeler-Alm and Shapiro 1992; Parsons, Ledbetter et al. 1994; Nett, Davidson et al. 2009). Dual-specificity kinases do exist in *T. brucei* and belong to the CMGC family, the largest kinase family identified in bloodstream T. brucei, 18 of 42 having a homologous conserved regulatory tyrosine residue (Parsons, Worthey et al. 2005). Both experimental and bioinformatic analysis clearly suggest the involvement of tyrosine phosphorylation in the many cell processes requiring signaling. We hypothesize that using the tyrosine kinase inhibitors SU14813 and Lapatinib will aid in revealing the connection between tyrosine phosphorylation and important processes in the cell such as cell growth, shape and endocytosis.

RESULTS

The Tyrosine Kinase Inhibitors Lapatinib and SU14813 inhibit cell growth in T. brucei

To determine whether or not Lapatinib and SU14813 had an effect on the growth of *T. brucei*, cell growth assays were performed. Because of the discovery of kinase homology between mammalian tyrosine kinase domains and *T. brucei*, it was hypothesized that particular tyrosine kinase inhibitors would inhibit tyrosine kinase-like domains in *T. brucei* (Table 2.1). In addition other tyrosine kinase inhibitors have been shown to kill *T. brucei* (Hardin 2009). Both lapatinib and SU14813 inhibited cell growth with IG_{50} between 1-2 micromolar (Figure 2.1). Lapatinib seemed to be the more potent drug as it completely killed *T. brucei* at 5 micromolar. This data suggests that there is a relationship between cell growth and tyrosine signaling in *T. brucei*.

Lapatinib changes the shape of T. brucei

It was previously discovered that *T. brucei* exposed to AEE788, a TKI, rounded up (Hardin 2009). To investigate whether this phenomenon also occurred upon treatment with the TKIs lapatinib and SU14813, DIC images of inhibitor-treated cells were obtained. Lapatinib begin changing the shape of *T. brucei*, to a rounded shape, as early as 30 minutes compared to DMSO treated cells at the same time (Figure 2.2B). Cells treated with SU14813 caused no change in morphology. The same concentration was used with both inhibitors; even after 1 hour of exposure, the morphology of cells treated with SU14813 remained similar to control cells (Figure 2.2C).

Flow cytometry was also used to reaffirm the findings of the DIC images. In the first experiment 2 x 10^6 cells/ml were treated with each TKI (5 micromolar) or DMSO in serum-free media and samples collected at 15, 30 and 60 minutes (Figure 2.3). A cell shape change could only be seen with lapatinib-treated cells. In the next experiment 10^7 cells/ml were treated with inhibitor (10 micromolar) or DMSO and samples removed at 15, 30 and 60 minute time points. Light scatter plots were constructed in order to determine a shift in the forward scatter of cells, which would indicate a change in shape. As seen in the DIC images lapatinib-treated cells shifted towards the left of the X-axis (forward scatter) and SU14813 did not display any shift. Specifically, median log values of the forward scatter at 1 hour were 377.4, 279.16 and 345.51 for DMSO, lapatinib and SU14813 respectively (Figure 2.4D). These data reveal a connection between cell shape and EGFR-like kinases in *T.brucei*.

Lapatinib Reduces Tyrosine Phosphorylation of Proteins in T. brucei

Because the mechanism of tyrosine kinase inhibitors is directed towards preventing phosphorylation, we hypothesized that lapatinib and SU14813 would have an effect on the tyrosine phosphorylation of proteins in *T. brucei*. To test this idea *T. brucei* was treated with lapatinib and SU14813 at 0, 5, 10 and 15 micromolar and the antibody PTyr100 was used to probe for tyrosine phosphorylation on western blots.

Lapatinib reduces tyrosine phosphorylation of proteins in *T. brucei* compared to DMSO treated cells (Figure 2.5A). In addition the intensity of two proteins increased after lapatinib treatment. On the other hand SU14813 did not display as strong of an effect on the decrease of tyrosine phosphorylation (Figure 2.5B). Hsp70 protein levels remained the same at all concentrations tested for both lapatinib and SU14813 suggesting that this effect in tyrosine phosphorylation is specific to inhibition of tyrosine kinase-like activity in *T. brucei*.

Lapatinib Reduces Receptor-Mediated Endocytosis in T. brucei

T. brucei relies on endocytosis for its nutrients, maintenance of cell volume and elimination of host immune system molecules (Morgan, Hall et al. 2002). In addition clathrin-mediated endocytosis, which is involved in receptor-mediated endocytosis, is essential in *T. brucei* (Allen, Goulding et al. 2003). Important components of clathrin-mediated endocytosis are identified as being tyrosine phosphorylated in *T. brucei* (Hardin 2009). Therefore tyrosine phosphorylation may be important in the regulation of receptor-mediated endocytosis in *T. brucei*.

To test this hypothesis the uptake of transferrin was tested in inhibitor-treated versus DMSO treated cells. Lapatinib inhibited endocytosis of transferrin at concentrations as low as 0.25 micromolar (Figure 2.6A), showing more of an increase in inhibition at 1 micromolar. On the other hand SU14813 failed to show any inhibition at either 0.25 or 1 micromolar (Figure 2.6B). Endocytosis was reduced 50% in lapatinib treated cells at 0.25 micromolar and 90% at 1 micromolar (Figure 2.6C). This data suggests that receptor-mediated endocytosis is regulated by tyrosine signaling in *T. brucei*.

DISCUSSION

Growth Inhibition

As seen in Figure 2.1 when bloodstream *T. brucei* is treated with lapatinib and SU14813 the cells exhibit an IG_{50} (concentration of inhibitor that inhibits growth of 50% of cells) between 1-2 micromolar. Despite having similar IG_{50} lapatinib seemed to have a more profound effect on cell growth as complete inhibition of growth was encountered at 5 micromolar.

SU14813 is a broad spectrum receptor tyrosine kinase inhibitor; it acts on the tyrosine kinases vascular endothelial growth factor, platelet-derived growth factor, KIT and FLT3, which trigger pathways involved with cell growth and survival in mammalian cells (Patyna, Laird et al. 2006). Lapatinib is a specific receptor tyrosine kinase inhibitor and only acts on the mammalian epidermal growth factor receptor also involved in cell growth, survival and intracellular trafficking (Medina and Goodin 2008). Lapatinib inhibited cell growth in a HER2-stimulated breast cancer cell line and the gastric cell lines SNU-216 and NCI-N87 with an IC_{50} between 0.01-0.02 micromolar (Kim, Kim et al. 2008).

Although there are a lack of known receptor tyrosine kinases in *T. brucei* these inhibitors remain effective; perhaps the target of the inhibitors in *T. brucei* has an effect on other downstream players in the signaling pathway eventually leading to cell death. This may be explained by referring to the mammalian EGFR kinase pathway; ligand binding causes receptor autophosphorylation, which recruits proteins with SH2 domains to bind to the receptor. These proteins recruit other proteins with SH3 domains eventually activating Ras. Ras then activates Raf, which phosphorylates MEK, activating MAPK. MAPK cascade homologues exist in *T. brucei*; MAPK plays an important role in the regulation of transcription factors. In fact an ERK-like MAPK is proposed to be involved in the proliferation of bloodstream form trypanosomes, possibly controlling cell cycle and subsequently the growth rate of the parasite (Hua and Wang 1994). Although SH2 and PTB (phosphotyrosine-binding) domains do not exist in the *T. brucei* genome 13 phosphorylated tyrosine residues on bloodstream form *T. brucei* protein kinases have been identified (Nett, Martin et al. 2009). KFR1, an ERK-like MAPK, is suggested to play

a role in proliferation of bloodstream *T. brucei*; upon infection interferon gamma is released from mammalian cells, increasing the proliferation of bloodstream *T. brucei* clearly linking signal transduction to cell growth (Hua and Wang 1997). Therefore direct or indirect inhibition of cellular signaling could have an effect on preventing cell growth. The MAPK family couples extracellular stimuli and intracellular responses. Therefore this family is important in *T. brucei* as it undergoes various environmental conditions during its life cycle. Based on these findings, it is possible for lapatinib and SU14813 to inhibit their target effecting downstream signaling, such as MAPK, explaining the deregulation of transcription factors of the cell cycle eventually killing the cell.

Dual specificity kinase families, such as DYRKs, STE7 and CLKs exist in *T. brucei* (Parsons, Worthey et al. 2005). Cell division cycle 2-related protein kinases CRK1, CRK2 and CRK3 are tyrosine phosphorylated as well as serine and/or threonine phosphorylated, corresponding to the human CDK1. The CDKs (cyclin dependent kinases) are a large group of kinases identified in the genome of *T. brucei* (Naula, Parsons et al. 2005). CDKs play an important role in controlling cell growth and cell division. In mammalian cells Wee1, a dual-specificity tyrosine kinase, phosphorylates CDK1 inactivating its cyclin-CDK complex and subsequently preventing cell cycle progression (Krupa, Preethi et al. 2004). A Wee1 homologue is found in *T. brucei*; therefore this same type of cell cycle regulation can occur and effect cell survival.

DYRKs activate themselves by autophosphorylation of an essential tyrosine residue and subsequently phosphorylate their substrates on serine and theronine residues (Kentrup, Becker et al. 1996). Perhaps the inhibitors are affecting these kinases, which would cause other signaling cascades to be involved including serine/threonine kinases. More experiments pertaining to the cell cycle will help elucidate the exact role these inhibitors play in *T. brucei's* killing.

Cell Morphology

As seen in Figure 2.2 a cell shape change is demonstrated within 30 minutes of treatment with lapatinib. SU14813 does not exhibit this same cell shape change at either 30 or 60 minutes of treatment. Flow cytometry analysis of the cell shape of inhibitor-treated versus DMSO-treated cells displays the idea of a morphological change in lapatinib-treated cells. A light scatter plot was used to evaluate the cell shape represented by forward scatter. As seen in Figure 2.4 at each time point the difference in cell shape between DMSO-treated and lapatinib treated cells was clearly demonstrated. On the other hand this phenomenon was not present in the SU14813-treated cells. The median log of the forward scatter of lapatinib-treated cells is decreased indicating a smaller cell at the 15, 30 and 60 minute time points (Figure 2.4D). The light scatter plot demonstrates about a 24% and 26% decrease in the median log of the forward scatter with lapatinib-treated cells at 15, 30 and 60 minute time points respectively. A cell shape change with lapatinib-treated cells was not noticeable at 15 minutes with DIC. Out of 100 lapatinib-treated cells counted only 10% displayed a cell shape change at 30 minutes and 20% at 60 minutes. This discrepancy in cell shape percentage could possibly be explained by dying cells, as light scatter plots do not differentiate between living or dying cells, only cell shape change.

A similar experiment as the aforementioned was performed, but treating 2 x 10^6 cells in serum-free media with 5 μM of the TKIs (Figure 2.3). The light scatter plot displays similarities to the DIC images as a cell shape change is only seen with lapatinib-treated cells. In addition it may suggest more of an evident cell shape change at each time point compared to the similar experiment performed in media containing serum. It is possible that the TKIs attach to the proteins in serum media; therefore in serum-free media more of an effect could be observed as more TKI is available to act on the cell.

The cell shape of *T. brucei* is dependent on its cytoskeleton, which is composed of microtubules (Angelopoulos 1970). Since lapatinib causes a cell shape change and inhibits phosphorylation of some proteins it could be postulated that proteins involved in the formation of

the cytoskeleton could be tyrosine phosphorylated. Tubulin is tyrosine phosphorylated in many organisms; in-vitro studies using the insulin receptor kinase of rat placental membranes demonstrate that tubulin phosphorylation everywhere except the carboxyl-terminal, permits polymerization (Wandosell, Serrano et al. 1987; Maness and Matten 1990; Ley, Verbi et al. 1994). Tubulin has also been noted to be a substrate for the JAK2 tyrosine kinase in eukaryotic cells (Ma and Sayeski 2007). When the anti-phosphotyrosine antibody 4G10 was used in bloodstream trypanosomes important cytoskeletal structures, the basal body and flagellum were heavily stained, once again suggesting the involvement of tyrosine-associated proteins (Nett, Martin et al. 2009). When bloodstream T. brucei cells are treated with AEE788, a tyrosine kinase inhibitor, alpha and beta-tubulin is identified as a protein that is possibly tyrosine phosphorylated or associated with tyrosine phosphorylated proteins (Hardin 2009). This evidence as well as the display of lapatinib-treated cells exhibiting a circular cell shape change suggests a link between tyrosine associated proteins and/or cell signaling involving tyrosine kinases and cellular morphology.

Tyrosine Phosphorylation

Lapatinib decreases protein tyrosine phosphorylation (astericks Figure 2.5A). When comparing the inhibitor-treated lanes to the DMSO-treated lanes a few proteins have decreased in intensity and even disappeared. SU14813 does not exhibit this same decrease in tyrosine phosphorylation in *T. brucei* (Figure 2.5B). Both inhibitors had no effect on the polypeptide levels of HSP70, indicating that the drug particularly inhibits tyrosine phosphorylation in *T. brucei*.

Tyrosine phosphorylation of RTKs or non-RTKs are largely involved in cell signaling in eukaryotes. When the EGFR inhibitor, Tyrphostin AG1478, is used on monocytes TrkA (nerve growth factor receptor) is not phosphorylated and thus inactive; when the NGF receptor inhibitor K252a is used EGFR phosphorylation is prevented (El Zein, D'Hondt et al.). This type of crosstalk between receptors displays the importance and regulation of tyrosine phosphorylation

as it relates to cell signaling. Despite the lack of receptor tyrosine kinases in *T. brucei* it should be expected that tyrosine phosphorylation of some proteins are effected due to the evidence of tyrosine phosphorylated proteins as well as tyrosine kinase activity (Parsons, Valentine et al. 1991; Wheeler-Alm and Shapiro 1992; Das, Peterson et al. 1996). In addition decreased phosphorylation of inhibitors targets as well as proteins downstream has been shown in gastric cancer and tumor cell lines treated with lapatinib and SU14813 respectively noting the effectiveness and specificity of each inhibitor (Patyna, Laird et al. 2006; Kim, Kim et al. 2008).

Tyrosine phosphorylation of two proteins is increased in lapatinib-treated cells (Figure 2.5A). This phenomenon could possibly be explained by referring to the Src kinase. When the Src kinase is phosphorylated on its tyrosine residue the protein is in an inactive confirmation; depohosphorylation activates the protein (Roskoski 2004). If a TKI inhibits phosphorylation of the Src kinase, promoting its kinase activity certain proteins downstream from the target will continue to be phosphorylated and in turn exhibit a tyrosine phosphorylation increase as compared to Src in its phosphorylated inactive state.

The lack of a decrease of tyrosine phosphorylation of proteins in SU14813-treated cells can most likely be explained by SU14813's targets in *T. brucei* being less in existence when compared to lapatinib's targets. Despite the kinase domain relatedness chart including strong E-values for the Pfam VEGFR domain when compared to the EGFR domain, SU14813 may simply inhibit less abundant proteins, unidentifiable on a western blot. Perhaps a more specific approach such as protein immunoprecipitation of SU14813-treated cells compared to control cells would give a clue into what proteins are dephosphorylated. In addition higher concentrations of SU14813 may be necessary to demonstrate an effect; the concentrations of both inhibitors remained similar in order to make a comparison between the two. Keep in mind that 5 micromolar of lapatinib completely killed *T. brucei*, while treatment with SU14813 did not have this same outcome (Figure 2.1).

Receptor-mediated Endocytosis

Lapatinib inhibited uptake of transferrin (Figure 2.6). At .25 micromolar the median log of Alexafluor488 was less than DMSO (control) cells, showing an even greater reduction in endocytosis at 1 micromolar (Figure 2.6C). SU14813 showed no inhibition at .25 or 1 micromolar (Figure 2.6C)

T. brucei relies on endocytosis not only to maintain its nutritional requirements, but to avoid the host's immune system. Endocytosis and tyrosine phosphorylation is connected in many eukaryotes (Grygoruk, Fei et al. ; Jurd, Tretter et al. ; Shi, Notenboom et al.). Invasion of host cells is also linked to tyrosine phosphorylation; genistein, a tyrosine kinase inhibitor prevented 50% penetration of Toxoplasma gondii into macrophages (Ferreira, De Carvalho et al. 2003).

The proteins clathrin and epsin were identified as being tyrosine phosphorylated proteins (Hardin 2009). These proteins participate in clathrin-mediated endocytosis, which is essential in T. brucei (Allen, Goulding et al. 2003). This data along with the inhibition of transferrin uptake by lapatinib suggests a link between tyrosine phosphorylation and regulation of endocytosis.

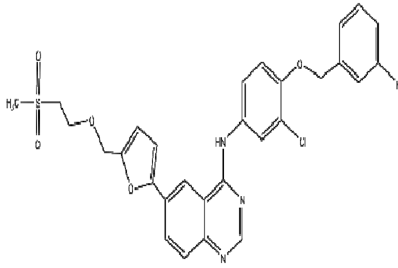
Table 2.1 Identification of Putative Tyrosine Kinases in the *T. brucei* Genome. A genome-wide search for the EGFR, c-Abl, c-Src and VEGFR kinase domains in the parasite proteome was performed by BLAST analysis. Next, PFAM was used to confirm protein kinase domains in the proteins discovered (not shown). The systematic ID of the 12 best candidates 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 different kinase domains is present.

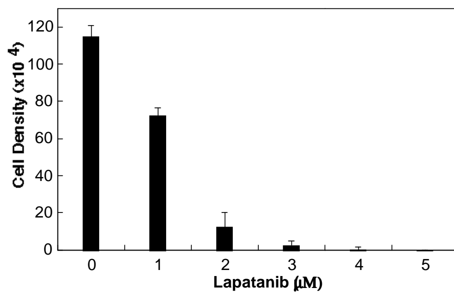
Systematic ID	PTK (E-value)*1	Homology to Specific Kinase Domain (P(N))			
		EGFR*2	c-Abl*3	c-Src*4	VEGFR*5
Tb10.406.0580	342-604 (4.4×10^{-41})	**1.7×10^{-11}**	5.0×10^{-9}	**6.0×10^{-22}**	**2.1×10^{-18}**
Tb10.61.2330	4-253 (4.3×10^{-35})	2.4×10^{-10}	**2.8×10^{-14}**	**1.1×10^{-20}**	**1.8×10^{-18}**
Tb927.5.2820	29-294 (2.6×10^{-32})	**2.7×10^{-11}**	**1.2×10^{-13}**	5.7×10^{-19}	**3.8×10^{-19}**
Tb927.6.2030	240-496 (1.5×10^{-29})	4.2×10^{-9}	4.2×10^{-9}	**3.8×10^{-21}**	1.8×10^{-15}
Tb10.70.7860	4-258 (2.9×10^{-29})	6.8×10^{-9}	1.3×10^{-10}	5.4×10^{-18}	7.4×10^{-16}
Tb927.8.5730	20-271 (5.0×10^{-29})	**1.2×10^{-12}**	3.7×10^{-10}	5.0×10^{-17}	**7.3×10^{-17}**
Tb927.7.6680	608-881 (6.1×10^{-28})	1.4×10^{-8}	1.5×10^{-6}	**2.4×10^{-20}**	1.7×10^{-8}
Tb11.02.2050	28-278 (1.3×10^{-27})	6.3×10^{-11}	**9.7×10^{-13}**	2.4×10^{-19}	1.4×10^{-15}
Tb927.7.3580	49-201 (3.6×10^{-27})	**3.4×10^{-14}**	**5.7×10^{-11}**	2.7×10^{-18}	**8.6×10^{-17}**
Tb927.3.3190	20-278 (1.8×10^{-26})	6.7×10^{-10}	**1.3×10^{-13}**	3.8×10^{-17}	5.1×10^{-15}
Tb10.70.5760	14-288 (1.8×10^{-25})	**1.8×10^{-11}**	2.4×10^{-10}	1.7×10^{-14}	1.5×10^{-7}
Tb11.46.0003	233-476 (3.9×10^{-25})	1.4×10^{-8}	4.8×10^{-8}	**1.7×10^{-19}**	2.5×10^{-14}

Table 2.1

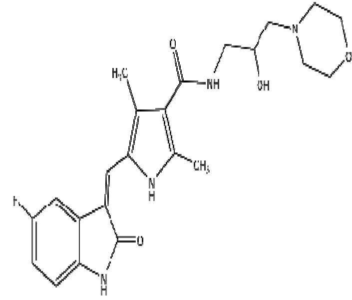
Figure 2.1: Tyrosine Kinase Inhibitors Lapatinib and SU14813 Inhibit Cell Growth in *T. brucei.* Different concentrations of **(A)** lapatinib or **(B)** SU14813 were added to T. brucei (10^4 cells/500 µl) and cultured for 48 hours. Control cells received DMSO, the solvent for the drugs. After 48 hours, cell density was determined by hemocytometer.

A. **Lapatinib**





B. **SU14813**



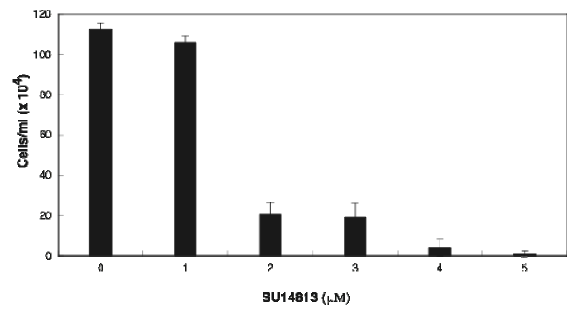
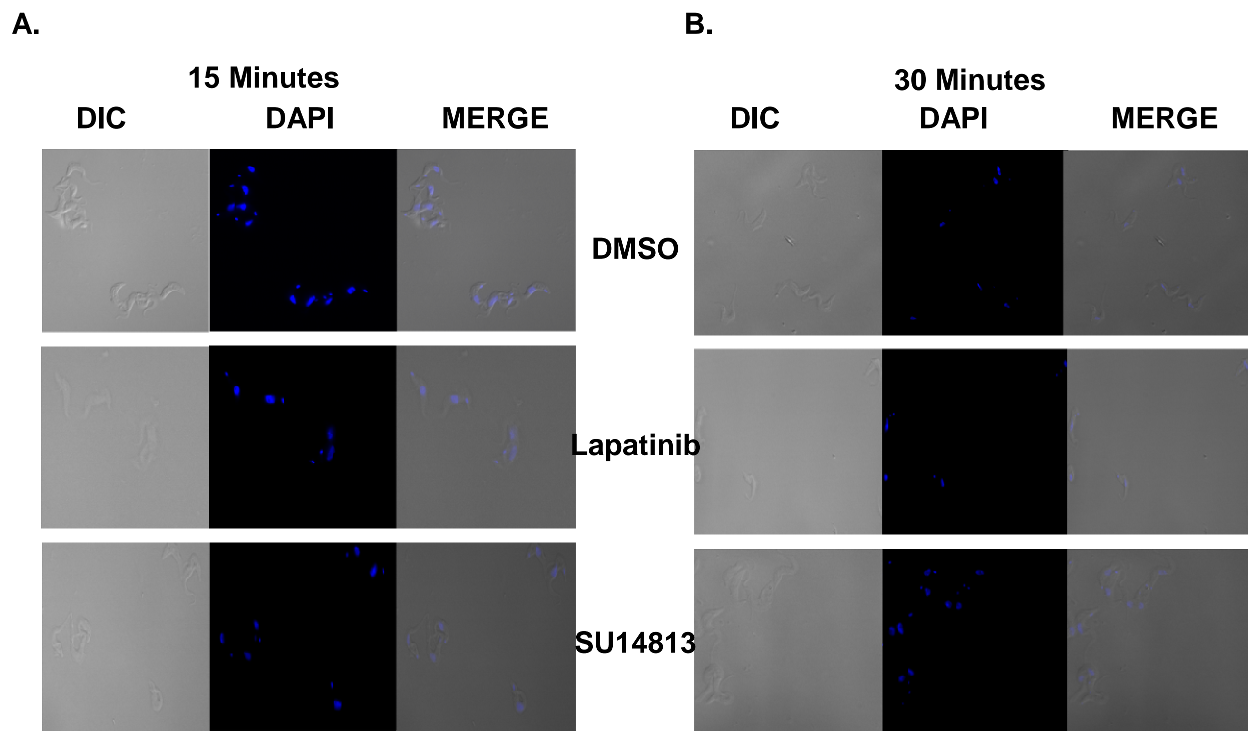


Figure 2.1

Figure 2.2: Lapatinib Changes Morphology of *T. brucei*. Bloodstream *T. brucei* (10^7 cells/ml) were treated with DMSO, Lapatinib (10 μM) or SU14813 (10 μM) at **(A)** 15, **(B)** 30 and **(C)** 60 minute time intervals. 2 x 10^6 cells were collected, fixed, mounted with Vectashield containing DAPI and visualized by DIC fluorescence microscopy.



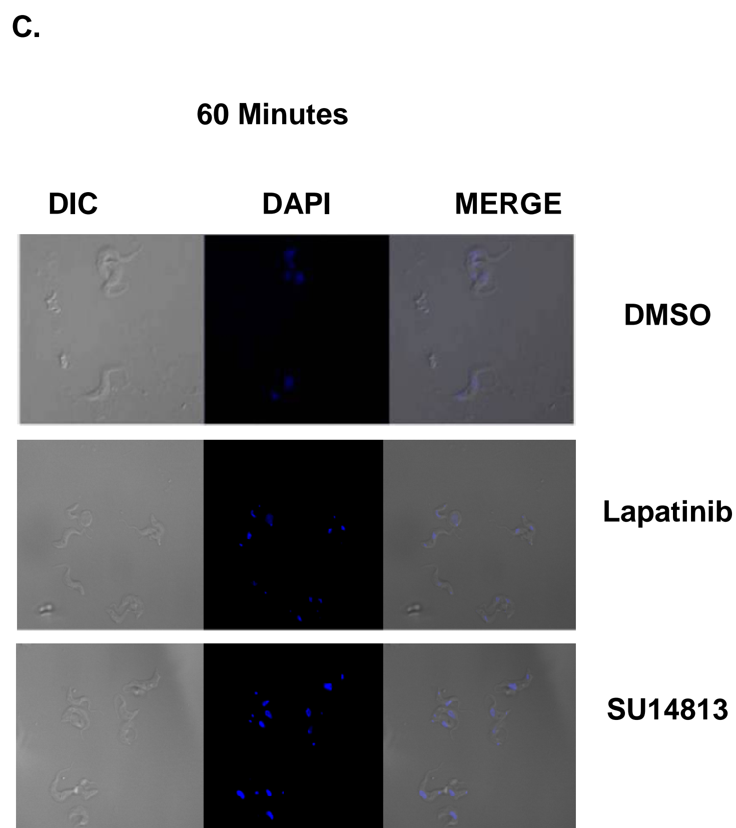
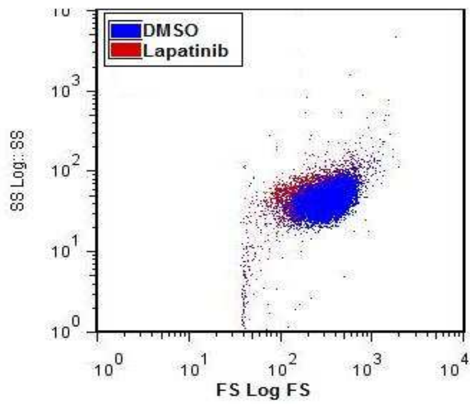
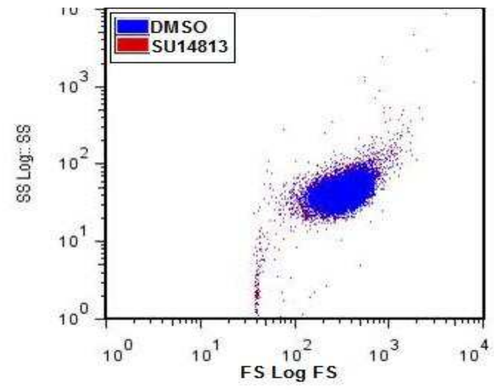


Figure 2.2

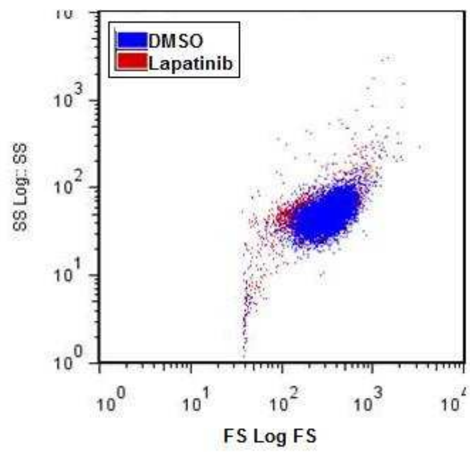
Figure 2.3: Light Scatter Plot displays Lapatinib-treated *T. brucei* cells in serum-free media change in shape. 2 x 10^6 cells/ml were treated with DMSO, Lapatinib (5 μM) or SU14813 (5 μM). At **(A)** 15, **(B)** 30, and **(C)** 60 minutes cells were collected and the forward scatter analyzed using flow cytometry.

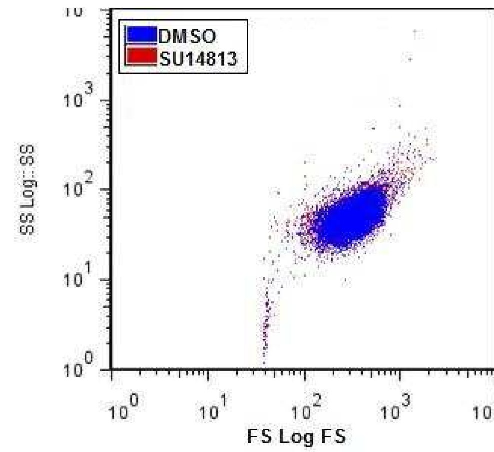
A.



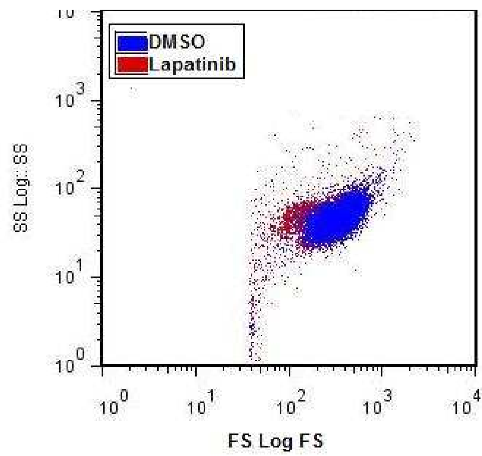


B.





C.



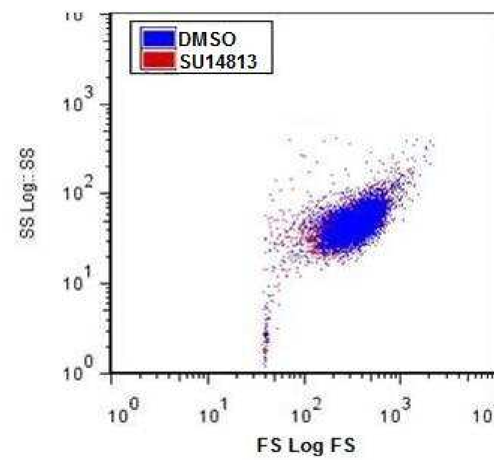
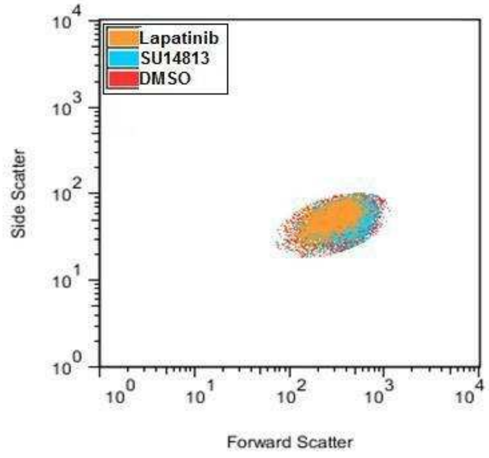


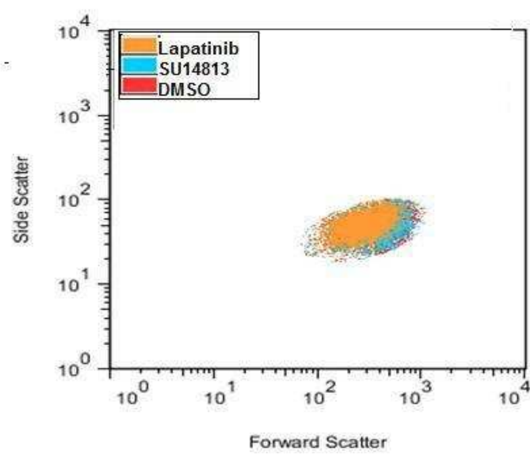
Figure 2.3

Figure 2.4: Light Scatter Plot confirms the Change in Cell Morphology of Lapatinib-Treated Cells in *T. brucei*. 10^7 cells were treated with DMSO, Lapatinib (10 μM) or SU14813 (10 μM). At **(A)** 15, **(B)** 30 and **(C)** 60 minute intervals cells were collected and the forward scatter was analyzed using flow cytometry. **(D)** Median log of the forward scatter is plotted for each time point.

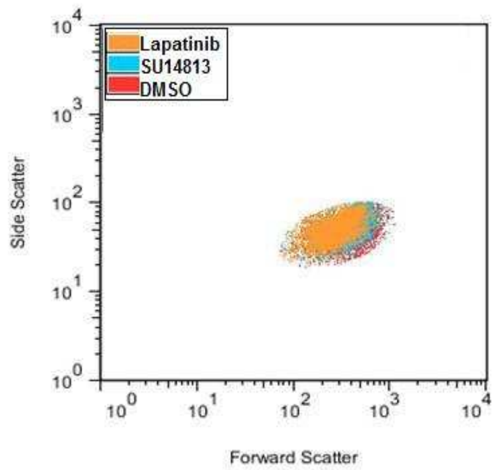
A.



B.



C.



D.

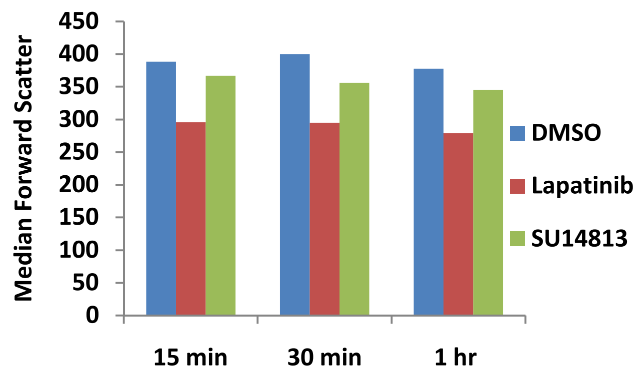
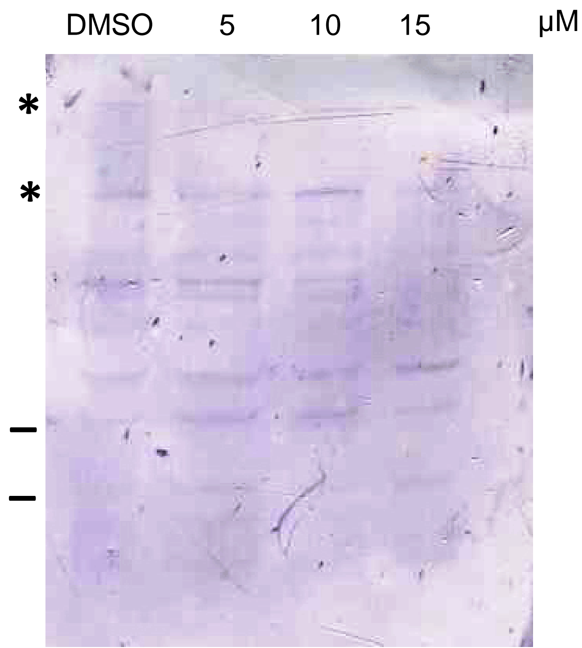
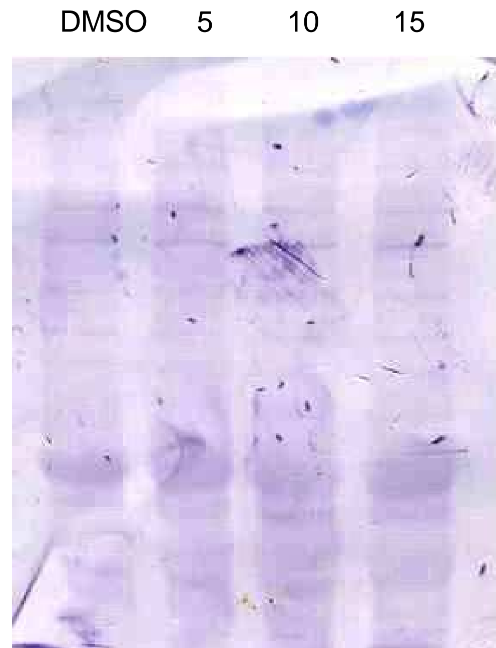


Figure 2.4

Figure 2.5: Lapatinib and SU14813 Reduces Tyrosine Phosphorylation of Proteins in *T. brucei*. Bloodstream *T. brucei* (10^7 cells/ml) were treated with DMSO, **(A)** Lapatinib and **(B)** SU14813 (0, 5, 10, 15 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-Ptyr100) or HSP70. An (*****) indicates decreased phosphotyrosine (pTyr) in the band after treatment; (**-**) indicates increased pTyr in the band.

A. **B.**



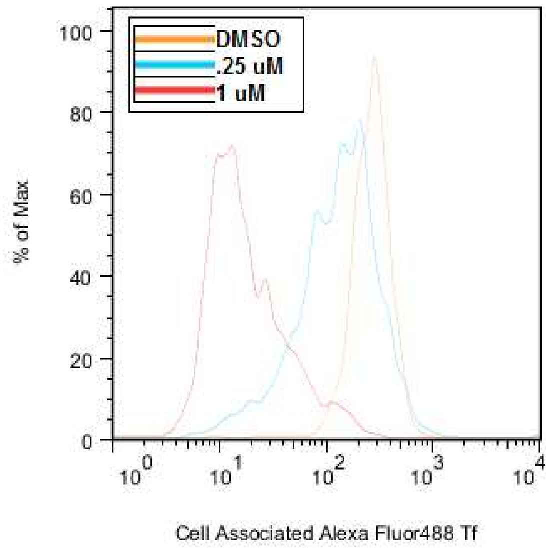


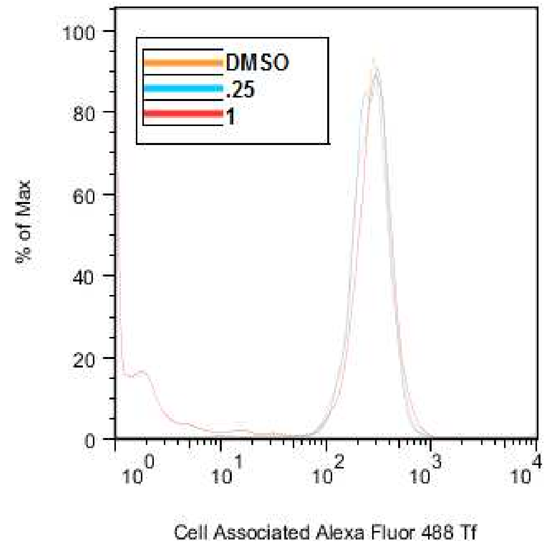
HSP70

Figure 2.5

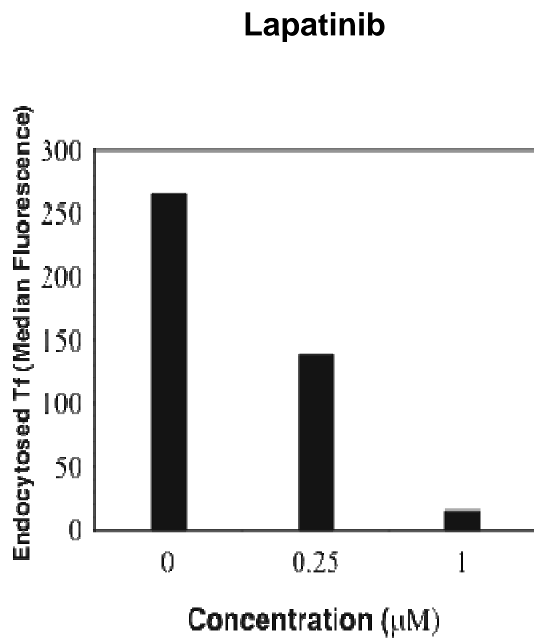
Figure 2.6: Lapatinib Inhibits Endocytosis of Transferrin in *T. brucei*. Bloodstream *T. brucei* (10^4 cells/ml) were treated with DMSO, **(A)** Lapatinib or **(B)** SU14813 at .25 µM or 1 µM for 30 minutes in HMI-9 medium. Transferrin-Alexa Fluor 488 (25 µg) was added, the cells incubated for 15 minutes in serum-free HMI-9 medium and the relative fluorescence measured using flow cytometry. **(C)** Median fluorescence intensity is plotted for each treatment described in panel A and B.

A. **B.**





C.



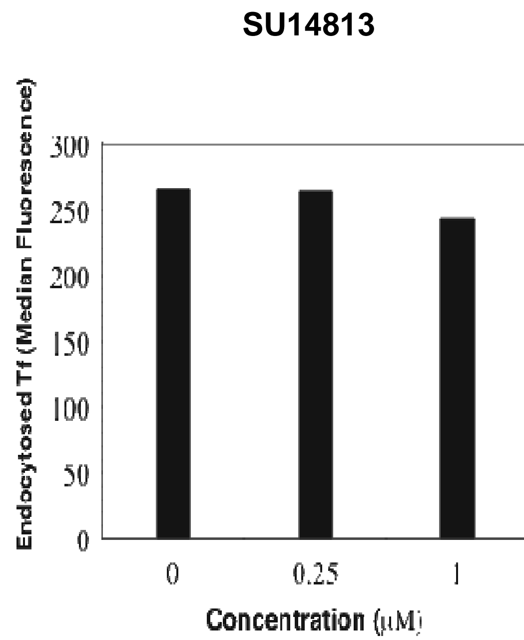


Figure 2.6

Conclusion

Although it seems as if the VEGFR domain is more homologous in regards to the kinase relatedness E-value in T. brucei (Table 2.1), lapatinib exhibited more of an effect among the criteria tested. Lapatinib's action of displaying a decrease in the tyrosine phosphorylation of some proteins, inhibition of receptor-mediated endocytosis and displaying a cell shape change suggests that this inhibitor is more effective in T. brucei than SU14813. Perhaps the criteria tested were not specific targets of SU14813 in T. brucei; morphological changes or tyrosine phosphorylation of abundant proteins are not determinants of SU14813 actions. Since both lapatinib and SU14813 have similar IG_{50} values (Figure 2.1) it can be concluded that both have an inhibitory growth effect on T. brucei. Other assays could be explored to determine the exact target of SU14813 in T. brucei. Immunoprecipitation experiments could identify specific proteins and/or cellular events related to each inhibitor.

Cells treated with lapatinib displayed a rounded cell phenotype and a decrease in tyrosine phosphorylation of proteins. The cell shape experiments were done at 10 micromolar, which was also a concentration tested in the tyrosine phosphorylation western blot. The display of a decrease in tyrosine phosphorylation at this concentration and the phenotypic change in T. brucei suggests a relationship. This connection confirms the existence of tyrosine phosphorylated proteins that are important in cytoskeleton maintenance in T. brucei. SU14813 targets several different kinase domains while lapatinib focuses on one. Perhaps the specificity versus the generality of the targets speaks to why lapatinib displays a greater effect on the criteria tested. In other words SU14813 would have to be used in higher concentrations in order to effectively bind to all of the kinase domains it inhibits, as opposed to lapatinib. Both lapatinib and SU14813 are competitive ATP inhibitors; they compete with ATP for access to the intracellular portion of the kinase domain. The cellular ATP levels in T. brucei are about 3-5 mM (Nolan and Voorheis 1992). Lapatinib would have to compete less with ATP binding on its one receptor. Contrarily SU14813 would have to compete for ATP binding on all four of its

receptors. If similarities of these domains exist in T. brucei (refer to table 2.1) this could explain SU14813's lack of action in the change in cell morphology, endocytosis and phosphorylation assays.

Because receptor-mediated endocytosis was inhibited in lapatinib-treated cells and these cells also displayed a cell shape change upon treatment it can be suggested that an inhibition of receptor-mediated endocytosis leads to a cell shape change, being circular. RNAi of the clathrin heavy chain in T. brucei results in an enlarged flagellar pocket ("big eye") and a blockage of endocytosis (Allen, Goulding et al. 2003). Depletion of actin, which colocalizes with the endocytic pathway in T. brucei, results in cell division arrest, an enlarged flagellar pocket, loss of endocytic activity and eventual cell death (Garcia-Salcedo, Perez-Morga et al. 2004). After 6 hours cells treated with 20 micromolar of SU14813 do eventually demonstrate a cell shape change in T. brucei (data not shown). Perhaps SU14813 will display a greater effect on the reduction of endocytosis, but at higher concentrations. Future experiments can be performed to determine whether or not the block in transferrin endocytosis affects the cell shape or if some other pathway is involved that directly or indirectly affects the cell shape. This idea would assist in discovering the relationship among tyrosine signaling, endocytosis and cytoskeletal rearrangement.

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